Akustička svojstva i filogenetski odnosi glavoča porodice Gobiidae (Teleostei; Gobiiformes)

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BIOLOŠKI ODSJEK

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AKUSTIČKA SVOJSTVA I FILOGENETSKI ODNOSI GLAVOČA PORODICE GOBIIDAE (TELEOSTEI; GOBIIFORMES)

DOKTORSKI RAD

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DOKTORSKI RAD

Mentor:

Prof. dr. sc. Davor Zanella

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Sveučilište u Zagrebu

FACULTY OF SCIENCE

DEPARTMENT OF BIOLOGY

Sven Horvatić

ACOUSTIC PROPERTIES AND PHYLOGENETIC RELATIONSHIPS OF GOBIES FROM THE FAMILY GOBIIDAE (TELEOSTEI; GOBIIFORMES)

DOCTORAL DISSERTATION

Supervisor:

Prof. Davor Zanella

Zagreb, 2021

Ovaj doktorski rad izrađen je u Laboratoriju za kralješnjake Zoologijskog zavoda Biološkog odsjeka Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu pod vodstvom prof. dr. sc. Davora Zanelle u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Eksperimentalni dio ovog doktorskog rada obavljen je na Sveučilištu u Zagrebu, Prirodoslovno-matematičkom fakultetu, Biološkom odsjeku (Hrvatska), dok je dio bioakustičkih i anatomskih istraživanja obavljen na Sveučilištu Ca' Foscari u Veneciji (Italija) i Sveučilištu u Liègeu (Belgija).

Informacije o mentoru

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U Zagrebu je završio osnovnu i srednju školu. Maturirao je 1992. g. na V. gimnaziji, a iste je godine upisao studij biologije, smjer profesor biologije na PMF-u u Zagrebu. Diplomirao je 1997. g. Od 2000. g. zaposlen je u Zoologijskom zavodu kao znanstveni novak. Godine 2003. obranio je magistarski rad, a 2007. i doktorsku disertaciju. Na mjesto docenta izabran je 2009., 2013. u izvanrednog profesora, a 2019. godine u redovitog profesora. Usavršavao se iz ekologije slatkih voda (određivanje kvalitete vode pomoću biotičkog indeksa) i populacija riba riječnih tokova i jezera u Laboratoriju za ihtiologiju i plankton Sveučilišta u Trstu, boravio je u Tromsu, u Norveškoj na stručnom usavršavanju iz područja ihtiologije u sklopu projekta "Management of Freshwater Fisheries of Bordering Rivers". Do 2009. g. vodi praktikume na preddiplomskom studiju iz kolegija Kralježnjaci, kao i terensku nastavu iz zoologije. Od 2009. g. nositelj je ili sunositelj kolegija Vertebrata, Upravljanje prirodnim populacijama, Ekologija i zaštita prirode, Zoologija 3 (Kralježnjaci), Ihtiologija, Akvakultura i ribarstvo slatkih voda i Akvakultura za studente preddiplomskog i diplomskog studija različitih smjerova. Sudjeluje u terenskoj nastavi iz navedenih kolegija. Voditelj je jednog doktorskog rada, 15 diplomskih radova, dva magistarska i 19 završnih radova. Njegova znanstvena djelatnost obuhvaća istraživanja raznolikosti i staništa slatkovodnih riba Hrvatske, posebno endemskih vrsta (inventarizaciju i utvrđivanje stupnja ugroženosti te bioloških i ekoloških značajki pojedinih vrsta). Drugo je područje njegova znanstvenog rada usmjereno na proučavanje prirodnih populacija te njihovom upravljanju. Objavio je kao autor ili koautor 58 znanstvenih radova (47 u časopisima indeksiranim u CC, 11 u časopisima indeksiranim u drugim sekundarnim i tercijarnim bazama). Koautor je tri knjige te jednog poglavlja u knjizi. Sudjelovao je na međunarodnim skupovima s 46 priopćenja te domaćim skupovima s 12 priopćenja. Sudjelovao je na brojnim međunarodnim i domaćim skupovima sa 60-ak priopćenja. Aktivno je sudjelovao u izvedbi triju znanstvenih projekata. Bio je i suradnik na dva međunarodna projekta.

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Sveučilište u Zagrebu Doktorski rad

Prirodoslovno-matematički fakultet

Biološki odsjek

AKUSTIČKA SVOJSTVA I FILOGENETSKI ODNOSI GLAVOČA PORODICE GOBIIDAE (TELEOSTEI; GOBIIFORMES)

SVEN HORVATIĆ

Prirodoslovno-matematički fakultet, Biološki odsjek

Glavoči su najbrojnijih skupina riba zrakoperki. Također, oni su jedna od akustički najistraživanijih skupina zrakoperki današnjice, sa 23 poznate vrste koje se glasaju. U sklopu ove disertacije, u četiri znanstvene publikacije prvi je put istražena, upotrebom bioakustičkih metoda i analiza, sposobnost stvaranja zvukova i vokalni repertoar glavoča linije *Gobius Neogobius melanostomus*, *Neogobius fluviatilis* i *Ponticola kessleri*, kao i *Perccottus glenii* (Odontobutidae). Nadalje, ovom su disertacijom istražene anatomske strukture uključene u proces produkcije zvukova kod rotana, a rezultati su pokazali kako se mehanizam najvjerojatnije temelji na kontrakcijama kranijalno-pektoralnih mišića. Upotrebom molekularnih analiza koje uključuju metode filogenetske rekonstrukcije s mitohondrijskim i jezgrenim genetičkim biljezima, rekonstruirani su odnosi vokalnih glavoča linije *Gobius* te istražena korelacija između genetičke i akustičke divergencije. Rezultati ove disertacije naglašavaju kako su zvukovi glavoča specifični za pojedinu vrstu, dok njihova interspecijska akustička divergencija prati genetičku, što pokazuje kako evolucija zvukova i genotipova prati isti obrazac.

(79 stranica, 8 slika, 207 literaturnih navoda, jezik izvornika: hrvatski)

Ključne riječi: glavoči, bioakustika, linija *Gobius*, filogenija, zvukovi

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University of Zagreb Doctoral thesis
Faculty of Science
Department of Biology

ACOUSTIC PROPERTIES AND PHYLOGENETIC RELATIONSHIPS OF GOBIES FROM THE FAMILY GOBIIDAE (TELEOSTEI; GOBIIFORMES)

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Gobies are the most numerous fish group. They are also one of the most investigated acoustic cluster of actinopterygian fish nowadays, with 23 species vocally tested. In this dissertation, represented through four scientific publications, the acoustic communication and vocal repertoire was investigated in *Gobius*-lineage gobies *Neogobius melanostomus*, *Neogobius fluviatilis* and *Ponticola kessleri*, as well as in *Perccottus glenii* (Odontobutidae), using standardized bioacoustic methods and analyses. The presented dissertation investigated the anatomical structures involved in the sound production, and the conducted analyses indicate that the sonic mechanism could be based on the contractions of cranio-pectoral muscles. By using phylogenetic analyses with a multilocus approach, we reconstructed the interspecific relationships between vocal gobies from *Gobius*-lineage and correlated the acoustic and genetic divergences. Results of this dissertation emphasize that goby sounds are truly species-specific, while their interspecific acoustic divergence follows the genotypic divergence, which could indicate that acoustic signals in gobies from *Gobius*-lineage carry phylogenetic signal.

(79 pages, 8 figures, 207 references, original in Croatian)

Keywords: gobies, bioacoustics, *Gobius*-lineage, phylogeny, sounds

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2. **Horvatić S**, Bem L, Malavasi S, Marčić Z, Buj I, Mustafić P, Ćaleta M, Zanella D (2019) Comparative analysis of sound production between the bighead goby *Ponticola kessleri* and the round goby *Neogobius melanostomus*: Implications for phylogeny and systematics. Environmental Biology of Fishes 102: 727-739.
3. **Horvatić S**, Malavasi S, Parmentier E, Marčić Z, Buj I, Mustafić P, Ćaleta M, Smederevac-Lalić M, Skorić S, Zanella D (2019) Acoustic communication during reproduction in the basal gobioid Amur sleeper and the putative sound production mechanism. Journal of Zoology 309: 269-279.
4. **Horvatić S**, Malavasi S, Vukić J, Šanda R, Marčić Z, Ćaleta M, Lorenzoni M, Mustafić P, Buj I, Raguž L, Ivić L, Cavraro F, Zanella D (2021) Acoustic divergence is correlated with phylogenetic distance in European gobies (Gobiidae; *Gobius* lineage): evolutionary insights. Predano na recenziju u *Plos One*.

PROŠIRENI SAŽETAK

Glavoči (porodice Gobiidae i Gobionellidae, prema Thacker, 2009) su najbrojnija skupina riba zrakoperki, s oko 1600 opisanih vrsta te mnoštvom neotkrivenih i kriptičkih skupina rasprostranjenih u gotovo svim akvatičkim ekosustavima (Nelson, 2006; Fricke i sur., 2020). Oni naseljavaju najraznolikija vodena staništa, kao što su duboka i hladna mora, potoci i rijeke te prijelazne bočate lagune i estuarije, a u plitkom infralitoralu dominiraju po brojnosti i raznolikosti vrsta. Zbog svojih bioloških karakteristika koje uključuju pridneni ili kriptobentički način života, speleofilni tip reprodukcije i teritorijalnost s izraženom roditeljskom brigom za potomke, glavoči su iznimno zanimljivi modelni organizmi u laboratorijskim i *in situ* bioakustičkim eksperimentima. Upravo su zato jedna od akustički najistraživanijih skupina riba današnjice (uz Pomacentridae, Batrachoidiidae, Gadidiae, Serrasalmidae), s 23 dosad poznate vrste koje se glasaju (oko 1,2% ukupne brojnosti prema Fricke i sur., 2020) ispitane u bioakustičkim eksperimentima (Zeyl i sur., 2016). Do danas se većina znanstvene pozornosti u bioakustičkim ispitivanjima pridavala glavočima linije *Pomatoschistus* (rodovi *Knipowitschia*, *Economidichthys*, *Gobiusculus* i *Pomatoschistus*) i atlantsko-mediteranskim skupinama linije *Gobius* (*Zosterisessor*, *Padogobius* i *Gobius*), dok je znanje o akustičkim signalima, vokalnom repertoaru i etološkom kontekstu produkcije zvukova bilo znatno neistraženo kod pontokaspijskih vrsta i glavoča u širem smislu (Gobioidei). Multidisciplinarna istraživanja navode kako vokalni glavoči iz dviju različitih porodica, *Gobius paganellus* (Gobiidae) i *Pomatoschistus pictus* (Gobionellidae), imaju (uz male osteološke razlike) karakterističan plan građe i jednake anatomske strukture u glavenom dijelu i oplećju, zbog čega njihovi osnovni elementi za produkciju zvukova najvjerojatnije slijede sličan evolucijski obrazac koji potencijalno dijele s ostalim sestrinskim skupinama kao što su Odontobutidae, Butidae i dr. (Parmentier i sur., 2013, 2017). Produkcija zvukova, izuzev navedene dvije porodice, istražena je još samo kod jedne vrste iz porodice Odontobutidae (*Odontobutis obscura*), a bioakustičko istraživanje potvrdilo je kako mužjaci te vrste stvaraju pulsatilni tip zvuka pomoću ždrijelnih zuba (Takemura, 1983). Stoga je detaljna bioakustička i anatomska analiza trebala istražiti vokalni repertoar i anatomske strukture uključene u proces stvaranja zvukova kod bazalnih pripadnika glavoča u širem smislu, kao što je primjerice rotan *Perccottus glenii*. Nadalje, filogenetski odnosi glavoča i dalje su prilično neistraženi iako novija filogenetska istraživanja navode kako se europski glavoči dijele, uz porodice, i na nekoliko evolucijskih skupina ili linija (*Gobius* i *Aphia* unutar potporodice Gobiine; *Pomatoschistus* unutar potporodice Gobionelline, prema Agorreta i sur., 2013). Molekularne analize, tj. metode filogenetske rekonstrukcije temeljene na pristupu multilokus (više različitih mitohondrijskih i jezgrenih gena), naglašavaju kako unutar linije *Gobius* atlantsko-mediteranski rodovi *Gobius, Padogobius* i *Zosterisessor* dijele bliske sestrinske odnose s pontokaspijskom skupinom (rodovi *Neogobius, Ponticola, Proterorhinus* i dr.) rasprostranjenom u slijevovima Crnog, Azovskog i Aralskog mora te Kaspijskog jezera (Thacker i Roje, 2011; Agorreta i sur., 2013). Iako su odnosi ove skupine detaljnije istraženi koristeći isključivo molekularne ili morfološke metode, akustička komparativna analiza dosad nije provedena. Usto, niti jedno istraživanje dosad nije empirijski i kvantitativno povezalo akustičku divergenciju s genetičkom diversifikacijom kod vokalnih riba kako bi se istražilo slijede li one isti obrazac evolucije, tj. nose li zvukovi filogenetski signal zaslužan za razlikovanje vokalnih vrsta iz linije *Gobius*.

Cilj je ove disertacije bio proširiti znanje o vokalnom repertoaru (s akustičkim parametrima), anatomskim strukturama uključenima u proces stvaranja zvukova te filogenetskim odnosima vokalnih glavoča linije *Gobius* (Gobiiformes, Gobiidae) i rotana (Gobiiformes, Odontobutidae) koristeći tradicionalne bioakustičke metode i analize te metode filogenetske rekonstrukcije s više različitih genetičkih biljega (mtDNA i nDNA). U četiri znanstvene publikacije, cilj je disertacije bio istražiti i testirati ove znanstvene hipoteze: i. Filogenetska interspecijska analiza pontokaspijskih i atlantsko-mediteranskih glavoča iz linije *Gobius*, rekonstruirana na temelju kvalitativnih i kvantitativnih akustičkih parametara, upućuje na određen stupanj međusobne srodnosti između vokalnih vrsta, ii. Kod glavoča linije *Gobius* glasaju se samo mužjaci tijekom intraspecijskih ili interspecijskih interakcija, iii. Mehanizam za produkciju zvukova temelji se kod rotana *Perccottus glenii* (Odontobutidae) na kranio-pektoralnim mišićima, a pulsatilni zvukovi predstavljaju ancestralni/bazalni tip akustičkog signala kod glavoča u širem smislu (hipoteza prati Malavasi i sur. (2008)), iv. Interspecijski filogenetski odnosi devet vokalnih vrsta glavoča linije *Gobius* (rodovi *Neogobius*, *Ponticola*, *Zosterisessor*, *Gobius*, *Padogobius*), konstruirani na temelju šest kvantitativnih akustičkih parametara, preklapaju se s rezultatima molekularnih analiza, tj. zvukovi prenose filogenetski signal odgovoran za razlikovanje srodnih vrsta.

U ovoj disertaciji, predstavljenoj u četiri znanstvene publikacije, prvi je put istražena, upotrebom tradicionalnih bioakustičkih metoda i analiza, sposobnost stvaranja zvukova i vokalni repertoar (s akustičkim svojstvima) pontokaspijskih glavoča linije *Gobius* (Gobiiformes; Gobiidae) *Neogobius melanostomus*, *N. fluviatilis* i *Ponticola kessleri* te invazivne vrste rotana *Perccottus glenii* (Odontobutidae) (publikacije **I**, **II** i **III**). Nadalje, ova je disertacija istražila anatomske strukture uključene u proces produkcije zvukova kod rotana, pripadnika sestrinske porodice svih ostalih glavoča u širem smislu (Gobioidei). Rezultati su pokazali kako dosad anatomski ispitani vokalni glavoči (*G. paganellus* i *P. pictus*), uključujući i rotana, dijele zajedničke anatomske strukture mehanizma za produkciju zvukova, koji se temelji na kranijalno-pektoralnim mišićima (*m. levator pectoralis*) i njihovim kontrakcijama (publikacija **III**). Istodobno, upotrebom molekularnih analiza koje uključuju metode filogenetske rekonstrukcije s više različitih genetičkih biljega (mtDNA i nDNA), rekonstruirani su filogenetski odnosi vokalnih glavoča linije *Gobius* te je korištenjem komparativne akustičko-molekularne metode istraženo postoji li pozitivna korelacija između genetičke i akustičke divergencije (publikacija **IV**). Rezultati ove disertacije potvrdili su kako su zvukovi glavoča linije *Gobius* izrazito specifični za vrstu, dok njihova interspecijska akustička divergencija prati genetičku, što sugerira kako se evolucija zvukova najvjerojatnije mijenjala (i dalje se mijenja) u istom pravcu i jednakom stopom kao i genotipska (neovisno o tipu mtDNA ili nDNA genetičkih biljega) kod navedene skupine riba (publikacija **IV**).

Znanstveni je doprinos ove disertacije proširivanje znanja o vokalnom repertoaru i akustičkim svojstvima invazivnih pontokaspijskih vrsta rodova *Neogobius* i *Ponticola* te rotana *P. glenii*. Rezultati ove disertacije naglašavaju kako su glavoči izrazito vokalne ribe te kako se, uz standardnu morfološku i molekularnu filogeniju, zvukovi mogu, s velikom pouzdanosti, koristiti u rekonstrukciji filogenetskih odnosa glavoča linije *Gobius*. Osim toga, ova disertacija naglašava kako se akustička i genetička divergencija znatno preklapaju te slijede vrlo sličan evolucijski obrazac, što potvrđuje važnu ulogu akustičkih signala u divergenciji pojedinih sestrinskih i blisko srodnih europskih glavoča. Ovi rezultati otvaraju potencijalnu mogućnost budućim modernim istraživanjima koja bi se temeljila na automatiziranom prepoznavanju i monitoringu vokalnih glavoča koristeći akustičke baze podataka ili pasivne akustičke snimače. Istraživanje anatomskih struktura uključenih u produkciju zvukova rotana u sklopu ove disertacije naglašava kako je mehanizam za produkciju zvukova, tj. kranijalno-pektoralni mišići, kod glavoča najvjerojatnije evolucijski konzerviran te kako potonji ne koriste druge strukture (npr. plivaći mjehur) u procesu stvaraju zvukova. Stoga ova disertacija znatno doprinosi shvaćanju i proširivanju znanja o načinu na koji glavoči produciraju zvukove tijekom interspecijskih ili intraspecijskih interakcija, što izravno utječe na njihove biološke i ekološke životne karakteristike. Ova disertacija naglašava kako su zvukovi, producirani tijekom reproduktivnih i agresivnih interakcija u sklopu životnog ciklusa, iznimno važan dio komunikacijskog procesa kod glavoča. Zbog toga bi buduća istraživanja blisko povezana s monitoringom ili eradikacijom invazivnih glavoča (kao što su rodovi *Neogobius*, *Ponticola* i *Perccottus*) trebala iskoristiti i implementirati znanja o akustičkoj komunikaciji glavoča radi njihove biokontrole ili potencijalne eradikacije koristeći akustičke zamke/oznake ili druge alate, kao što je već predloženo (Isabella-Valenzi i Higgs, 2016).

THESIS SUMMARY

Gobies (families Gobiidae and Gobionellidae; according to Thacker, 2009) are the most numerous groups of actinopterygian fishes with approximately 1.600 described species, while many undiscovered and cryptic taxa are distributed in almost every aquatic ecosystem (Nelson, 2006; Fricke et al., 2020). Gobies inhabit diverse aquatic habitats, ranging from cold and deep seas, over streams and rivers, to brackish lagoons and estuaries, and in shallow infralittoral they dominate in the abundance and multiformity. Due to their biological characteristics, which include benthic or cryptobenthic lifestyle, speleophylic reproduction and prominent (male) territoriality, gobies are interesting model organisms for laboratory and *in situ* bioacoustic experiments. Therefore, together with other fish groups (Pomacentridae, Batrachoidiidae, Gadidiae, Serrasalmidae), they are commonly used fish in bioacoustic experiments, with 23 species (~1,2% overall species abundance accrding to Fricke et al., 2020) investigated so far (Zeyl et al., 2016). Until now, most of the scientific attention has been given to sand gobies from *Pomatoschisus*-lineage (genus *Knipowitschia*, *Economidichthys*, *Gobiusculus* and *Pomatoschistus*) and Atlantic-Mediterranean gobies from *Gobius*-lineage (*Zosterisessor*, *Padogobius* and *Gobius*), while the knowledge regarding the acoustic communication, vocal repertoire and acoustic parameters of other interesting groups, like the Ponto-Caspian gobies and Odontobutidae (gobies in a wider sense), are mostly lacking. Multidisciplinary studies indicate that vocal gobies from two different goby families, *Gobius paganellus* (Gobiidae) and *Pomatoschistus pictus* (Gobionellidae), posses characteristics and fairly similar body *bauplan* (structural plan) and anatomical structures in the cranio-pectoral part of their body, therefore emphasizing that gobies (together with other primitive groups of Gobioidei like Odontobutidae) could share similar building elements of their sound production mechanism, making their evolutionary pattern quite recognizable and predictable (Parmentier et al., 2013, 2017). Sound production, outside gobies in the narrow sense (Gobiidae and Gobionellidae), has been investigated in only one additional family named sleepers (Odontobutidae), and the study proposed that *Odontobutis obscura* could produce pulsatile sounds thanks to rapid pharyngeal teeth collision (Takemura, 1983). Therefore, due to the lack of knowledge about the vocal repertoire and precise anatomical structures involved in sound production in sleepers, a detailed study was necessary to investigate these two aspects of acoustic communication in another sleeper, *Perccottus glenii*. Furthermore, even though phylogenetic relationships between certain goby groups are still largely undefined, some studies propose that European gobies can be divided into several

lineages, namely *Gobius*- and *Aphia*- within the subfamily Gobiine; *Pomatoschistus*- within Gobionelline, according to Agorreta et al. (2013). Molecular analyses, based on the phylogenetic methods with the multilocus approach, indicate that within *Gobius*-lineage Atlantic-Mediterranean taxa *Gobius*, *Padogobius* and *Zosterisessor* share a close relationship with Ponto-Caspian group (genus *Neogobius*, *Ponticola*, *Proterorhinus*, etc.) widespread in Pontian drainage (Aral, Azov and the Black Sea) and Caspian Sea (Thacker and Roje, 2011; Agorreta et al., 2013). Even though molecular and morphological studies have been conducted in certain *Gobius*-lineage species, comparative acoustic-genetic research has not been performed for *Gobius*-lineage gobies or any other teleost group so far. Also, no study has tested empirically and quantitatively the correlation between acoustic diversification and genetic divergence to observe whether they follow the same pattern of evolution, i.e. does the sound carry phylogenetic signal responsible for the vocal taxa discrimination.

This dissertation aimed to broaden the scientific knowledge about the acoustic repertoire (and acoustic parameters), anatomical structures involved in the sound production process and phylogenetic relationships of vocal *Gobius*-lineage gobies (Gobiiformes, Gobiidae) together with amur sleeper *Perccottus glenii* (Gobiiformes, Odontobutidae) by using standardized bioacoustic methods and molecular analyses of phylogenetic inference combined with multilocus approach (mtDNA and nDNA). Trough four original scientific publications, this dissertation tried to answer the following questions: i.) How phylogenetically close are Ponto-Caspian gobies with the Atlantic-Mediterranean taxa considering the quantitative and qualitative acoustic parameters, and are only the males vocal sex during intersexual interactions? ii.) Is the sound production mechanism in *P. glenii* based on the cranio-pectoral muscle contractions and is the pulsatile sound ancestral among gobies in a wider sense (Gobioidei) according to the proposed hypothesis by Malavasi et al. (2008)? iii.) Is there an overlap between the interspecific acoustic divergence of *Gobius*-lineage gobies (genus *Neogobius*, *Ponticola*, *Zosterisessor*, *Gobius*, *Padogobius*), constructed on six quantitative acoustic parameters, with genetic distance, i.e. do sounds in gobies carry phylogenetic signals responsible for the differentiation of investigated vocal gobies?

In this doctoral dissertation, represented by four scientific publications, for the first time by using bioacoustic methods and analyses, the acoustic communication and vocal repertoire of Ponto-Caspian gobies from *Gobius*-lineage (Gobiiformes; Gobiidae) *Neogobius melanostomus*, *N. fluviatilis* and *Ponticola kessleri*, as well invasive odontobutid *Perccottus glenii* (Odontobutidae) were tested (publication **I**, **II** and **III**). In addition, this dissertation investigated the anatomical structures closely related to cranio-pectoral girdle in *P. glenii* which could be responsible for the sound production in this basal Gobioid goby. The results of this dissertation have shown that, together with previously investigated vocal gobies (*G. paganellus* i *P. pictus*), *P. glenii* shares a similar cranio-pectoral structural plan, which is based on *levator pectoralis* muscle for which it was hypothesized to be responsible for sound production (publication **III**). By using molecular analyses of phylogenetic inference combined with a multilocus approach (mtDNA and nDNA genetic markers), it was possible to reconstruct the phylogenetic relationships between investigated vocal gobies from *Gobius*-lineage. In addition, the phylogenetic analysis allowed us to correlate the genetic distance between nine investigated *Gobius*-lineage gobies with interspecific acoustic divergence (publication **IV**). The results indicated that the sounds in *Gobius*-lineage gobies are species-specific, while their acoustic interspecific differentiation follows genotypic divergence (irrespective of genetic marker) which suggests that two traits evolve in the same manner (publication **IV**).

The scientific contribution of this doctoral dissertation is the broadening of the scientific knowledge about the acoustic communication and vocal repertoire in invasive Ponto-Caspian gobies from genus *Neogobius* and *Ponticola*, together with amur sleeper *P. glenii*. Results of this dissertation strongly suggest that the gobies are highly vocal fish, and together with a standardized method for inferring relationships like morphological or molecular phylogeny, sounds could (as proven by this dissertation) to a certain degree reveal the patter of interspecific divergence between vocal *Gobius*-lineage gobies. In addition, the observed correlation between acoustic divergence and genetic distance emphasizes that the sounds have a prominent role in the diversification (and possibly speciation) process of closely related *Gobius*-lineage gobies. These observations present the possible significance of the acoustic signals in indirect and passive usage of the sounds in monitoring or eradication programs of invasive gobies like *Neogobius melanostomus*, *N. fluviatilis*, *P. kesssleri* and *P. glenii*. Anatomical investigations confirm that gobies in the wider sense, including odontobutid *P. glenii*, relly on the similar communication pathways, and that the mechanism could be evolutionary conserved (since it is probably based on cranio-pectoral muscle contractions) exploiting already present structures for the sound production through the process of exaptation. Therefore, this dissertation contributes significantly to our understanding of the multimodal communication system in gobies, which also strongly broadens our knowledge about their general biological or ecological life traits. In addition, since the gobies communicate acoustically during the reproductive seasons, this dissertation emphasizes that the future studies should utilize the information regarding the sound communication in *Gobius*-lineage gobies (i.e. invasive species from genera *Neogobius*, *Ponticola* and odontobutid *Perccottus*) and implement it in the future monitoring or eradication programs based on acoustic traps/markers like successfully proposed by (Isabella-Valenzi and Higgs, 2016).

1. UVOD

1.1. *Glavoči, najbrojnija skupina riba zrakoperki*

Ribe su najbrojnija i najraznolikija skupina kralješnjaka, ponajprije zbog svoje spektakularne morfološke i fenotipske varijabilnosti, ali i opsežnoga geografskog areala te svojih bioloških, često odvedenih, karakteristika (Nelson, 2006, 2016). Kako bismo razumjeli njihovu evoluciju, na početku je potrebno definirati naizgled jednostavan pojam „riba“ i odrediti skup karakteristika koje ih definiraju. Mnogi autori u termin „riba“ često uključuju mnogo nesrodnih skupina kralješnjaka u jedinstvenu neformalnu skupinu. Berra (2001) u svom djelu *„Freshwater fish distribution“* iznosi nekoliko kriterija po kojima se može jasnije definirati „riba“, ali sam autor nailazi na mnogo iznimki koje otežavaju naizgled jednostavnu diferencijaciju. Naime, posve logične pretpostavke kako sve ribe žive u vodi, dišu isključivo škrgama, imaju ljuske na površini tijela, pokreću se perajama te su poikilotermni organizmi, ostaju neutemeljene upravo zbog njihove iznimne raznolikosti i plastičnosti, koje pokazuju kako su se ribe danas prilagodile najraznolikijim uvjetima u okolišu te se fenotipski prilično izmijenile (npr. ribe roda *Periophthalmus* Bloch i Schneider, 1801 mogu preživjeti određeno vrijeme izvan vode; dvodihalice roda *Protopterus* Owen, 1839 imaju plućno krilo, čime su sličnije tetrapodnim kralješnjacima; somovi i jegulje imaju golu kožu bez ljusaka; ribe roda *Apterichtus* Duméril, 1806 nemaju peraje; brzi plivači kao što su tune i neki morski psi imaju tjelesnu temperaturu nešto višu od okolnog medija; Berra, 2001). Nelson (2006) pak simplificira pojam te predlaže ovaj opis: *„riba je svaki akvatički kralješnjak koji diše škrgama tijekom cijelog života s tjelesnim privjescima u obliku peraja“*. Prateći taj kriterij, termin „riba“ predstavlja polifiletsku skupinu kralješnjaka i uključuje, uz hrskavičnjače i ribe zrakoperke i primitivnije oblike kao što su paklare i sljepulje, daleke rođake recentnih „riba“, ali i dvodihalice i latimerije (mesoperke) koje su filogenetski vrlo bliske tetrapodima (Pough i sur., 2009). Kao što je već navedeno, mnogo različitih nesrodnih skupina moglo bi se smatrati „ribama“ u širem smislu. Ipak, pojedini ihtiolozi, tj. stručnjaci koji istražuju ribe, danas termin „riba“ primjenjuju u određenim i specifičnim situacijama, referencirajući se na monofiletsku skupinu zrakoperki – Actinopterygii (Nelson, 2006). Takav je pristup primijenjen i u ovoj disertaciji u kojoj se pojam „riba“ odnosi samo na zrakoperke.

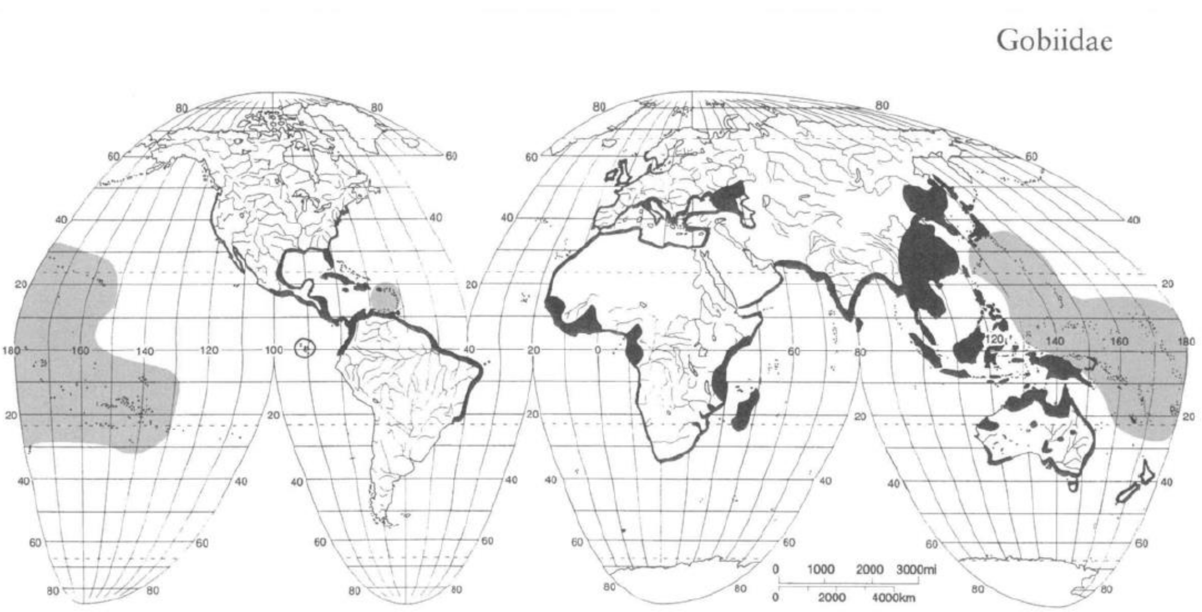
Recentne zrakoperke, u odnosu na druge kralješnjake, dominiraju u gotovo svim vodenim ekosustavima, naseljavajući najraznolikija vodena staništa, od malih potoka na Tibetu na visinama od 5200 m pa do velikih dubina u oceanima ispod 7000 m (Nelson, 2006; Pough i sur., 2009). Neke su zrakoperke čak napustile vodeni medij te mogu preživjeti na kopnu dulje vrijeme, kao što su pripadnici linije *Periophthalmus* (Nelson, 2006), druge žive u iznimno ekstremnim staništima (vrsta tilapije *Oreochromis alcalicus* (Hilgendorf, 1905) živi u jezerima na temperaturi od oko 42 °C, a vrste roda *Trematomus* Boulenger, 1902 žive na – 2 °C ispod ledenog pokrova na Antartici). Ukratko, zrakoperke imaju karakteristične pokretne i zrakaste peraje (grč. *actino* = šipčica, *ptero* = peraja), umjesto tvrdih ili mesnatih peraja, kakve nalazimo kod morskih pasa i mesoperki. Fleksibilne peraje omogućile su im nevjerojatnu preciznost i usmjeren manevar pri pokretanju, hranjenju, reprodukciji, itd. (Pough i sur., 2009). Tijekom svoje evolucijske povijesti zrakoperke su prošle intenzivnu evolucijsku radijaciju i divergenciju u morskim i u slatkim vodama, a danas gotovo da ne postoji vodeno stanište u kojem nije zabilježena barem jedna vrsta zrakoperke (Nelson, 2006, 2016). One su ujedno i najbrojnija skupina kralješnjaka s oko 36.000 recentnih vrsta, što čini više od polovine sveukupno opisanih kralješnjaka (Nelson 2006; Fricke i sur., 2020). Brzina i stopa opisa novih vrsta drastično se mijenjala tijekom godina, na što upućuje činjenica kako je deseto izdanje *Systema Naturae* (Linnaeus, 1758) navodilo svega 478 vrsta riba. Samo se u 2019. godini otkrila i opisala 351 nova vrsta riba, a prosječan broj iznosi oko 200 opisanih vrsta godišnje, računajući od 2001. godine (Fricke i sur., 2020). Svjetski oceani zauzimaju oko 97% ukupnog volumena vode na Zemlji, dok slatkovodni ekosustavi (ponajprije rijeke i jezera) jedva 0,0093%, a ostatak vode vezan je u ledenjacima, tlu i atmosferi (Horn, 1972). S obzirom na to, zanimljivo je kako je od ukupnog broja opisanih riba njih oko 18.000 isključivo slatkovodno (Fricke i sur., 2020), što potvrđuje kako oko 50% svih riba živi u rijekama i jezerima koji čine samo 0,01% ukupne vode na Zemlji (Horn, 1972). Još zanimljivije zvuči činjenica da prosječno jednu morsku vrstu riba nalazimo u otprilike 113.000 km^3 morske vode, a slatkovodnu u samo 15 km^3 slatke vode (Horn, 1972). Razlog navedene primijećene diskrepancije u broju slatkovodnih i morskih riba vjerojatno proizlazi iz činjenice kako su ekološke niše u slatkovodnim ekosustavima dugo bile ili djelomično jesu izolirane te su iznimno raznolike (Cohen, 1970). Međutim, uz sve poznato, evolucija i podrijetlo riba i dalje ostaju prilično neistraženo i kompleksno područje znanosti. Zasad je poznato kako najraniji fosili riba koštunjača (*Osteichthyes*) datiraju još iz kasnog silura (oko 420 milijuna godina, https://www.britannica.com/animal/fish; Pough i sur., 2009), zrakoperki iz devona (oko 400 milijuna godina, Pough i sur., 2009), dok se tijekom razdoblja krede (mezozoik; 144 – 66 milijuna godina) pojavljuju njihovi recentni oblici (Near i sur., 2013). Tijekom kenozoika dolazi do ubrzane evolucije i znatne raznolikosti zrakoperki (Near i sur., 2013), koja je vjerojatno bila omogućena znatnim promjenama i specijalizacijama koštanih dijelova lubanje i aparata za hranjenje (uključujući čeljusti), zajedno s razvojem pokretljivih i fleksibilnih peraja (Pough i sur., 2009). Zrakoperke se danas koriste u znanstvenim istraživanjima kao modelni organizmi u raznim ekološkim, etološkim, evolucijskim i biogeografskim studijama, a od neizmjerne su važnosti za ljude i kao važan izvor hrane i proteina širom svijeta od početka ljudske povijesti (Nelson, 2006). Zbog svoje uloge u ekosustavu i važnosti za ljude dio su internacionalnih sporazuma i dogovora, koriste se kao indikatori onečišćenja i narušenosti vodenih ekosustava, a imaju i izraženu rekreacijsku te ekonomsku ulogu (Nelson, 2006).

Glavoči (u užem smislu, pripadnici porodica Gobiidae i Gobionellidae, filogenija prema Thacker, 2009) predstavljaju jednu od najraznolikijih skupina riba unutar reda Gobiiformes (Thacker, 2009; Bentacur i sur., 2013), ali i jednu od najstarijih skupina zrakoperki budući da su se pojavili u eocenu (prije 51 – 55 milijuna godina; Near i sur., 2013; Thacker, 2014). Među ostalim, oni su i najbrojnija skupina riba općenito, s gotovo 1250 validnih vrsta opisanih do danas, iako njihov broj izrazito varira od autora do autora te se nove vrste svakodnevno opisuju (Berra, 2001; Nelson, 2006; Thacker, 2009; Near i sur, 2013; Fricke i sur., 2020). Široko su rasprostranjeni (**Slika 1**), a najčešće naseljavaju plitka morska staništa (infralitoral) gdje su najbrojnija skupina riba (Nelson, 2006), bočate estuarije ili lagune te slatkovodne rijeke, jezera i krške potoke u umjerenim, tropskim i suptropskim područjima, s posebnim naglaskom na tropski indopacifički pojas gdje su glavoči izrazito brojni (Berra, 2001; Nelson, 2006; Thacker, 2009, 2015). Zanimljivo je kako nekoliko vrsta naseljava naizgled nepovoljna vodena staništa kao što su hladna mora oko Norveške i Islanda te subpolarne potoke u Sibiru (Berra, 2001; Kovačić i Svensen, 2019). Na koraljnim grebenima glavoči su jedna od najbrojnijih skupina riba i čine oko 20% ukupne raznolikosti vrsta i 35% ukupnog broja riba (Winterbottom i sur., 2011).

Prema načinu života glavoči zauzimaju gotovo sve ekološke niše u vodenom mediju. Oni su većinom pridnene ili bentičke ribe koje su svojim navikama blisko vezane za plitko morsko ili slatkovodno dno (većina vrsta živi na dubinama od 2 do 10 metara) iako postoji i nekoliko pelagičkih oblika (npr. *Aphia minuta* (Risso, 1810), *Crystallogobius linearis* (Düben, 1845), *Pseudaphya ferreri* (Buen i Fage, 1908) (Kovačić i Patzner, 2011)), dok su neke i batofilne vrste, kao što je primjerice *Gobius roulei*, vrsta zabilježena na dubinama od oko 400 metara (Kovačić i Patzner, 2011). Neke vrste, kao što su glavoči linije *Periophthalmus* (rodovi *Boleophthalmus* Valenciennes, 1837, *Periophthalmus*, *Periophthalmodon* Bleeker, 1874 i *Scartelaos* Hamilton, 1822) prilagodile su se amfibiotskom načinu života, tj. izlaze iz vode u potrazi za hranom, kreću se uz pomoć prsnih peraja po plitkom muljevitom dnu te se nakon nekog vremena provedenog vani vraćaju u svoja muljevita vodena staništa (Nursall, 1981;

Berra, 2001; Nelson, 2006). Zanimljiv primjer su i slatkovodni glavoči roda *Clamydogobius* Whitley, 1930, koji naseljavaju arteške izvore i lokve u pustinjama središnje Australije te predstavljaju odvojenu slatkovodnu skupinu u odnosu na svoje morske pretke (Larson, 1995). Pojedine vrste žive u simbiotskom odnosu s drugim životinjama (npr. spužve, rakušci, ježinci; najpoznatiji primjer vrsta *Crytocentrus steinitzni* (Klausewitz, 1974) i dekapodni rakušac roda *Alpheus* Fabricius, 1798 (Preston, 1978; Karplus, 1979)), dok su vrste roda *Gobiosoma* Girard, 1858 „čistači“ te se hrane ektoparazitima drugih tropskih riba na koraljnim grebenima (Limbaugh, 1961; Losey, 1987; Nelson, 2006). Glavoče se većinom može naći na mekanom sedimentnom supstratu kao što su muljevita ili pjeskovita dna, dok znatan broj vrsta živi i na tvrdom supstratu (kamenje, stijene, antropogeni materijal) te u špiljama (Kovačić i Patzner, 2011). Također, neke vrste su kriptobentičke (tj. žive u pukotinama, šupljinama ili ispod raznog materijala, prema Miller, 1979, 1996), što otežava njihovo istraživanje *in situ* (Kovačić i Patzner, 2011). Fenotipski, glavoči su izrazito raznolika skupina, no sve ih karakterizira nekoliko zajedničkih morfoloških i bioloških osobina. Glavoči su male ribe čija prosječna duljina tijela rijetko prelazi više od 20 centimetara (cm) ukupne duljine (TL), dok većina vrsta doseže duljine od 5 do 10 cm (najmanji glavoč na svijetu je s Filipina *Pandaka pygmaea* Herre, 1927, čija ukupna duljina ne prelazi 1,3 cm, dok je najveći glavoč *Gobioides broussenetii* Lacepède, 1800 iz Karipskog mora, koji doseže veličine do 50 cm; Miller, 1986; Berra, 2001; Kovačić i Patzner, 2011). Većina glavoča nema plivaći mjehur (tj. hidrostatski organ za održavanje u vodenom stupcu povezan s probavnim sustavom) i kompletno razvijen sustav bočne pruge na tijelu (skupina osjetilnih tjelešaca koja registriraju pokrete i pritisak uz bočne strane tijela ribe), dok su navedene strukture prisutne kod većine današnjih riba (Berra, 2001; Nelson, 2006; Kottelat i Freyhof, 2007). Smatra se kako je nedostatak plivaćeg mjehura prilagodba na bentički način života, dok je bočna pruga djelomično reducirana i ograničena samo na glavu u obliku kanala i vanjskih nizova osjetilnih papila, koji čine važan skup dijagnostičkih morfoloških karakteristika za razlikovanje vrsta i rodova (Miller, 1986). Nadalje, gotovo svi glavoči imaju adhezivni (trbušni) disk nastao spajanjem trbušnih (*pterygia ventralia*) peraja, a smatra se kako im on, zbog podtlaka koji stvara, omogućuje olakšano održavanje u pridnenim dijelovima (Berra, 2001; Nelson, 2006). Tijekom reproduktivne sezone glavoči su izrazito teritorijalne ribe koje prezentiraju stereotipno ritualno udvaranje i intraseksualnu kompeticiju među mužjacima za ženku i gnijezdo (ljušture školjkaša, šupljine, pukotine, antropogeni materijal i dr.), odakle privlače partnerice na mrijest (Lindström i Hellström, 1993; Malavasi, 2003, 2009). Kod većine vrsta ženka polaže jaja na gornju površinu gnijezda (tzv. speleofilna reproduktivna taktika), a mužjak ga priprema i čuva od drugih potencijalnih udvarača te se brine o mladima (roditeljska briga) dok se ne izlegnu iz jaja (Tavolga, 1956; Torricelli i sur., 1985; Lindström i Hellström, 1993).

Pokazalo se kako se današnji žarišni centar raznolikosti (eng. *hot-spot*) glavoča nalazi u Indo-australskom području (plitka tropska područja, tj. otočne skupine koje se pružaju u smjeru juga od Filipina, preko Indonezije do Nove Gvineje) iako je nekoliko skupina prisutno u Mediteranskom moru, Pacifičkom oceanu (istočni dio), Karipskom moru te u Atlantskom oceanu (istočno i zapadni dio) (Thacker, 2015). Fosilni nalazi pokazuju kako su se pripadnici podreda Gobioidei (glavoči u širem smislu, porodice Odontobutidae, Ryachthydidiae, Eleotridide, Butidae, Gobionellidae i Gobiidae, prema Thacker, 2009) razvili u slatkim, bočatim i rubnim morskim staništima tijekom kenozoika, s nekoliko primjeraka fosila poznatih iz eocena, oligocena i miocena u Europi (Gierl i sur., 2013; Prikryl, 2014). U mediteranskom području glavoči (Gobiidae i Gobionellidae) su najbrojnija skupina riba s oko 60 zabilježenih vrsta (Kovačić, 2020), dok u crnomorskom području obitavaju 33 vrste, a u sjeveroistočnom (SI) dijelu Atlantskog oceana 47 vrsta (za sve reference, vidi Kovačić i Patzner, 2011). Filogenija i sistematika glavoča i dalje ostaje djelomično nerazriješena, uz prisutnost mnogih fenotipski (morfološki) izrazito sličnih vrsta (od kojih neke još uvijek čekaju determinaciju i znanstveni opis) koje dodatno otežavaju ionako detaljne i kompleksne analize (Kovačić i Patzner, 2011). Poznato je kako glavoči u širem mediteranskom području (Mediteransko i Crno more te SI Atlantik) naseljavaju najraznolikija morska staništa, od plitkih obalnih ekosustava do velikih dubina preko 300 m (Miller, 1986). Od ukupnog broja, 27% vrsta naseljava vodeni stupac od površine do 2 m dubine, 31% naseljava pojas od 2 do 10 m, a samo 13% vrsta živi na dubinama većim od 50 m (Kovačić i Patzner, 2011). Većinom su bentičke ribe blisko vezane uz dno, pri čemu 60% vrsta preferira mekana dna (pijesak, mulj, sitni šljunak) u odnosu na tvrdi supstrat ili špilje (Kovačić i Patzner, 2011). Jedna je od najmanjih vrsta navedenog područja *Speleogobius trigloides* Zander i Jelinek, 1976 (do 2.26 cm TL), a jedna od najvećih *Gobius cobitis* Pallas, 1814 (do 27 cm TL), pri čemu samo 34% vrsta naraste iznad 10 cm TL (Miller, 1986; Kovačić i Patzner, 2011). Po brojnosti i ukupnoj raznolikosti vrsta sjeverni dio Mediteranskog mora (zajedno s Jadranskim morem; broj vrsta iznosi između 46 i 43 vrste) i Levantsko područje (istočni Mediteran s 29 do 37 vrsta) razlikuju se od središnjeg i zapadnog Mediterana (do 12 vrsta), dok se Crno more izdvaja zbog specifične zajednice i sastava vrsta u odnosu na druga područja (33 vrste). U SI Atlantiku živi manji broj vrsta u odnosu na Atlantski obalni dio (Kovačić i Patzner, 2011). Kovačić i Patzner (2011) navode kako je upravo sjeverni Mediteran najbrojniji vrstama i mediteranskim endemima, a 2/3 (14 vrsta) svih mediteranskih endema egzistira u njegovim sjeverozapadnim dijelovima i Egejskom, Jonskom i Jadranskom moru s naglaskom na Jadran koji s 22 mediteranske endemske (morske) vrste dominira u odnosu na ostala područja. Zapažena brojnost i bogatstvo vrsta u Mediteranu posljedica je najvjerojatnije geoloških, hidroloških i klimatskih događaja na tom području, na što dodatno upućuje i broj endemskih vrsta (samo šest u SI Atlantiku nasuprot 24 u Mediteranu). Pretpostavlja se kako su današnje mediteranske endemske vrste vjerojatno evoluirale od svojih predaka iz Atlantskog oceana koji su nakon mesinske krize saliniteta (MSC) za vrijeme miocena (presušivanje Mediteranskog mora u razdoblju 5,9 − 5,3 milijuna godina; Hsü i sur., 1977; Rögl, 1998; Krijgsman i sur., 1999; Quinard i Tomasini, 2000) mogle migrirati kroz Gibraltarski tjesnac te proći u Mediteransko more, koje je poslužilo kao refugij toplovodnim vrstama tijekom glacijalnih razdoblja (Briggs, 1974; Almada i sur., 2001). Među ostalim, temperaturne fluktuacije tijekom glacijalnih i interglacijalnih razdoblja u pleistocenu (2,5 − 0,011 milijuna godina) mogle su utjecati na diversifikaciju i evoluciju tadašnje faune riba mediteranskog područja (uključujući i glavoča) zahvaljujući ponovnoj invaziji i rekolonizaciji Mediterana atlantskim vrstama (Almada i sur., 2001). U odnosu na morske vrste, mediteransko područje (uključujući Crno more i Kaspijsko jezero) naseljava 50 vrsta slatkovodnih glavoča zabilježenih u rijekama, potocima, jezerima i izvorima, sa slijevovima Jadranskog i Egejskog mora (od sjeverne Italije, južno preko Grčke do zapadne Anatolije) kao dominantnim žarišnim centrima njihove raznolikosti (Miller, 2003, 2004; Šanda i Kovačić, 2009; Freyhof, 2011). Zbog svoje raznolikosti i širokog areala pojedini autori (Miller, 1986, 2003, 2004; Smirnov, 1986) smatraju kako u europskim vodotocima i širem mediteranskom području (uključujući SI Atlantik, Mediteransko more i Crno more) postoje dvije skupine glavoča s alopatrijskom (diskontinuiranom) distribucijom: atlantsko-mediteranska (SI Atlantik i Mediteransko more) i pontokaspijska (Crno, Aralsko i Azovsko more i Kaspijsko jezero s pritocima). Atlantsko-mediteranska skupina predstavlja skupinu rodova (za ovu disertaciju najbitniji *Gobius* Linnaeus, 1758 i *Zosterisessor* Whitley, 1935, koji se danas smatra mlađim sinonimom roda *Gobius*), čiji su filogenetski odnosi i sistematski položaj kompleksniji i zamršeniji u odnosu na pontokaspijske vrste. Pontokaspijske vrste su monofiletska skupina glavoča sastavljena od vrsta rodova *Babka* Iljin, 1927, *Benthophiloides* Beling i Iljin, 1927, *Benthophilus Eichwald, 1831, Caspiosoma* Iljin, 1927, *Mesogobius* Bleeker, 1874, *Neogobius* Iljin, 1927, *Ponticola* Iljin, 1927, *Proterorhinus* Smitt, 1900 (Miller, 2003, 2004; Neilson i Stepien, 2009a; Medvedev, 2013), od kojih su neke vrste alohtone i invazivne u europskim vodotocima i sjevernoj Americi (npr. *Neogobius melanostomus* (Pallas, 1814), *N. fluviatilis* (Pallas, 1814), *Ponticola kessleri* (Günther, 1861) (Charlebois i sur., 1997; Stepien i Tumeo, 2006; Neilson i Stepien, 2009a)). One su, zajedno s mediteranskim *Padogobius* Berg, 1932 rodom, pleizomorfne s eurihalinim mediteranskim rodom *Zosterisessor* u redukciji ili gubitku slobodnih šipčica prsnih peraja (Miller, 2004), dok, kao i *P. nigricans* (Canestrini, 1867), imaju iznimno povećan broj kralješaka (29 − 35) i reduciran plivaći mjehur u odnosu na mediteranske rodove (Miller, 2003, 2004).



Slika 1. Rasprostranjenost glavoča u slatkovodnim (crno) i morskim (sivo) ekosustavima diljem svijeta (preuzeto iz Berra, 2001). Napomena: autor uključuje sve glavoče u užem smislu unutar porodice Gobiidae (Gobiiformes; Gobioidei), a oni su se poslije modernim molekularnim analizama razdvojili na dvije zasebne porodice (Gobiidae i Gobionellidae prema Thacker, 2009, 2015).

1.2. *Pregled najvažnijih filogenetskih istraživanja europskih glavoča linije Gobius (Gobiiformes, Gobiidae)*

Iako su iznimno brojna skupina riba, glavoči se često izostavljaju iz evolucijskih i biogeografskih studija (Bellwood i Wainwright, 2002; Cowman, 2014), ponajprije zbog iznimne, ali i kompleksne raznolikosti (fenotipska, morfološka, ekološka, etološka, itd.), zamršene taksonomije (pogotovo bliskih vrsta, od kojih neke još uvijek čekaju na svoj opis), male veličine tijele te skrovite naravi, tj. kriptobentički način života (Thacker, 2011, 2015). Razvojem novih molekularnih metoda, u odnosu na tradicionalne morfološke i morfometrijske analize, kompleksna slagalica povezana s evolucijom mnogih vrsta i njihovim filogenetskim odnosima počela se djelomično sastavljati (Thacker, 2009). Donedavno su glavoči smatrani podredom unutar grgečki (Gobioidei; Perciformes), te su zajedno s podredovima Percoidei i Labroidei činili 3/4 ukupne brojnosti svih opisanih grgečki (Berra, 2001; Nelson, 2006). U ovoj disertaciji pratit će se filogenetski odnosi i taksonomski položaj glavoča koje predlažu Thacker (2009) i Betancur-R i sur. (2017), prema kojima se glavoči izdvajaju od grgečki, a red Gobiiformes postaje dio subdivizije Percomorphaceae (serija Gobiaria, Betancur-R i sur., 2017) unutar kojega se prepoznaje podred Gobioidei sa šest porodica: Rhyacichthyidae, Odontobutidae, Butidae, Eleotridae, Gobionellidae i Gobiidae (Thacker, 2009). Također, Agorreta i sur. (2013) grupiraju sve glavoče unutar jedne porodice Gobiidae, ali prepoznaju unutar nje nekoliko nezavisnih evolucijskih linija, od kojih je za ovu disertaciju najvažnija linija *Gobius*. Treba imati na umu kako se, prema Thackeru (2009), glavoči dijele na dvije porodice (Gobiidae i Gobionellidae), dok Agorreta i sur. (2013) prepoznaju samo jednu porodicu (Gobiidae) s nekoliko potporodica, a za nas su najvažnije Gobiinae i Gobionellinae. Kao što je već navedeno, u ovoj disertaciji slijedit će se sistematika porodica koju predlaže Thacker (2009), dok će se nazivi linija preuzeti od Agorreta i sur. (2013).

Jedno od prvih interspecijskih istraživanja filogenetskih odnosa glavoča mediteranskog područja objavili su Penzo i sur. (1998). Cilj je tog istraživanja bio analizirati sekvence 12S i 16 S rRNA mitohondrijskih gena za trinaest vrsta glavoča (rodovi *Gobius, Padogobius, Pomatoschistus* Gill, 1863, *Knipowitschia* Iljin, 1927, *Zebrus* de Buen, 1930, *Zosterisessor*) kako bi se istražili odnosi među pojedinim vrstama te kako bi se razriješilo pitanje kada se prvi put javlja preferencija na slatkovodno stanište kod proučavanih vrsta (tri su vrste u analizi bile isključivo slatkovodne), uzimajući u obzir geološki kontekst šireg mediteranskog područja. Prijašnja su istraživanja navodila kako se preferencija na slatkovodna staništa razvila nekoliko puta nezavisno, upućujući na polifiletske odnose među talijanskim glavočima (Miller i sur., 1994). Metodama najveće parsimonije (MP) i metodom genetske udaljenosti (NJ), rezultati istraživanja pokazali su kako su se istraživane vrste grupirale u dvije grupe (eng. *clade*) na filogenetskom stablu: prva obuhvaćajući vrste iz rodova *Knipowitschia* i *Pomatoschistus*, a druga grupirajući sve ostale vrste (rodovi *Gobius, Padogobius, Zebrus, Zosterisessor*). U prvoj su se grupi oba roda *Knipowitschia* i *Pomatoschistus* pokazala parafiletskima, kao i rod *Gobius* iz druge grupe jer su se sve morske vrste s preferencijom na muljevito/pjeskovito stanište (*Gobius niger* Linnaeus, 1758, *G. auratus* Risso, 1810, *G.*

bucchichi Steindachner, 1870, i *Zosterisessor ophiocephalus* (Pallas, 1814)) grupirale zajedno. Također, u drugoj grupi rod *Padogobius* pokazao se parafiletskim zato što se *P. nigricans* grupirao kao sestrinska vrsta s *G. paganellus* Linnaeus, 1758, dok je *P. bonelli* (Bonaparte, 1846) prikazan bazalnom vrstom u odnosu na prijašnje dvije. *Zebrus zebrus* (Risso, 1827) sestrinska je vrsta u odnosu na *G. paganellus* + *Padogobius* spp. grupu (Penzo i sur., 1998). Dobiveni rezultati pokazali su kako se preferencija na slatkovodno stanište talijanskih glavoča (*Orsinigobius punctatissimus* (Canestrini, 1864), *Padogobius nigricans* i *P. bonelli*) razvila nekoliko puta (polifiletsko podrijetlo) iako je odnos između *P. nigricans* i *G. paganellus* ostao nerazriješen, odnosno iako morfološki slični, *P. nigricans* i *P. bonelli* pokazali su se kao nesestrinske vrste (Penzo i sur., 1998). Ti su rezultati pokazali kako je preferencija na slatku vodu najvjerojatnije posljedica jednog od dvaju scenarija: preferencija se javlja nekoliko puta nezavisno kod vrsta roda *Padogobius* ili samo jednom, kod pretka grupe *G. paganellus* + *Padogobius* spp., nakon čega se sekundarno gubi kod *G. paganellus* koji je eurihalina/morska vrsta (Penzo i sur., 1998). Stoga je bliska filogenetska srodnost *Z. ophiocephalus* s drugim vrstama roda *Gobius*, zajedno s navedenim slučajem *G. paganellus* + *Padogobius* spp., pokazala kako je rod *Gobius* najvjerojatnije parafiletskog podrijetla (McKay i Miller, 1991; Penzo i sur., 1998). Autori su zaključili kako je inicijalno razdvajanje dviju grupa (glavoči pijeska i *Gobius*, + *Padogobius* + *Zosterisessor*) vjerojatno rezultat odvajanja Parateisa (Pontokaspijsko područje) od mediteranskog oceana Tetisa prije 10 −12 milijuna godina (Steininger i Rögl, 1984). Prilagodba istraživanih glavoča na slatkovodno stanište rezultat je vjerojatno mesinske krize saliniteta u Mediteranu (prije 5,5 milijuna godina) i razdoblja poznatog kao „Lago Mare“ (Hsü i sur., 1977) ili zapadnog širenja azijske ihtiofaune kroz Panonski kanal prije 24 milijuna godina u miocenu (Banarescu, 1992).

Radi rješavanja filogenetskih odnosa i sistematskog položaja vrsta iz podreda Gobioidei, Thacker (2009) provodi detaljno molekularno istraživanje i analizu DNK sekvenci četiri mitohondrijska biljega (COI, ND1, ND2 i cyt *b*) izoliranih iz jedinki glavoča reda Gobiiformes, a u analizu uključuje podatke o nekoliko njihovih fenotipskih i ekoloških karakteristika. Rezultati tog istraživanja pružili su dublji uvid u zamršenu sistematiku glavoča, naglašavajući kako bi se redu Gobiiformes trebala vratiti taksonomska jednakost u odnosu na Perciformes (tj. glavoče odvojiti od grgečki u zasebnu taksonomsku cjelinu, tj. red) te kako unutar navedenog reda postoje tri odvojene grane (podreda), od kojih monofiletski podred Gobioidei obuhvaća šest porodica (Odontobutidae, Rhyacichthyidae, Eleotridae, Butidae, Gobiidae i Gobionellidae) u odnosu na prije predloženih devet (Miller, 1973; Springer, 1983;

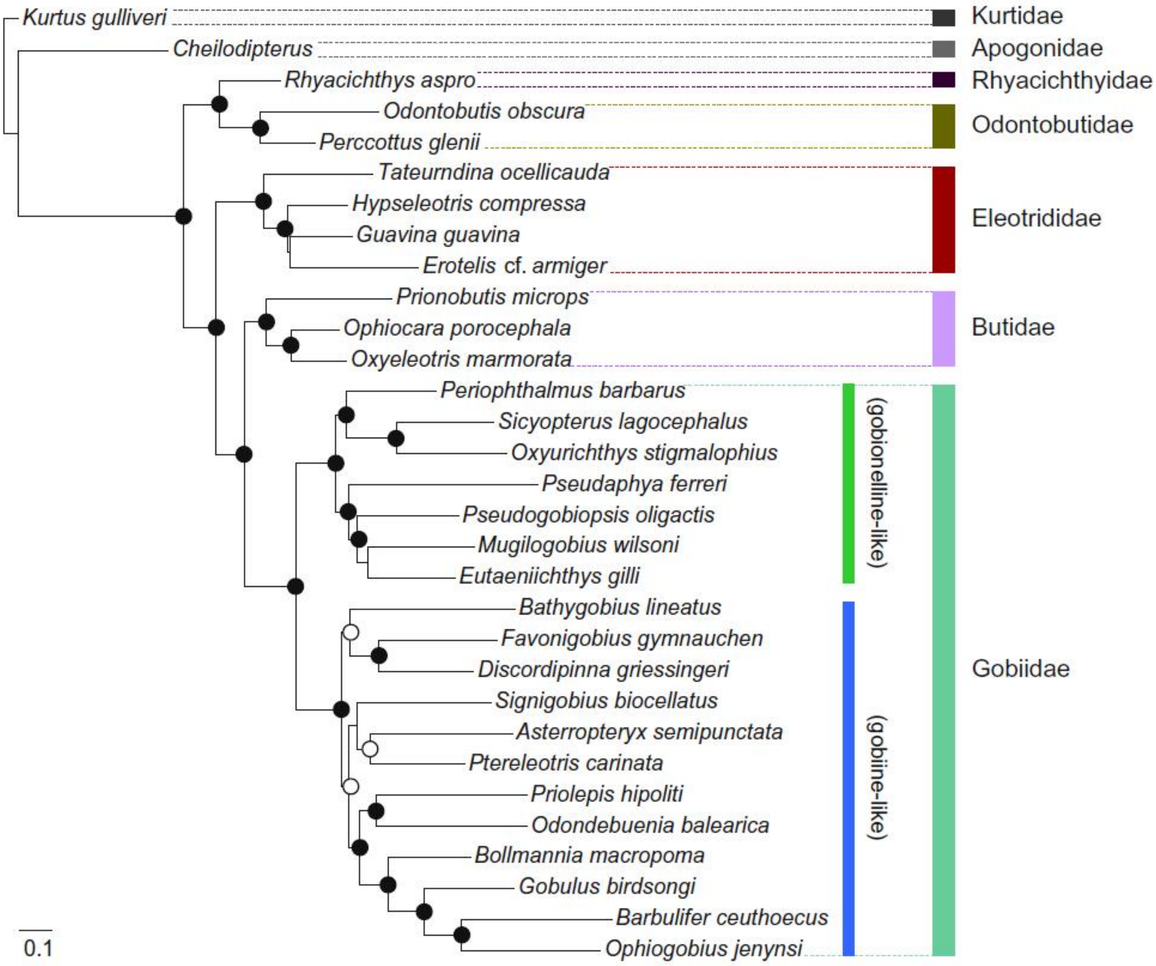
Hoese, 1984; Hoese i Gill, 1993; Johnson i Brothers, 1993; Thacker, 2000). Provedeno je istraživanje doprinijelo sveukupnom znanju o filogenetskim odnosima pojedinih skupina glavoča te simplificiralo zamršenu sistematiku grupirajući neke manje skupine (potporodice) u više taksonomske cjeline (porodice). Uz to, rezultati su pokazali kako su dvije najbrojnije i najraznolikije skupine unutar Gobioidei upravo Gobiidae i Gobionellidae (glavoči u užem smislu), od kojih brojnija Gobiidae obuhvaća većinom morske vrste, a Gobionellidae slatkovodne i bočate taksone (Thacker, 2009).

Neilson i Stepien (2009a) istražili su interspecijske filogenetske odnose i sistematski položaj pontokaspijskih vrsta (skupina Benthophilinae Iljin 1927) koristeći analizu DNK sekvenci za dva mitohondrijska (cyt *b* i COI) i dva jezgrena (RAG1 i S7) gena uz pomoć metoda filogenetske rekonstrukcije: metoda najveće parsimonije (MP), metoda najveće vjerodostojnosti (ML) i Bayesova metoda (BI). Dodatni su ciljevi istraživanja bili istražiti monofiletsko podrijetlo pontokaspijskih vrsta, objasniti ekološku prirodu njihova invazivnog karaktera te uklopiti hipotezu o njihovoj evoluciji u geološke događaje i paleoklimatske promjene navedenog područja (Aralsko, Azovsko i Crno more te Kaspijsko jezero s pritocima, prema Reid i Orlova, 2002). Istodobno, istraživanje je provedeno kako bi se pontokaspijski glavoči, prije svrstani u jedinstveni rod *Neogobius* sensu Berg (1949), razdvojili, prateći taksonomske norme, u odgovarajuće sistematske kategorije (rodove i vrste). Kombinirani filogenetski rezultati, temeljeni na četiri gena, pokazali su kako su pontokaspijski glavoči zaista monofiletska skupina unutar porodice Gobiidae, unutar kojih se razdvajaju tri skupine visoke podržanosti (>85%): Neogobiini (vrste roda *Neogobius*), Benthophilini (rodovi *Benthophilus* i *Caspiosoma*) te Ponticolini (*Proterorhinus*, *Mesogobius*, *Babka* i *Ponticola*). Na filogenetskom stablu skupina Neogobiini pokazala se sestrinskom grupom drugoj grupi sastavljenoj od vrsta grupe Benthophilini i Ponticolini (95% ML bootstrap). Također, detaljna je analiza divergencije (na cyt *b* koristeći ML) pokazala kako su se pontokaspijski glavoči odvojili od ostatka Gobiidae prije oko 39 milijuna godina, s naglaskom na tome kako se posljednji zajednički predak vrsta rodova *Neogobius*, *Babka*, *Proterorhinus* i *Ponticola* razdvojio prije oko 10 milijuna godina kada su se razdvojili Pontokaspijski i Panonski bazeni (Reid i Orlova, 2002; Neilson i Stepien, 2009a). Razdvajanje Crnog mora od Kaspijskog jezera (prije 5,8 – 5 milijuna godina) u isto vrijeme kao i mesinska kriza saliniteta u Paleomediteranu i crnomorskom slijevu, dovelo je do razdvajanja vrsta unutar skupine Neogobiini. Rezultati molekularne analize navode kako su se nakon 5,0 milijuna godina sve skupine Benthophilinae najvjerojatnije razdvojile, nakon čega je počela interspecijska evolucija pojedinih rodova, i morskih i slatkovodnih (Reid i Orlova, 2002; Neilson i Stepien, 2009a).

Thacker i Roje (2011) analizirali su, kao dopunu prijašnjem istraživanju (Thacker, 2009), izolirani set DNK sekvenci za pet gena (tri mtDNA – CO1, ND1 i ND2 i dva nDNA – RAG2 i Rho) za 100 vrsta iz porodice Gobiidae (potporodice Gobiinae, Microdesmidae, Ptereleotridae, Kraemeriidae i Schindleriidae) radi dobivanja boljeg uvida u njihove filogenetske odnose. Provedeno istraživanje uključilo je u molekularne analize genski materijal gotovo svih poznatih grupa Gobiidae, uzimajući u obzir i američke, pontokaspijske, atlanstko-mediteranske vrste. Rezultati provedenog istraživanja pokazali su kako unutar porodice Gobiidae postoje tri monofiletske grupe (eng. *clades*) i trinaest linija (eng. *lineages*), čiji su međusobni odnosi na filogenetskom stablu slabo poduprti, ali je zato unutar linija poduprtost grananja iznosila 80% (*boostrap*) ili više (Thacker i Roje, 2011). Zanimljivo je kako su se unutar treće i ujedno najveće linije grupirali rodovi Novog svijeta i Indonezije, ali i atlantski, pontokaspijski i mediteranski rodovi (Thacker i Roje, 2011). Detaljniji je uvid u filogenetske odnose otkrio kako na filogenetskom stablu pontokaspijski glavoči čine sestrinsku grupu s atlantsko-mediteranskim vrstama (rod *Gobius* i *Zosterisessor*) s čvorom visoke podržanosti (80%), dok afrički rodovi *Coryogalops* Smith, 1958 i *Caffrogobius* Smitt, 1900 čine sestrinsku grupu s već navedenom skupinom (Thacker i Roje, 2011). Autori naglašavaju kako navedena grupa (pontokaspijske i atlantsko-mediteranske vrste) čini monofiletsku skupinu, a rezultati molekularnog istraživanja koje su proveli Neilson i Stepien (2009a) uputili su na sličan scenarij, s odnosima među pojedinim pontokaspijskim vrstama malo drugačijim u odnosu na Thacker i Roje (2011) (unutar skupine Benthophilinae Neogobiini čini bazalnu skupinu, dok su Ponticolini i Benthophilini sestrinske skupine). Ti su rezultati objašnjeni u svjetlu geološke prošlosti i događaja šireg mediteranskog područja te naglašavaju kako su mesinska kriza saliniteta (tj. isušivanje mediteranskog područja i formiranje slanih i slatkih jezera diljem mediteranskog i pontokaspijskog područja prije 5,9 – 5,3 milijuna godina) i naknadan dotok morske vode kroz Gibraltarski tjesnac (prije 5,3 milijuna godina) imali znatan utjecaj na diversifikaciju vrsta navedenog područja (Penzo i sur., 1998; Simonović, 1999; Neilson i Stepien, 2009a).

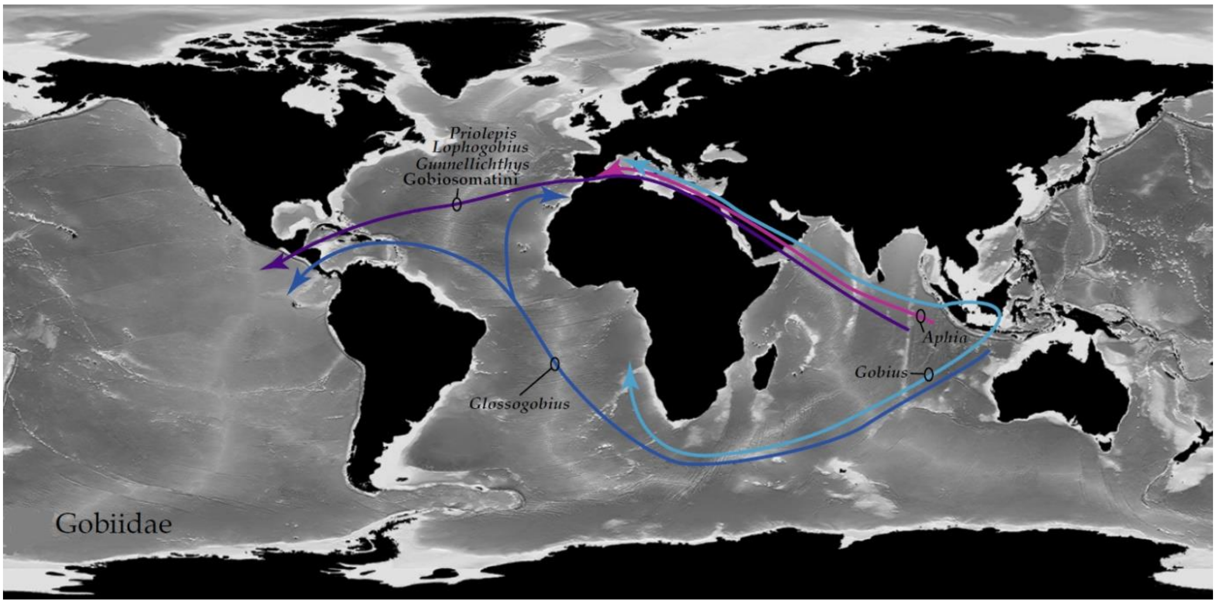
Nadalje, Agorreta i sur. (2013) analizirali su sekvence pet genetičkih biljega (dva mtDNA – 12S/16S i cyt *b* - i tri nDNA – zic1, sreb2, RAG1) od 222 vrste podreda Gobioidei (glavoči u širem smislu) te pokušali detaljnije rekonstruirati njihove filogenetske odnose koristeći ML i BI metode, s posebnim naglaskom na europske vrste i filogenetske odnose među njima.

Slično kao i Thacker (2009), rezultati molekularnog istraživanja Agorreta i sur. (2013) pokazali su kako je podred Gobioidei monofiletskog podrijetla te kako stabla upućuju na slične topologije, tj. filogenetski najbazalnije porodice među Gobioidei su upravo Rhyacichthyidae i Odontobutidae koje na filogenetskom stablu predstavljaju sestrinsku grupu za sve ostale porodice unutar Gobioidei (**Slika 2**). Naknadno se na filogenetskom stablu nakon Rhyacichthyidae i Odontobutidae odvojila porodica Eleotridae, a nakon nje Butidae koja se pokazala sestrinskom grupom porodici Gobiidae, unutar koje su autori prepoznali dvije visokopodržane podgrupe/potporodice (Gobiinae i Gobionellinae, Agorreta i sur., 2013). Uz to, rezultati navedenog istraživanja detaljnije su istražili filogenetske odnose među pojedinim europskim skupinama glavoča (36 rodova) koji se, s obzirom na molekularne analize temeljene na pet gena, grupiraju u tri odvojena evolucijska klastera: liniju *Pomatoschistus,* liniju *Aphia* Risso, 1827, i liniju *Gobius* (prva je dio podgrupe Gobionellinae, dok su zadnje dvije dio potporodice Gobiinae, Agorrera i sur., 2013). Većina se europskih glavoča, zajedno s afričkim skupinama (*Sufflogobius* Smith, 1956, *Gorogobius* Miller, 1978, *Corcyrogobius* Miller, 1972, *Chromogobius* Buen, 1930 i dr.), grupirala u liniju *Gobius*, što potvrđuje kako nekoliko istočno atlantskih tropskih rodova dijeli bliske filogenetske odnose s europskim vrstama, ali i da afrički glavoči prema Thacker i Roje (2011) ne predstavljaju monofiletsku skupinu (Agorreta i sur., 2013). Kao i u prijašnjim analizama (Neilson i Stepien, 2009a; Thacker i Roje, 2011, Tornabene i sur., 2013), pontokaspijski glavoči pokazali su se, na temelju provedenih analiza, monofiletskom skupinom blisko povezanom s europskim rodovima *Gobius* i *Zosterisessor* (Agorreta i sur., 2013). Također, rezultati provedenog istraživanja pokazali su kako je linija *Gobius*, uklanjanjem enigmatičnog taksona *Kraemeria* Steindachner, 1906 iz analiza, blisko povezana s rodom *Priolepis* Valenciennes, 1837 rodom (Pacifički ocean) i skupinom Gobiosomatini (Karipsko more) (Agorreta i sur., 2013).



Slika 2. Filogenetsko stablo (filogram) vrsta unutar podreda Gobioidei rekonstruirano na temelju pet različitih genetičkih biljega (dva mitohondrijska i tri jezgrena) metodom najveće vjerodostojnosti (ML). Na stablu se odvaja pet porodica unutar podreda Gobioidei (Odontobutidae, Rhyacichthyidae, Eleotridae, Butidae, Gobiidae), dok su glavoči u užem smislu podijeljeni na dvije potporodice unutar Gobiidae (Gobiinae i Gobionellinae). Ispunjeni čvorovi na podržanost ML (BP > 70%), dok neispunjeni čvorovi na podržanost ML (BP < 70%). Skala predstavlja supstitucije po mjestu na grani stabla. Preuzeto iz Agorreta i sur., 2013.

Biogegrafske karakteristike te migracijske rute glavoča tijekom kenozoika (Gobiiformes, Gobioidei) istražene su u Thacker (2015) filogenetskim istraživanjem mitohondrijskih ND1, ND2, COI i niklearnih RNF, Rho, RAG2 gena, za ukupno 83 glavoča u užem smislu (Gobiidae i Gobionellidae) s *Oxyeleotris lineolata* (Steindachner, 1867) (Butidae) kao vanjskom grupom (eng. *outgroup*), radi temporalnog (vremenskog) kalibriranja pojedinih grupa i određivanja njihova ancestralnog areala. Također, u istraživanju su molekularnim podacima integrirani i podaci o recentnoj distribuciji i fosilnim nalazima glavoča kako bi se rekonstruirali povijesni pravci i rute invazije pojedinih grupa i rodova glavoča. Prema literaturi, tijekom kenozoika postojalo je nekoliko vrućih točaka povezanih s uzajamnom rapidnom diversifikacijom i radijacijom koralja i glavoča (zapadni Tetis – Mediteran, zapadni Indijski ocean, Indopacifik) (Wood, 1999; Bellwood i Wainwright, 2002; Renema i sur., 2008). Dobiveni rezultati molekularnog istraživanja Gobiidae i Gobionellidae, s topologijama na filogenetskom stablu i odnosima među pojedinim grupama, nisu znatno odstupali od prijašnjih istraživanja (Thacker i Roje, 2011; Thacker, 2013, 2014; Agorreta i sur., 2013), a filogenetsko je istraživanje pokazalo kako je linija *Gobius* (mediteranski i pontokaspijski glavoči) blisko povezana (sestrinski položaj) s američkom skupinom Gobiosomatini. Vremenski kalibrirana filogenija pokazala je kako su glavoči porodica Gobiidae i Gobionellidae najvjerojatnije evoluirali u razdoblju od oko 12 – 13 milijuna godina tijekom eocena, od otprilike 48,7 pa do 36,2 milijuna godina, s ancestralnin arealom za Gobiidae + Gobionellidae smještenim u Indopacifiku (gdje su i danas najbrojnija skupina), a ne u Mediteranu ili zapadnom Indijskom oceanu (Thacker, 2015). Prema rezultatima istraživanja, smatra se kako je za skupine unutar Gobionellidae evolucijsko podrijetlo vezano za Mediteransko more, dok je linija *Pomatoschistus* do danas zadržala takvu rasprostranjenost. Za skupine Gobiidae, ancestralnim arealom predložen je Indopacifik, dok je linija *Gobius* najvjerojatnije evoluirala na području zapadnog Indijskog oceana (Thacker, 2015; **Slika 3**). Nakon evolucije u Indopacifiku glavoči su najvjerojatnije rapidno migrirali te naselili istočni Tihi ocean, Atlantski ocean te Mediteransko more; skupina Gobiidae stigla je do Atlantskog preko zapadnog Tethys oceana, gdje je naknadno izumrla, dok se skupina Gobionellidae nije proširila iz oceana Tetisa dalje od SI Atlantika (Thacker, 2015).



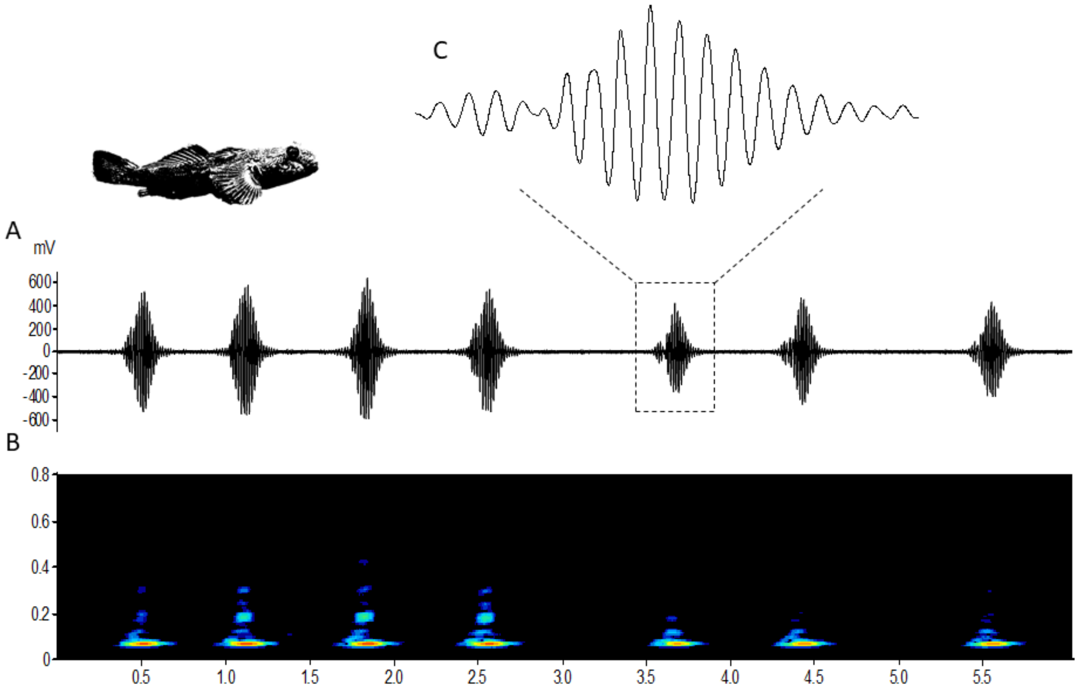
Slika 3. Geografska karta koja prikazuje globalne migracijske rute linija (eng. *lineages*) unutar porodice Gobiidae, s ancestralnim mjestom nastanka smještenim u Indopacifiku. Linija *Gobius* naselila je područja izvan Indopacifika (Mediteransko more, sjeveroistočni i jugoistočni Atlantski ocean, zapadni Indijski ocean) iako geološka i hidrološka karta globalnoga kontinentalnog područja u kenozoiku nije izgledala kao što izgleda danas (Indijski ocean i Mediteransko more bili su spojeni, kao i zapadni Atlantski i istočni Tihi oceani). Preuzeto iz Thacker, 2015.

1.3. *Bioakustika, znanost o zvukovima u prirodi*

Komunikacijski signal je bilo kakva promjena/signal jedinke odašiljača koja izaziva odgovor u ponašanju (tj. etološkom kontekstu) jedinke primatelja (Otovic i Partan, 2009). Većina životinja komunicira koristeći istovremeno nekoliko različitih signala (kemijskih, zvučnih, vizualnih itd.) pomoću različitih senzornih kanala, a taj je proces poznat pod nazivom „multimodalna“ ili „multisenzorna komunikacija“ (Otovic i Partan, 2009). Mnoge životinje, uključujući sisavce, ptice, žabe, ali i kukce, ribe i paukove, stvaraju zvukove (tj. akustičke signale) kako bi prenijele važne informacije ili usmjerile svoje ponašanje, a modulacijom svojih akustičkih parametara reguliraju sadržaj, tj. skup informacija koje taj zvuk prenosi (Simmons, 2003; Ladich, 2015; Suthers i sur., 2016; Ladich i Winkler, 2017). Zvukovi, kao sredstvo komunikacije, prenose ključne biološke informacije za jedinku, kao što su lokacija (hrane, partnera, predatora, objekta u okolišu), identitet (vrste, jedinke ili spola), socijalni status (dominantnost ili podređenost), motivacija (strah, bijes, spremnost za parenje) i dr. (Simmons, 2003). Ukratko, zvuk je fizički poremećaj u nekom mediju (vodi ili zraku) nastao kao rezultat pomicanja molekula zbog mehaničke aktivnosti (Simmons, 2003).

Najjednostavnije objašnjeno, zvuk se može vizualizirati kao niz periodičkih i sinusoidnih promjena tlaka vode ili zraka tijekom vremena (**Slika 4**). Zbog fizičkog poremećaja uzorkovanog npr. glasnicama u grkljanu ili bubnjem, molekule u mediju se zbijaju (kondenzacija) ili šire (rarefrakcija) oko svojega ravnotežnog položaja (eng. *equilibrium*), šireći se u smjeru longitudinalnih valova (Beeman, 1998; Simmons, 2003). U blizini izvora zvuka molekule u zraku/vodi fizički se pomiču od svog ravnotežnog položaja u obliku gibanja čestica (eng. *particle motion*), što se naziva komunikacija u blizini polja, dok se one čestice (kondenzacije i rarefrakcije) koje se šire daleko od mehaničkog izvora zvuka šire kroz medij u obliku valova pritiska/tlaka (eng. *pressure waves*) te predstavljaju komunikaciju u daljini polja (na udaljenostima od izvora zvuka većim od 1 − 3 valne duljine, komunikacija u daljini polja dominira u odnosu na komunikaciju u blizini polja; Simmons, 2003). Navedene razlike imaju važno biološko značenje budući da neke životinje (pogotovo kralješnjaci) komuniciraju na velikim udaljenostima (šišmiši, kitovi, i dr.), pri čemu se koriste valovi pritiska koji se dalje šire kroz medij, a mogu ih detektirati organi za sluh (uši), dok kod riba i žaba slušni organi detektiraju oba tipa širenja čestica (Simmons, 2003; Ladich, 2014; Ladich i Schulz-Mirbach, 2016; Ladich i Winkler, 2017). Neki su od najbitnijih parametara zvuka (tzv. akustičke varijable), važnih za životinjsku komunikaciju i prijenos informacija: frekvencija (u Hercima, Hz), amplituda (dB), faza, period (milisekunda), valna duljina (cm) i trajanje (sekunda). Odnosi među pojedinim parametrima imaju ključnu biološku važnost za životinje budući da još uvijek nije poznato npr. u kojem su odnosu frekvencija i period, tj. kako životinje percipiraju zvuk: u obliku broja ciklusa (kondenzacije i rarefrakcije) u određenom periodu (frekvencija) ili prema trajanju pojedinog ciklusa (period) (Simmons, 2003). Nadalje, ne reagiraju sve životinje na isti spektar frekvencija. Primjerice, ljudi registriraju zvukove frekvencije od 20 do 20.000 Hz (zvuk frekvencije ispod 20 Hz naziva se infrazvuk, a iznad 20.000 Hz ultrazvuk), slonovi registriraju zvukove ispod 20 Hz, dok sluh šišmiša i kitova registrira zvukove frekvencije i do 150.000 Hz koji se koristi kao sonar za eholokaciju (Simmons, 2003, Ladich i Winkler, 2017). Nadalje, valna duljina (λ), kao jedna od važnih prostornih parametara zvuka koja se odnosi na udaljenost u prostoru između dva susjedna brijega zvuka, razlikuje se za zvukove stvorene u zraku (gdje je brzina širenja zvuka zbog gustoće medija 343 m s^{-1}) u odnosu na one stvorene u vodi (brzina zvuka iznosi 1.500 m s^{-1}, uz minimalno prigušenje s udaljenosti), zbog čega je logično da će akvatičke životinje (kitovi i perajari) koristiti upravo zvukove manjih frekvencija (ali i dulje valne duljine) u odnosu na šišmiše (Simmons, 2003; Ladich i Winkler, 2017). Au i Hastings (2008) navode kako je upravo zvuk, zbog svoje sposobnosti dalekog raspršivanja i prelaska velikih udaljenosti, kao i male apsorpcije, idealan tip komunikacijskog signala u vodenim ekosustavima. Navedene razlike u valnoj duljini imaju znatan utjecaj na sluh akvatičkih životinja i na lokalizaciju zvuka, ponajprije zbog odnosa valne duljine i razlike u udaljenosti slušnih organa (tj. ušiju), zbog čega neke životinje ne mogu registrirati zvukove velikih valnih duljina (Simmons, 2003).

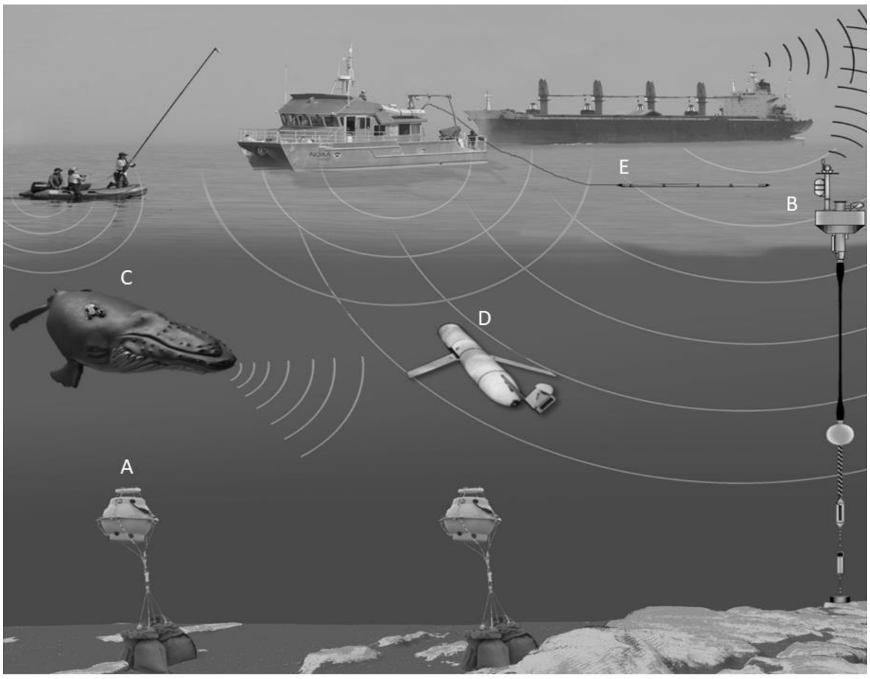
Bioakustika (od grč. *bios* – život; *akoustikos* – slušno, čujno) je interdisciplinarna grana znanosti koja povezuje mnoge druge znanstvene discipline (zoologiju, animalnu fiziologiju, etologiju, akustiku – fiziku, i dr.), a proučava životinjske zvukove (uključujući i ljudske), tj. njihovo stvaranje, širenje (disperziju) i primanje (recepciju), utjecaj okoliša na akustičku komunikaciju, njegovu funkcionalnu povezanost s akustičkim parametrima životinjskih zvukova te, u novije vrijeme, utjecaj antropogene buke na fiziologiju i komunikaciju životinja (http://www-3.unipv.it/cibra/edu_underwaterbioacoustics_uk.html; Amorim i sur., 2018; De Jong i sur., 2018). Bioakustika proučava i fiziološke procese povezane sa stvaranjem i primanjem zvuka te istražuje kako funkcioniraju organi za primanje zvukova kod raznih životinja (većinom unutarnje uho, ali i kosti, čeljusti, otoliti itd. (Ladich i Schulz-Mirbach, 2016)). Razvoj bioakustike kao znanstvene discipline blisko je povezan s razvojem moderne tehnologije, pogotovo one za dokumentaciju zvukova (Beeman, 1998). Važna je prekretnica u tehnologiji (te ujedno i u bioakustici) postignuta oko 1950-ih godina, kada su razvijeni uređaji za snimanje, vizualizaciju i mjerenje akustičkih signala (mikrofon, hidrofon, osciloskop i spektrograf), dok je rapidni napredak u znanosti postignut razvojem digitalnih računala i naprednih matematičkih programa za modeliranje, prikaz i automatsku analizu zvukova (Beeman, 1998). Podvodna akustika je podgrana znanstvene discipline akustike koja proučava i istražuje širenje zvukova pod vodom, a razvila se isključivo zahvaljujući sonaru koji omogućuje detekciju objekata pod vodom i procjene dubine vode radi lakše navigacije (http://www-3.unipv.it/cibra/edu_underwaterbioacoustics_uk.html). Iako se većina tehnologije povezane s pasivnim sonarima i alatima za podvodno snimanje (npr. hidrofon) razvila zahvaljujući vojsci, u novije se vrijeme pasivni sonari koriste za detekciju i praćenje životinjskih zvukova, ponajprije onih koje stvaraju kitovi, ali i ribe, radi procjene njihove biomase te mjerenja globalne prosječne temperature i njenih fluktuacija u okolišu (http://www-3.unipv.it/cibra/edu_underwaterbioacoustics_uk.html; Urick, 1983; Au, 1993; Richardson i sur., 1995). U novije vrijeme razlikujemo dva tipa podvodne akustike: a.) aktivnu, koja koristi snop zvučnih valova stvorenih pomoću električnih pretvarača (eng. *beamers*) i primjenjuje akustičke metode raspršenja (eng. *scattering methods*) na organizmima radi monitoringa i vizualizacije riba i ribljih populacija na određenim dubinama i b.) pasivnu, koja se temelji na hidrofonu te tehnikama snimanja i slušanja zvukova riba (i drugih vokalnih akvatičkih životinja) radi dobivanja detaljnih informacija o njihovoj distribuciji i ponašanju (Mann i sur., 2008). Kao osnovna pretpostavka pasivne akustike navodi se sposobnost ribe da producira zvuk te je kao takva podosta ograničena na one vrste koje se aktivno (ili pasivno) glasaju, ali i na lokaciju i vrijeme kada se one glasaju u svome okolišu i staništu (Mann i sur., 2008). Aktivna akustika znatno je revolucionalizirala komercijalni ribolov tako da je omogućila korištenje sonara za ehosondiranje (sustav u kojem jedna sonda generira zvukove, dok jedan prijamnik ili više njih „sluša“, tj. hvata jeku koja nastaje odbijanjem od površina) kako bi se lokalizirale razne vrste riba (Sund, 1935; Balls, 1948). Dvije su osnovne mjere koje primjena ehosondara omogućuje povratni radarski signal jedinki koje su sondirane (tj. njihova gustoća iz koje se izračunava i brojnost) te volumen povratnog radarskog signala (Mann i sur., 2008). Pasivna akustika istražuje zvukove riba i njihov etološki kontekst koji je većinom povezan s agresivnim interakcijama i reprodukcijom (udvaranje i parenje) radi dobivanja informacija kada se odvija mrijest pojedinih vrsta te koliko je jedinki prisutno na određenom području (Mann i sur., 2008). Dok je u aktivnoj podvodnoj akustici sonar najbitniji tehnološki dio opreme, u pasivnoj je akustici hidrofon već desetljećima dio standardne aparature za dokumentaciju zvukova i prostorno-temporalnog položaja vokalnih riba (Mann i sur., 2008). Dok su sonari postali široko primjenjiv dio opreme za procjenu brojnosti riba, pasivna akustika, tj. hidrofoni, to dosad još uvijek nije postigla. Razlog je vjerojatno taj što sustav za snimanje nije niti kompaktan niti komercijalno dostupan te što se podaci dobiveni pasivnom akustikom ne mogu primijeniti u „stručne svrhe“ (procjena brojnosti riba, količina jaja i fekunditet ženki itd.) koje bi koristili agencije i konzervacijske tvrtke te zbog velike količina danas neizbježne antropogene buke koja otežava posao bilježenja i interpretacije podataka (Mann i sur., 2008). Pasivna akustika, kao primjenjiva i relativno jeftina metoda, danas se koristi širom svijeta u laboratorijske svrhe, u sklopu kojih stručnjaci pokušavaju dokumentirati i opisati zvukove riba u kontroliranim (laboratorijskim) uvjetima, istražiti etološki kontekst produkcije zvukova i fiziološke odgovore jedinki na akustičku produkciju. Kao što je već navedeno, osnovni je dio opreme u eksperimentima pasivne akustike hidrofon, koji pretvara zvukove ribe u električni impuls (napon, tj. razliku električnog potencijala u milivoltima) koji se poslije može obrađivati, analizirati te slušati u stvarnom vremenu (Mann i sur., 2008). Metode obrade zvuka podrazumijevaju pretvaranje analognog signala u digitalni te generiranje triju najvažnijih prikaza za opisivanje zvuka: spektrograma/sonograma (promjena frekvencije zvuka i temporalnih karakteristika tijekom trajanja zvuk), oscilograma (promjena trajanja zvuka u odnosu na amplitudu) i spektra frekvencije (promjena frekvencije zvuka u odnosu na amplitudu) (Mann i sur., 2008; Fine i Parmentier, 2015). Istodobno, sustav za pohranu podataka (većinom audiosnimke .wav formata) bitan je dio opreme koji može izravno biti povezan s hidrofonom ili bežičnom vezom ili, alternativno, putem prijenosnog računala (laptop s internom memorijom) koji omogućuje praćenje zvukova u stvarnom vremenu (Mann i Lobel, 1995). Negativne su strane pasivne akustike velike količine generiranog, sirovog materijala, tj. audiosnimki na kojima se ne nalaze ciljani zvukovi te nemogućnost povezivanja zvuka s videosnimkom kako bi se utvrdila vrsta životinje koja je proizvela zvuk (ako se hidrofoni postavljaju na morsko dno bez vizualnog nadzora kao što je to u laboratorijskim eksperimentima), tako da bi ubuduće napredne metode trebale razviti automatizirani sustav kojim bi se omogućilo automatsko identificiranje vrsta (i jedinki) životinja koje stvaraju zvuk i mjerenje njihovih akustičkih parametara (Mann i sur., 2008; Fine i Parmentier, 2015). Budući da većina riba stvara tihe zvukove (maksimalna zabilježena amplituda zvukova sjenki iz porodice Sciaenidae iznosi 160 dB re: 1 Pa na 1 m udaljenosti) niske frekvencije (do 2 kHz), njihov spektar preklapa se sa spektrom frekvencija okolne, često antropogene buke (brodovi, seizmička istraživanja), što dodatno otežava njihovo bilježenje (Amorim, 2006; Mann i sur., 2008; Ladich, 2014; Fine i Parmentier, 2015).



Slika 4. Primjer isječka audiosnimke (.wav format) na kojem se vidi niz tonalnih zvukova proizvedenih od mužjaka glavočića okrugljaka (*Neogobius melanostomus*). A. niz zvukove se

prikazuje u obliku oscilograma (vrijeme u sekundama vs. amplituda u miliVoltima), dok se na B. oni prikazuju spektrogramom ili sonogramom (frekvencija u kHz vs. vrijeme u sekundama). Jačina intenziteta boje upućuje na količinu akustičke energije prisutne u zvuku (oko 90 Hz), koja je prisutna do otprilike 500 Hz. C. prikazuje detaljnu strukturu tonalnog zvuka na kojem se vidi kako je zvuk sastavljen od periodičkih sinusoidalnih valova. Autor: Horvatić, S.

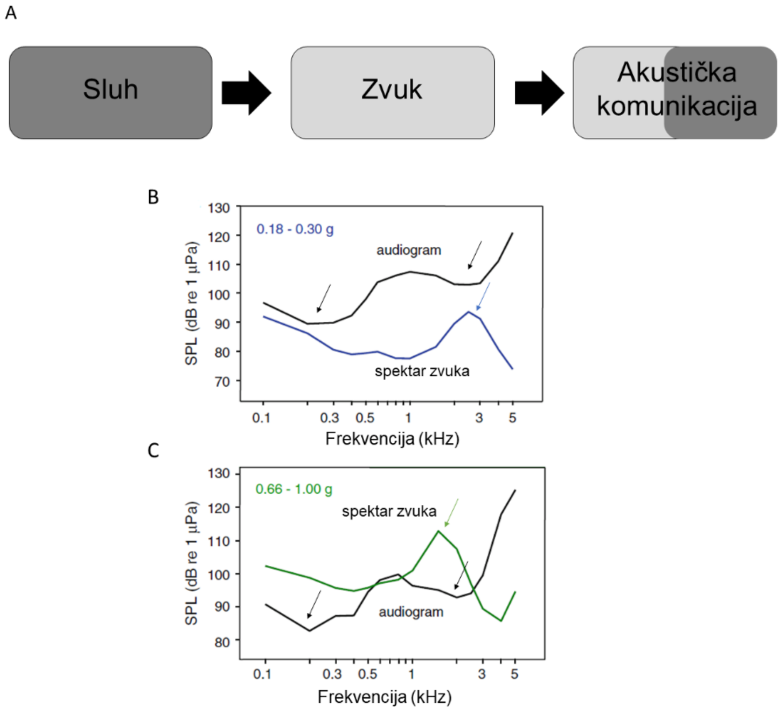
Kao izvedena grana iz podvodne akustike razvila se i podvodna bioakustika, interdisciplinarna grana koja istražuje podvodne zvukove i akustičke parametre životinja i njihovo akustičko ponašanje radi praćenja (monitoringa) te utjecaj okolišnih parametara (klima, morske struje, temperatura itd.) na produkciju i širenje zvukova, ali i razvoj novih tehnologija i instrumenata za njihovo podvodno širenje i praćenje (http://www-3.unipv.it/cibra/edu_underwaterbioacoustics_uk.html). Ubrzan razvoj i znanstvena popularnost podvodne bioakustike može se pripisati hidrofonu (podvodnom mikrofonu), sofisticiranoj napravi koja omogućuje snimanje, detekciju i pohranu životinjskih zvukova. Ukratko, hidrofon registrira nadolazeći zvučni val te ga pretvara u električni signal (zbog čega se može smatrati električnim pretvaračem), dok je većina današnjih hidrofona omnidirektnog smjera, odnosno mogu registrirati zvukove iz svih smjerova te registrirati širok spektar frekvencija (od nekoliko pa da 100 kHz) (http://www-3.unipv.it/cibra/edu_underwaterbioacoustics_uk.html). Današnja je primjena hidrofona raznolika, a većinom se koriste u eksperimentima pasivne akustike ili u obliku dipolarnog transekata tijekom uzorkovanja morskih staništa, u kojima se nekoliko hidrofona spaja u dugačak niz (sustav prijamnika) razdvojenih svega nekoliko metara, čime se može odrediti smjer i vremenski dolazak (s vremenskim zakašnjenjem) zvuka svakom pojedinačnom hidrofonu. Ako se povežu s uređajem za pohranu podataka velike veličine (ili u novije vrijeme imaju ugrađenu memorijsku karticu u svom hardveru) i automatsku analizu zvukova, hidrofoni služe kao iznimno prigodno autonomno sredstvo namijenjeno za pasivnu svrhu (npr. mogu se spustiti na morsko dno te određeno vrijeme skupljati i pohranjivati podatke o vokalnim aktivnostima životinja (http://www-3.unipv.it/cibra/edu_underwaterbioacoustics_uk.html; Luczkovich i sur., 2008; Mann i sur., 2008) (**Slika 5**).



Slika 5. Primjena raznih metoda podvodne akustike (aktivna i pasivna akustika) za detekciju, dokumentaciju i monitoring životinjskih zvukova u Atlantskom oceanu te procjenu utjecaja antropogenih zvukova i buke na akvatičke životinje. Zvukovi se bilježe pasivnom metodom pomoću autonomnih snimača (montiraju se na morsko dno ili u vodeni stupac; A i B) ili se registriraju aktivno, pomoću akustičkih oznaka (eng. *acoustic tags*; C), podvodnom jedrilicom (eng. *autonomous underwater glider*; D) i sustavom hidrofona povezanim u niz (eng. *array of hydrophones*; E). Preuzeto i prilagođeno s https://www.fisheries.noaa.gov/new-england-mid-atlantic/endangered-species-conservation/passive-acoustic-research-atlantic-ocean.

1.4. *Bioakustika riba*

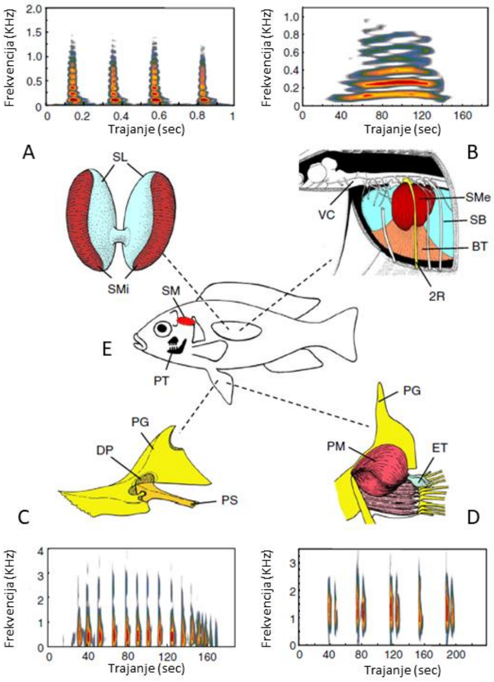
Bioakustika riba posebna je grana bioakustike (i podvodne akustike) koja istražuje produkciju i detekciju zvukova riba, funkcionalnu povezanost akustičke komunikacije i sluha (orijentacije) te evolucijske sile i selektivne pritiske koji utječu na razvoj navedenih struktura kod riba (Ladich, 2014). Iako je sasvim logično zaključiti kako su organi za stvaranje zvukova (sonički ili vokalni organi), koji su kod riba najraznolikiji u odnosu na sve kralješnjake, povezani s akustičkom komunikacijom, i dalje je nedovoljno poznato što to zapravo ribe slušaju osim zvukova srodnih i nesrodnih vrsta (Ladich, 2014, 2019; Parmentier, i Fine, 2016; Parmentier i sur., 2017; Fine i Parmentier, 2017). Također, rezultati mnogih istraživanja pokazuju kako su se sluh i komunikacija zvukovima kod riba razvili sasvim neovisno, na što upućuje velika raznolikost i broj različitih mehanizama za stvaranje zvukova (Ladich, 2015; Wysocki i Ladich, 2002). Taj se paradoks može objasniti sljedećim primjerom. Istražujući vokalni repertoar ribe *Trichopsis vittata* (Cuvier, 1831), Henglmüller i Ladich (1999) i Wysocki i Ladich (2001) otkrili su kako mlade jedinke *T. vittata* (do 0,3 g) stvaraju već nakon 87. dana razvoja zvukove nazvane „*prasak*“ zvukovima (eng. *bursts*) pomoću posebno građenih tetiva prsnih peraja koje pri produkciji zvukova vibriraju kao žice na gitari. Kada su usporedili audiogram (slušnu krivulju koja upućuje na područja najistančanijeg sluha) sa spektralnim vrijednostima frekvencije produciranih zvukova (akustička energija prisutna na pojedinim frekvencijama) mladih i odraslih jedinki (**Slika 6**), autori su uvidjeli kako se dvije krivulje u mlađim uzrasnim kategorijama ne preklapaju (do 0,3 g), dok je to slučaj kod odraslih riba (od 0,6 g), što ih je navelo na zaključak kako se mlade jedinke međusobno ne čuju te ne mogu komunicirati zvukovima (Wysocki i Ladich, 2001). Iz toga su Wysocki i Ladich (2001) zaključili kako se ontogenetski kod *T. vittata* (a vjerojatno i svih ostalih riba) najprije razvija sluh, a naknadno sposobnost za produkciju akustičkih signala, što im tek poslije omogućuje intraspecijsku razmjenu informacija pomoću zvukova, tj. akustičku komunikaciju (**Slika 6.**).



Slika 6. Razvoj akustičke komunikacije kod zrakoperke *Trichopsis vittata*. A. Shematski prikaz ontogenetskog razvoja akustičke komunikacije kod zrakoperke *T. vittata*. Inicijalno, kod malih se jedinki *T. vittata* razvija sluh na određene frekvencije zvuka (B. – crna krivulja), nakon čega se poslije javlja sposobnost jedinki za produkciju akustičkih signala (B. – plava krivulja). Tek se u kasnijim fazama ontogenetskog razvoja audiogram najveće osjetljivosti zvuka (C. – crna krivulja) poklapa sa

spektrom najveće frekvencije (i amplitude) zvukova (C. – zelena krivulja). Preuzeto iz Ladich, 2015. Crne strelice na audiogramu B. i C. prikazuju područje najboljeg sluha, a plava/zelena strelica označuje područje najveće energije zvuka pri određenim frekvencijama (spektar snage, DF). DF – dominantna frekvencija (kHz)

Iako je kod šaranki malo vrsta vokalno, tj. samo nekoliko vrsta stvara zvukove, one imaju napredne strukture za poboljšavanje sluha (npr. Webberov uređaj), zbog čega se smatraju specijalistima. S druge strane, glavoči nemaju dodatne slušne strukture, a gotovo su sve istraživane vrste vokalne (Ladich, 2014). Sposobnost recepcije zvukova kod svih kralješnjaka temeljena je na osjetnim stanicama s osjetnim dlačicama u unutarnjem uhu iako su se dodatne slušne strukture namijenjene primanju zvukova te mehanizmi za njihovu produkciju najvjerojatnije razvili nezavisno kod danas opisanih riba (Ladich i Popper, 2004; Ladich, 2000, 2017). Kod riba nalazimo najraznolikije mehanizme za stvaranje zvukova (zbog čega se često nazivaju kukcima među kralješnjacima) te, za razliku od ostalih kopnenih kralješnjaka gdje se mehanizam temelji isključivo na vibraciji membrana (npr. glasnica u grkljanu ili pjevalu), kod riba ne postoji jedinstveni primarni organ za stvaranje zvukova (Ladich i Fine, 2006; Ladich, 2014; Parmentier i Fine, 2016). Međutim, akustička komunikacija istražena je samo kod riba zrakoperki, dok je za paklare, morske pse i dvodihalice podosta toga nepoznato, uz tek nekoliko istraživanja provedenih do danas, zbog čega se smatra kako je akustička komunikacija kod riba i tretrapodnih kralješnjaka rezultat konvergentne evolucije (Thompson, 1968; Ladich i Fine, 2006; Bass i Chagnaud, 2012; Christensen i sur., 2015). Jedan su od prvih popisa akustički aktivnih riba i njihovih zvukova objavili, za potrebe američke mornarice, Fish i Mowbray (1970), a rezultati su pokazali kako većina riba stvara specifične zvukove ne uzimajući u obzir ekološki kontekst (položaj ribe i interakcije s drugim jedinkama) pri produkciji zvukova. Uz to, samo je nekoliko istraživanja pokušalo rekonstruirati filogenetske odnose između vokalnih vrsta riba (Pomacentridae, Gobiidae, Batrachoididae) koristeći samo akustičke parametre u analizama (Malavasi i sur., 2008; Parmentier i sur., 2009; Rice i Bass, 2009), a rezultati tih istraživanja pokazali su kako neke vrste imaju specifične zvukove koji prenose filogenetski signal (tj. skup specifičnih informacija koji otkrivaju filogenetsku pripadnost pojedine vrste) zaslužan za njihovu diferencijaciju od ostalih, često genetski srodnih vrsta. Nasuprot tome, kod nekih skupina, kao što su ribe klaunovi (Amphiprioninae, Pomacentridae), zvukovi ne odvajaju srodne vrste, najvjerojatnije zbog selektivnog utjecaja veličine tijela i konteksta produkcije zvukova (samo teritorijalni) na akustičke, ponajprije spektralne, parametre, zbog čega se maskira njihov filogenetski signal (Colleye i Parmentier, 2012). Ukratko, nekoliko je glavnih tipova mehanizama za stvaranje zvukova kod riba iako ih je, zbog svoje iznimne raznolikosti, teško striktno definirati (**Slika 7**): 1.) sonički („bubnjajući“) mišići povezani s plivaćim mjehurom (direktni – unutarnji mišići koji se povezuju sa stjenkom plivaćeg mjehura (**Slika 7A**); indirektni – vanjski mišići vezani za kost te povezani s plivaćim mjehurom; mišići koji dovode do vibracije mjehura bez funkcionalne veze s njegovom stijenkom, već su povezani tetivama ili koštanim pločama **Slika 7B**); 2.) prsne peraje i oplećje (stridulacijski zvukovi stvaraju se pri prelasku bodlje prsne peraje preko oplećja kao kod npr. somovki, a mišići imaju dodatnu funkciju u plivanju), **Slika 7C**; kod vrsta roda *Trichopsis* dvije tetive vibriraju tijekom pomicanja peraja, **Slika 7D**; kod peševa cijelo oplećje vibrira kontrakcijama posebnih mišića pri produkciji zvukova, **Slika 7E**); 3.) mehanizmi koji ne pripadaju nijednoj od tih dviju grupa (ždrijelni zubi (**Slika 7E**) i ostale strukture). Zvukovi riba stvoreni navedenim mehanizmima sastoje se od pulseva (ili niza pulseva), čija akustička energija ovisi o tipu mehanizma koji ih je generirao: zvukovi stvoreni plivaćim mjehurom imaju harmonijsku strukturu, a njihova akustička energija na sonogramu ne prelazi više od 500 Hz, s vrijednostima fundamentalne frekvencije (oko 200 Hz) jednake rapidnim kontrakcijama mišića plivaćeg mjehura; bubnjajući (eng. *drumming*) zvukovi koji traju od nekoliko milisekundi do nekoliko minuta kao kod vrste *Portichthys notatus*; stridulatorni zvukovi stvoreni prsnim mehanizmom (peraje i oplećje) čija akustička energija na sonogramu prelazi 1 kHz; niskofrekvencijski zvukovi (< 200 Hz) stvoreni ždrijelnim zubima ili vibracijama oplećja; tonalni zvukovi stvoreni rapidnim i kontinuiranim serijama mišićnih kontrakcija (fundamentalna frekvencija i stopa mišićnih kontrakcija se preklapaju), s vrijednostima energije oko 200 – 300 Hz (za sve reference, pogledati Ladich, 2014). Anatomski, svi kralješnjaci, uključujući i ribe, imaju organe za recepciju zvukova, ali nema svaka vrsta i organe za njihovo stvaranje. Morfološki, organi za sluh, tj. recepciju, mogu se podijeliti na isključivo unutarnje uho i dodatne periferne organe za primanje zvukova ili poboljšavanje sluha povezane s unutarnjim uhom (Ladich, 2014). Unutarnje uho, kao zajednički organ svih kralješnjaka, sastoji se od tri polukružna kanalića (osim kod sljepulja i paklara gdje se sastoji od dva kanalića) i tri vrećice, od kojih svaka posjeduje karbonatni otolit (slušni kamenčić) prislonjen na niz trepetljikinih stanica. Budući da su ribe jednake gustoće kao okolni medij, zvučni val prolazi kroz njih i one se kreću u istom smjeru kao nadolazeći val. Jedina teža struktura koja zaostaje za tim pokretima i dovodi do nagibanja trepetljika jest otolit (grč. ὠτο-, ōto- uho; λῐ́θος, líthos, kamen), zbog čega se stvara razlika u njihovu pomaku u odnosu na zvučni val, što se poslije u mozgu registrira kao niskofrekvencijski zvuk (Hawkins, 1993; Popper, 2011; Ladich, 2014). Kod pojedinih riba postoji funkcionalna (direktna ili indirektna) veza unutarnjeg uha sa strukturom ispunjenom plinom (plivaći mjehur), što im omogućuje da detektiraju promjene u akustičkom tlaku (od nekoliko Hz do nekoliko kHz) koje se u obliku vibracija plivaćeg mjehura, kod nekih preko koštanih struktura (Webberov aparat), prenose do unutarnjeg uha (Fay, 1988; Ladich, 2004), dok neke ribe, kao pripadnici skupine Anabantoidei, imaju suprabranhialnu komoru (Hawkins, 1993). Odgovor na pitanje zašto su se mehanizmi za stvaranje zvukova razvili kod većine bentičkih, ali ne i kod svih pelagičkih skupina riba nalazi se najvjerojatnije u njihovim životnim navikama; da bi riba bila vokalna i stvarala zvukove, ona mora braniti limitirani resurs (npr. teritorij, hranu itd.) od potencijalnih napadača te privlačiti partnere (većinom ženke) u nastambu ili gnijezdo (pogotovo kod speleofilnih ili litofilnih vrsta), zbog čega im zvukovi služe kao sredstvo zastrašivanja u slučaju agresivnih interakcija ili omogućuju veći reproduktivni uspjeh, tj. fitnes za vrijeme reproduktivnih interakcija (Ladich i Myrberg, 2006; Ladich, 2014). Kod nekih manjih porodica riba (Doradidae, Bagridae, Pimelodidae, Batrachoididae, Gadidae, Sciaenidae, Holocentridae, Pomacentridae i Carapidae) sve su vrste, ili gotovo sve, vokalne, dok je kod velikih skupina, kao što su šaranke (Cyprinidae), većina vrsta nijema s nekoliko izuzetaka (Johnston i Johnson, 2000; Holt i Johnston, 2014; Fine i Parmentier, 2015).



Slika 7. Raznolikost mehanizama za stvaranje zvukova kod riba i spektrogrami/sonogrami zvukova stvorenih tim mehanizmima. A. unutarnji mišići (SMi) koji se povezuju sa stijenkom plivaćeg mjehura (SL) kod *Halobatrachus didactylus*; B. vanjski mišići (SMe) koji su vezani na drugo rebro (2R) te na tetivu (BT) na ventralnoj strani plivaćeg mjehura kod piranje *Serrasalmus rhombeus*; C. stridulacijski mehanizam kod somića kod kojeg se dorzalni izdanak (DP) prsne bodlje (PS) tare po oplećju (PG); D. poboljšane tetive prsne peraje (ETs) koje vibriraju kao žice gitare kod *Trichopsis vittata*; E. mehanizam ždrijelnih zuba (PT) kod češljoustki, sunčanica i dr. te mehanizam temeljen na vibracijama oplećja pomoću soničkih mišića (SM) kod peševa. SB – plivaći mjehur, VC – kralješnica, BT – tetiva, PM – prsni mišić. Preuzeto iz Ladich (2014).

1.5. *Pregled najvažnije literature o bioakustici glavoča*

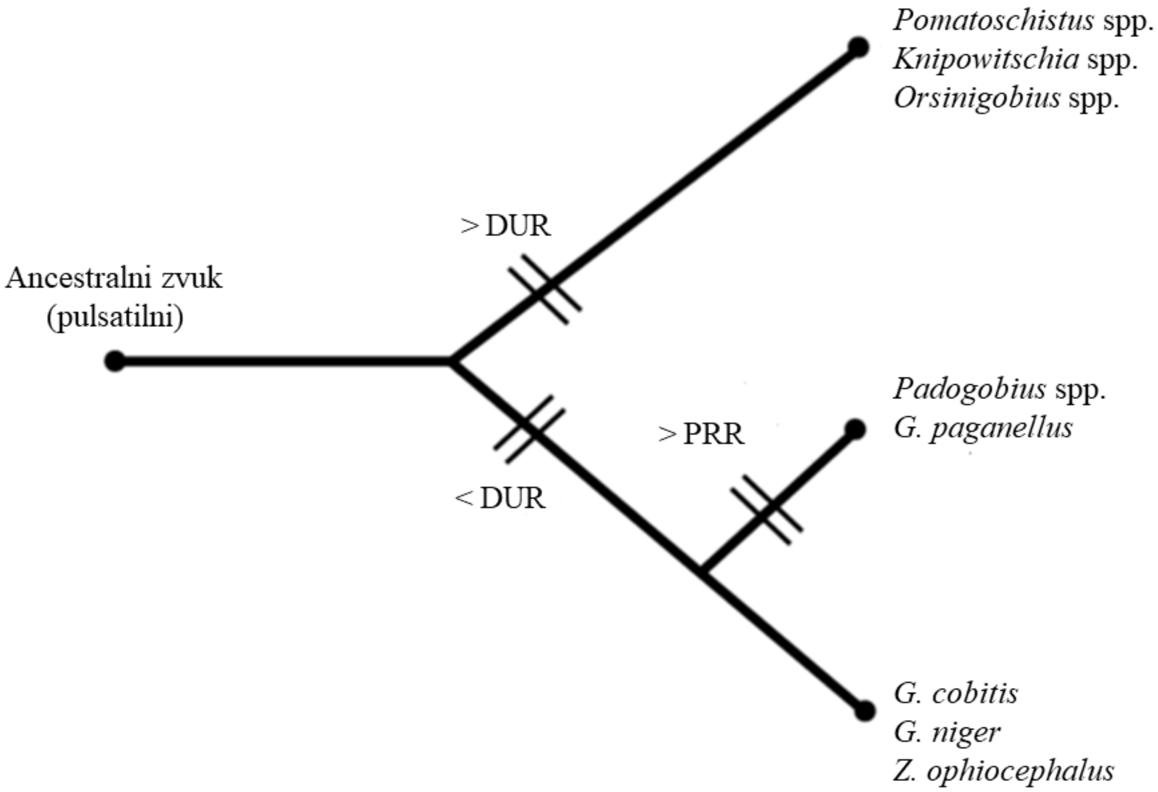
Glavoči (Gobiidae i Gobionellidae) su jedna od najistraživanijih skupina vokalnih riba (zajedno s Pomacentridae, Batrachoidiidae, Gadidiae, Serrasalmidae) iako je zanimljivo kako nemaju nikakve specijalizirane mehanizme ili strukture za akustičku komunikaciju i primanje zvukova (npr. neke vrste nemaju čak ni plivaći mjehur (Ladich, 2014; Zeyl i sur., 2016)). Plivaći mjehur predložen je kao osnovni element mehanizma za produkciju zvukova kod mnogih skupina riba (Ladich i Fine, 2006), dok se kod glavoča pokazalo kako vrste koje imaju (*Padogobius bonelli, Pomatoschistus minutus* (Pallas, 1770), *Gobius cruentatus* Gmelin, 1789) ili nemaju (*Padogobius nigricans, Neogobius melanostomus*) navedenu strukturu i dalje imaju sposobnost vokalizacije (Zeyl i sur., 2016). Do danas su 24 vrste (oko 1,2% ukupne brojnosti, Fricke i sur., 2020) istražene u bioakustičkim eksperimentima, sa samo jednom vrstom (*Economidichthys pygmaeus* (Holly, 1929)) koja se nije glasala tijekom istraživanja (Gkenas i sur., 2010; Zeyl i sur., 2016). Zajedno s drugim vokalnim skupinama riba (Cottidae i Percidae) glavoči dijele konvergentne životne navike (bentički način života i speleofilni tip reprodukcije), zbog čega su jako dobri modelni organizmi u eksperimentima pasivne akustike, evolucijske biologije ili akustike općenito (Zeyl i sur., 2016). Oni stvaraju niskofrekvencijske zvukove (oko 200 – 300 Hz) tijekom agresivnih ili reproduktivnih interakcija, dok su od posebne važnosti akustički signali stvoreni tijekom parenja, za koje se smatra kako povećavaju reproduktivni uspjeh (tj. fitnes) mužjaka zbog utjecaja seksualne selekcije na akustička svojstva (Zeyl i sur., 2016). Eksperimenti povezani s anatomskom strukturom glavoča pokazali su kako plivaći mjehur najvjerojatnije nije uključen u proces produkcije zvukova (Lugli i sur., 2003; Parmentier i sur., 2013), a zbog morfoloških sličnosti kranio-pektoralnih mišića (onih vezanih za lubanju i prsne peraje) glavoča i peševa (Parmentier i sur., 2013; Colleye i sur., 2013), zajedno s dokumentiranim pokretima glave (klimanje) i prsnih peraja tijekom produkcije zvukova, smatra se kako se mehanizam za stvaranje zvukova temelji na kontrakcijama navedenih mišića. Naime, kako bi poboljšali transmisiju niskofrekvencijskih zvukova kroz akustički ograničeni okoliš (plitka voda ponaša se kao filtar za zvukove niskih frekvencija (Rogers i Cox, 1988; Mann, 2006)), glavoči iskorištavaju dva ekološka faktora: njihovi niskofrekvencijski zvukovi gotovo savršeno odgovaraju „tihom prozoru“ u frekvencijskom spektru okolišne buke (oko 150 – 300 Hz, Lugli i sur., 2003; Lugli, 2010) te amplificiraju frekvencije zvukova nastambama u kojima se razmnožavaju (Lugli, 2012, 2013, 2014). Zvukovi glavoča mogu se podijeliti s obzirom na vrijeme kada ih stvaraju, npr. tijekom agresivnih interakcija (mužjak – mužjak ili mužjak – ženka), za vrijeme privlačenja ženke koja je izvan nastambe (eng. *courtship*) i tijekom parenja u nastambi (eng. *pre-spawning* i *spawning* Lugli i sur., 1997; Lugli i Torricelli, 1999; Johnston i Johnson, 2000; Myrberg i Lugli, 2006). Prethodno spomenuti zvukovi ne šire se daleko u plitkim (obalnim) vodama, zbog čega se ne koriste za dalekometnu komunikaciju (Zeyl i sur., 2016). Od ukupnog broja istraživanih glavoča, njih 16 stvara isključivo pulsatilne zvukove u agresivnim ili reproduktivnim interakcijama, dok je kod ostalih vrsta akustička struktura zvukova tonalnog karaktera (udvaranje) ili kao dio kompleksnog zvuka (Lugli i sur., 1997). Kod malog broja vrsta zvukovi znaju biti popraćeni tupim zvukovima (eng. *thump sounds*) stvorenim tijekom udvaranja izvan nastambe kada mužjaci prilaze ženki (Amorim i Neves, 2007; Malavasi i sur., 2009). Većina vokalnih glavoča pripada porodicama Gobiidae i Gobionellidae (filogenija prema Thacker, 2009; Thacker i Roje, 2011), dok je jedina vokalna vrsta izvan navedenih skupina *Odontobutis obscura* (Temminck i Schlegel, 1845) (Odontobutidae) za koju su dokumentirani isključivo pulsatilni zvukovi (Takemura, 1984). Takvi rezultati, kao i veća učestalost pulsatilnih zvukova u odnosu na tonalne, naveli su neke autore na zaključak kako se sposobnost za produkciju zvukova kod glavoča u širem smislu (podred Gobioidei) javlja filogenetski dosta rano i kako su tonalni zvukovi odvedeno svojstvo budući da je porodica Odontobutidae sestrinska skupina svim drugim porodicama podreda Gobioidei (uključujući Gobiidae i Gobionellidae; Thacker, 2009; Agorreta i sur., 2013; Zeyl i sur., 2016). Slijedeći navedenu akustičku hipotezu, tonalni zvukovi mogli su potencijalno nastati od pulsatilnih tako da je u zvuku nestao period između dva pulsa, zbog čega se oni ponavljaju velikom brzinom, a zvukovi kratko traju te se moduliraju u frekvenciji (Malavasi i sur., 2008).

Jedno su od prvih detaljnih istraživanja vokalnog repertoara glavoča mediteranskog područja proveli Torricelli i sur. (1990). Autori su snimili zvukove te kvantitativnom i kvalitativnom analizom opisali strukturu akustičkih signala teritorijalnih mužjaka slatkovodnog glavočića (*Padogobius bonelli*) te dodatno istražili kakav utjecaj okolišni (temperatura vode) i individualni faktori imaju na zvukove i njihova svojstva, pod hipotezom da kod ektotermnih organizama imaju znatan utjecaj na fiziologiju. *Padogobius bonelli* slatkovodna je vrsta autohtona za sjevernu Italiju (porječje rijeke Po), Švicarsku, Sloveniju i Hrvatsku, gdje naseljava male potoke s kamenim/šljunkovitim supstratom, a razmnožava se u kasno proljeće kada ženka polaže jaja na krov nastambe, a mužjak pokazuje paternalnu brigu za mlade (Miller, 2004). Zvukovi mužjaka *P. bonelli* bili su dokumentirani tijekom agresivnih (mužjak – mužjak) i reproduktivnih (udvaranje) interakcija, a kvalitativna je analiza pokazala kako se oni sastoje od rapidno produciranih pulseva, sa stopom ponavljanja koja se smanjuje kako zvuk odmiče kraju (Torricelli i sur., 1990). Nadalje, rezultati istraživanja pokazali su kako temperatura vode ima znatan utjecaj na pojedine akustičke parametre zvuka (stopu ponavljanja pulseva, modulaciju i trajanje) s posebnim naglaskom na stopu ponavljanja pulseva (prosječno oko 300 Hz) koja je negativno korelirana, dok je trajanje pozitivno korelirano s temperaturom. Veličina tijela (samim time i veličina mišića za stvaranje zvukova) nije bila znatno povezana s akustičkim parametrima, dok su agresivni zvukovi trajali dulje i imali manje vrijednosti stope ponavljanja pulseva (i fundamentalne frekvencije) od reproduktivnih zvukova, koji su se pokazali manje varijabilnima od agresivnih signala (Torricelli i sur., 1990). Autori zaključuju kako su male varijabilnosti reproduktivnih zvukova najvjerojatnije posljedica velike stereotipnosti signala uključenih u proces reprodukcije (Torricelli i sur., 1986; Torricelli i sur., 1990).

Lugli i sur. (1996) proveli su prvo komparativno akustičko istraživanje radi dokumentiranja i opisa akustičkih svojstava i zvukova triju talijanskih slatkovodnih glavoča (*Padogobius bonelli*, *P. nigricans* i *O. punctatissimus*) produciranih tijekom reproduktivnih intraspecijskih interakcija (udvaranje i parenje). Istovremeno, u sklopu laboratorijskih i *in situ* eksperimenata, autori su kvantitativno istražili etološki kontekst produkcije zvukova navedenih glavoča. Poznato je kako glavoči komuniciraju intraspecijski (mužjak – mužjak ili mužjak – ženka) koristeći istovremeno različite senzorne signale (vizualne, kemijske i akustičke, Tavolga, 1954), a kao osnovna pretpostavka provedenog rada bila je činjenica kako zvuk niskih frekvencija proizveden na malim udaljenostima potencijalno ima važnu ulogu u reprodukciji glavoča. Provedeno je istraživanje pokazalo kako je vokalni repertoar glavoča iznimno velik, a u slučaju slatkovodnog glavočića *P. bonelli* sastoji se od čak tri, akustički različita, zvuka (tonalni, pulsatilni i kompleksni) stvorenih tijekom različitih faza reprodukcije. Tonalni zvukovi *P. bonelli*, zabilježeni ovim i prijašnjim istraživanjima (Torricelli i sur., 1986; Torricelli i sur., 1990), stereotipni su signali kratkog trajanja (oko 200 – 300 ms), čiji zbijeni pulsevi odaju izgled sinusoidnog vala produciranog zvuka. Nadalje, pulsatilni („bubnjajući") zvukovi sastoje se od niza pulseva stvorenih u kratkom razdoblju (40 pulseva/sec.) koji traju između 50 i 800 ms te imaju maksimalne vrijednosti frekvencije oko 100 Hz. Kombinacijom navedenih zvukova, *P. bonelli* stvarao je kompleksne (dvokomponentne) zvukove koji se sastoje od početnog (pulsatilni, trajanje: 60 – 800 ms) i krajnjeg (tonalni, trajanje: 70 – 700 ms) dijela, kod kojega je većina akustičke energije koncentrirana na fundamentalnoj frekvenciji (Lugli i sur., 1996). Za razliku od *P. bonelli*, druge dvije vrste producirale su samo jedan tip zvukova: *K. puncatitssima* stvara samo pulsatilni tip (strukturalno sličan pulsatilnim zvukovima *P. bonelli*), karakteriziran s 40 pulseva/s, čije trajanje varira između 400 i 1000 ms; *P. nigricans* emitirao je samo kratke, stereotipne tonalne zvukove sinusoidalnog izgleda u trajanju 300 – 400 ms (Lugli i sur., 1996). S obzirom na etološki kontekst, tj. prisutnost ženke u blizini ili u gnijezdu, autori su signale podijelili na: 1. zvukove stvorene kada je ženka izvan gnijezda (eng. *pre-spawning*) i 2. zvukove stvorene kada je ženka u gnijezdu (eng. *spawning*, Lugli i sur., 1995, 1996). Zvukove prve grupe iz nastambe stvarali su mužjaci *P. nigricans* i *P. bonelli*, s naglaskom kako obje vrste stvaraju samo tonalne zvukove pri udvaranju maksimalnom stopom 60 – 90 zvukova/min. (Lugli i sur., 1996). Kada je ženka unutar gnijezda, mužjaci *P. bonelli* stvarali su pulsatilne i kompleksne zvukove tijekom cijelog njezina boravka, a prestaju tek kada ženka napusti gnijezdo. Budući da su se zvukovi stvarali tijekom cijelog razdoblja ženkina boravka u nastambi (a ne neposredno prije parenja), autori zaključuju kako oni nemaju funkciju u sinkronizaciji ispuštanja gameta (Lugli i sur., 1996). Kao i kod *P. bonelli*, mužjaci *O. punctatissiumus* stvarali su pulsatilne zvukove samo kada je ženka ušla u nastambu i trajali su sve dok je ona tamo boravila, sa stopom 2 – 10 zvukova/min u trenutku kada ženka zauzme ovipoziciju (pozicija za polaganje jaja, trbuhom okrenuta prema stropu nastambe). Kod obje vrste koje produciraju zvukove kada je ženka u nastambi (*P. bonelli* i *O. punctatissiumus*), najveći je broj zvukova mužjaka stvoren tijekom prvih 10 – 15 min nakon ovipozicije, a kako ženka polaže sve više jaja na strop nastambe, tako se produkcija zvukova smanjuje (Lugli i sur., 1996). Mužjaci *P. bonelli* nisu producirali zvukove jednom kada je ženka ušla ili boravila u gnijezdu, već su ih stvarali samo kada je ženka na ulazu ili kruži oko njega (Lugli i sur., 1996). Rezultati istraživanja upućivali su na to kako zvukovi kod glavoča ne sudjeluju u procesu sinkroniziranog izbacivanja gameta tijekom reprodukcije, već imaju „udvaračku" funkciju budući da se ne stvaraju samo netom prije parenja ili fertilizacije jaja, već ih neke vrste produciraju prije ovipozicije i tijekom reprodukcije (Lugli i sur., 1996).

Prvo detaljno multidisciplinarno istraživanje mehanizma za produkciju zvukova glavoča proveli su Parmentier i sur. (2013). Cilj je istraživanja bio, koristeći morfološko-eksperimentalne analize i rezultate nekoliko različitih metoda (elektromiografija, anatomija, superbrze kamere, histologija, bioakustika), ispitati koje strukture sudjeluju u procesu produkcije zvukova te koji mehanizam najbolje opisuje akustička svojstva kod morske vrste glavoča *Gobius paganellus*, koji stvara dva tipa zvukova (pulsatilni i tonalni; Malavasi i sur., 2008). *Gobius paganellus* velika je vrsta morskog glavoča koja je rasprostranjena od Škotske pa sve do Senegala i Crnog mora (Miller, 1986), a u provedenom pokusu autori su akustički istražili i snimili dvije populacije (Francuska i Italija) u laboratorijskim uvjetima koristeći metodu pasivne akustike te kvalitativnom i kvantitativnom bioakustičkom analizom istražili akustička svojstva zvukova. Rezultati su pokazali kako se populacije znatno razlikuju s obzirom na tip produciranog zvuka: u Francuskoj *G. paganellus* je stvarao samo pulsatilne zvukove (trajanje: 192 ms; 11 pulseva; trajanje pulsa: oko 20 ms; puls period: 60 Hz) popraćene pokretima bukalne, brahialne i operkularne regije, dok to nije bio slučaj s talijanskim jedinkama koje su stvarale samo tonalne zvukove (trajanje: 334 ms; 31 puls; puls period: 93 Hz) bez uočenih pokreta glave i ostalih regija, zbog čega se uloga pokreta glave odbacila kao potencijalni mehanizam. Razlike u tipu produciranog zvuka (Francuska – pulsatilni naspram Italija – tonalni) pripisala se razlici u dužini tijela između dviju populacija (Parmentier i sur., 2013). Rezultati superbrzih kamera (200 okvira/s) pokazali su kako su ribe iz Francuske stvarale brze pokrete glavom tijekom produkcije zvukova (faza pripreme i faza spuštanja glave ili „klimanje“ glavom), čiji ciklusi savršeno odgovaraju periodu svakog pulsa njihovih zvukova, dok navedeni pokreti nisu bili primijećeni kod talijanske populacije, najvjerojatnije zbog razlike u tipu zvuka između dviju populacija (Parmentier i sur., 2013). Anatomska je analiza pokazala kako se lubanja i okolne strukture *G. paganellus* ne razlikuju između dviju populacija te kako je ona tipična za ribe reda Perciformes (Vandewalle i sur., 1982; Barel, 1983; Liem, 1993). Upotrebom elektromiografije i elektronske mikroskopije, pokazalo se kako mišić *levator pectoralis*, koji se veže na lubanju i povezuje na dorzalni dio oplećja (kosti grlenjače), sudjeluje u procesu stvaranja zvukova kod *G. paganellus*. Autori su, koristeći anatomske analize, navedeni mišić podijelili na dva različita snopa koja se križaju na mjestu vezanja na grlenjači: *pars lateralis* (drži se kaudalnog dijela neurokraniuma, a veže se na rostralni, tj. ventralni dio grlenjače) i *pars medialis* (drži se ventralnog dijela neurokraniuma, a veže se dijagonalno na dorzalnoj strani grlenjače). S obzirom na dobivene rezultate anatomskih i bioakustičkih analiza, autori su predložili kako kontrakcijama navedenih mišića i vibracijama oplećja (*ossi radiales*) najvjerojatnije dolazi do vokalizacija kod jedinki *G. paganellus* (Parmentier i sur., 2013). Nadalje, histološka je analiza pokazala kako se poprečni presjek mišića *levator pectoralis* sastoji od tri zone bogate mitohondrijima i posebnog rasporeda miofibrila, dok je navedeni mišić tipične strukture i izgleda kao i ostali sonički mišići drugih vokalnih riba (najbrže kontrahirajući mišići među kralješnjacima; Loesser i sur., 1997; Ladich, 2001; Boyle i sur., 2013). Rezultati elektromiografije (EMG), tj. upotrebe para elektroda utaknutih u lijevi i desni mišić *levator pectoralis* kako bi se stimulirala njihova aktivnost, potvrdili su kako stopa kontrakcija mišića savršeno odgovara vrijednostima fundamentalne frekvencije te najvjerojatnije i određuje spektralne vrijednosti akustičkih signala (Parmentier i sur., 2013). Stoga su autori zaključili kako glavoči i peševi (Cottidae), zbog konvergentnih anatomskih, kinetičkih (pokreti glave, tj. klimanje glavom) i ekoloških svojstava (bentički način života i speleofilna taktika) dijele i jednaki mehanizam za stvaranje zvukova temeljen na kontrakcijama mišića *levator pectoralis* i okolnih struktura oplećja, odbacujući pretpostavku kako bi plivaći mjehur mogao imati ulogu u procesu glasanja (Parmentier i sur., 2013).

Jedno su od najvažnijih, a ujedno i prvo detaljnije interspecijsko istraživanje produkcije zvukova glavoča, proveli Malavasi i sur. (2008), u sklopu kojega su autori analizirali šest kvalitativnih i kvantitativnih akustičkih parametara zvukova (stvorenih u agresivnom i reproduktivnom kontekstu) za jedanaest vrsta mediteranskih glavoča rodova linije *Gobius* (*Padogobius, Zosterisessor, Gobius*; prema Agorreta i sur., 2013) i linije *Pomatoschistus* (*Pomatoschistus* i *Knipowitschia;* prema Agorreta i sur., 2013) te pokušali odrediti koliko se rezultati provedenih bioakustičkih analiza poklapaju s prijašnjim morfološkim i molekularnim istraživanjima (Miller, 1990; McKay i Miller, 1991, 1997; Miller i sur., 1994; Penzo i sur., 1998; Simonovic, 1999; Sorice i Caputo, 1999; Huyse i sur., 2004). Osnovna je hipoteza istraživanja bila da, zbog svoje velike raznolikosti i širokog vokalnog repertoara, glavoči mogu poslužiti kao idealni modelni organizmi u filogenetskim i bioakustičkim analizama koje istražuju utjecaj srodnosti ili adaptacija na evoluciju akustičke komunikacije. Rezultati su pokazali kako se, na temelju akustičkih parametara, sve vrste znatno razlikuju jedna od druge, što pokazuje kako su zvukovi specifični za vrstu (Malavasi i sur., 2008). Detaljna akustička i multivarijatna statistička analiza pokazale su kako su dva najvažnija akustička parametra zaslužna za razlikovanje vrsta upravo temporalna svojstva: trajanje zvuka (DUR) i stopa ponavljanja pulseva (PRR) (Malavasi i sur., 2008). Takvi rezultati poklapali su se s prijašnjim istraživanjima nekoliko vokalnih porodica riba Pomacentridae, Centrarchidae, i Cichlidae (Lobel, 1998; Amorim i sur., 2004; Myrberg i Lugli, 2006) koja su potvrdila sličnu ulogu temporalnih svojstava u diferencijaciji vrsta. Uz to, tri su se grupe vrsta mogle razlikovati s obzirom na dva najvažnija akustička parametra: tonalna grupa (visok PRR, kratko DUR; *Padogobius* spp. i *Gobius paganellus*), velika pulsatilna grupa (nizak PRR, kratko DUR; *Gobius* spp. i *Zosterisessor ophiocephalus*) i mala pulsatilna grupa (nizak PRR, dugačko DUR; *Pomatoschistus* spp. i *Knipowitschia* spp.). Na fenogramu konstruiranom na temelju akustičkih svojstava, glavoči pijeska (*Pomatoschistus*, *Orsinigobius* i *Knipowitschia*) odvojili su se u jednu, dok su se ostale velike vrste odvojile u drugu grupu, unutar koje su rodovi *Gobius* i *Padogobius* prikazani parafiletski budući da se *Padogobius nigricans* grupirao s *G. paganellus* i *G. cobitis,* a P. *bonelli* s *G. niger* (Malavasi i sur., 2008). Zanimljivo, rezultati istraživanja pokazali su kako su vrijednosti modulacije frekvencije (FM) kod većine vrsta bile blizu nule ili negativne, dok je jedino kod *G. paganellus* FM iznosila oko 25 Hz, što je sugeriralo kako su zvukovi navedene vrste znatno povišeni u frekvenciji (Malavasi i sur., 2008). Nadalje, analiza korelacije između akustičkih parametara (DUR i PRR) i veličine tijela te temperature pokazala je kako je DUR znatno (negativno) korelirana s veličinom tijela i PRR kod istraživanih vokalnih glavoča. Autori su naglasili kako negativna korelacija DUR (koja odvaja dvije grupe glavoča na fenogramu) i veličine tijela te njihova predvidljivost (male vrste – dugački zvukovi i obrnuto) može imati filogenetsko značenje zbog morfološkog ograničenja koje predstavlja za mehanizam produkcije zvukova; evolucijske (selektivne) sile povezane s razlikama u veličini tijela glavoča mogle bi nastati kao rezultat razlika u trajanju zvuka koje je potencijalno svojstvo za razlikovanje identiteta riba (Kihslinger i Klimley, 2002; Malavasi i sur., 2008). Istovremeno, rezultati bioakustičkog istraživanja poklapali su se djelomično s morfološkim i molekularnim analizama, što je autorima sugeriralo kako akustički signali (zajedno sa svojim kvantitativnim parametrima) potencijalno prenose filogenetski signal zaslužan za razlikovanje vrsta (Malavasi i sur., 2008). Kako bi dobivene rezultate autori objasnili u filogenetskom kontekstu, autori su razvili hipotezu u obliku grafičkog prikaza, o evoluciji akustičke komunikacije kod glavoča (**Slika 8**): pulsatilni zvuk najvjerojatnije predstavlja ancestralan tip (pleziomorfno svojstvo) koji se zadržao kod svih vokalnih glavoča pijeska, kao i kod nekih velikih vrsta (*Gobius, Zosterisessor, Padogobius*), dok su tonalni zvukovi kod *G. paganellus* i *Padogobius* spp., u parsimonijskom scenariju, odvedeni tip zvukova nastali od starijih pulsatilnih tako što se njihovo trajanje (DUR) smanjilo, omogućivši povećanje stope ponavljanja pulseva (PRR, zvukovi se u kratkom vremenu mnogo puta ponove, bez međuintervala) s obzirom na zapaženu negativnu međusobnu korelaciju DUR i PRR. Također, kako su zvukovi postali kraći s velikom stopom ponavljanja pulseva, tako su dobili mogućnost biti frekvencijski modulirani, što je naknadno otvorilo put za divergenciju bliskih vrsta (npr. *G. paganellus* od *G. cobitis* (Malavasi i sur., 2008)).



Slika 8. Dijagram koji prikazuje evoluciju akustičke komunikacije i razvoj zvukova kod mediteranskih glavoča (rodovi *Padogobius, Zosterisessor*, *Gobius, Knipowitschia, Orsinigobius* i *Pomatoschistus*). Prema navedenoj hipotezi, inicijalni je tip zvuka bio pulsatilni, koji je modulacijom dvaju najvažnijih akustičkih svojstva (DUR – trajanje; PRR – stopa ponavljanja pulseva) doveo do razvoja pulsatilnih/bubnjajućih zvukova kod skupine *Pomatoschistus* + *Orsinigobius* + *Knipowitschia,* tonalnih zvukova kod *Padogobius* spp. + *G. paganellus* te pulsatilnih zvukova kod preostale skupine. Na dijagramu, na mjestu gdje se povećava (>) PRR, javlja se ujedno i tonalna struktura zvukova. Preuzeto i prilagođeno iz Malavasi i sur. (2008)

PREGLED DISERTACIJE

Ova disertacija obuhvaća četiri izvorne znanstvene publikacije (**I** – **IV**) u kojima su navedeni ciljevi i znanstvene hipoteze.

Ciljevi ove disertacije:

1. Zabilježiti zvukove i kvantificirati temporalne i spektralne parametre akustičkih signala pontokaspijskih glavoča (Gobiidae) *Neogobius fluviatilis* Pallas, 1918, *N. melanostomus* (Pallas 1814) i *Ponticola kessleri* (Günther 1861), kao i rotana *Perccottus glenii* Dybowski 1877 (Odontobutidae) te detaljno opisati intraspecijsku akustičku varijabilnost zvukova istraživanih vrsta.

2. Istražiti interspecijsku akustičku varijabilnost vokalnih vrsta linije *Gobius*, tj. atlantsko-mediteranskih glavoča (rodovi *Gobius*, *Padogobius* i *Zosterisessor*) i pontokaspijskih vrsta (*Neogobius fluviatilis*, *N. melanostomus* i *Ponticola kessleri*), rekonstruirati hipotezu o evoluciji akustičke komunikacije glavoča linije *Gobius* te istaknuti najvažnija akustička svojstva koja diferenciraju vokalne vrste.

3. Istražiti etološki kontekst akustičke komunikacije i anatomske strukture povezane s potencijalnim mehanizmom za produkciju zvukova kod rotana *P. glenii* (Odontobutidae) te usporediti morfološke rezultate s predloženim mehanizmom kod ostalih ispitanih vrsta.

4. Istražiti genetsku srodnost vokalnih glavoča linije *Gobius*, rekonstruiranu na temelju DNK sekvenci četiri mitohondrijska i jezgrena biljega (cyt *b*, COI, Rho i RAG), te usporediti dobivene rezultate s provedenim akustičkim analizama kako bi se istražilo postoji li poklapanje akustičke divergencije s genetičkom.

Znanstvene hipoteze ove disertacije:

i. Filogenetska interspecijska analiza pontokaspijskih i atlantsko-mediteranskih glavoča iz linije *Gobius*, temeljena na kvalitativnim i kvantitativnim akustičkim parametrima, upućuje na određen stupanj međusobne srodnosti pojedinih vokalnih vrsta. Također, pretpostavlja se kako se vokalni repertoar vokalnih glavoča linije *Gobius* sastoji od malog broja različitih tipova zvukova.

ii. Kod glavoča linije *Gobius* glasaju se samo mužjaci tijekom intraspecijskih interakcija.

iii. Mehanizam za produkciju zvukova temelji se kod rotana *Perccottus glenii* (Odontobutidae) na kranio-pektoralnim mišićima, a samo pulsatilni zvukovi predstavljaju ancestralni tip akustičkog signala kod glavoča u širem smislu (Gobioidei).

iv. Interspecijski filogenetski odnosi devet vokalnih vrsta glavoča linije *Gobius* (rodovi *Neogobius, Ponticola, Zosterisessor, Gobius, Padogobius*), konstruirani na temelju šest kvantitativnih akustičkih parametara, preklapaju se s rezultatima molekularnih analiza, što pokazuje kako zvukovi prenose filogenetski signal odgovoran za razlikovanje srodnih vrsta.

Publikacije **I**, **II** i **III** blisko su povezane s prvim ciljem te odgovaraju na prvu i treću hipotezu ove disertacije. Publikacija **I** opisuje zvukove i kvantificira akustičke parametre vrste *Neogobius fluviatilis,* dotad jedne od tek nekoliko ispitanih vrsta glavoča s pontokaspijskog područja. Rezultati navedenog rada pokazuju kako su tonalni zvukovi zajednička karakteristika ispitanih pontokaspijskih vrsta te kako nisu samo mužjaci ti koji stvaraju komunikacijske zvukove, već i ženke koje imaju sposobnost produciranja akustičkih signala. Publikacija **II** dodatno produbljuje znanje o akustičkoj komunikaciji pontokaspijskih vrsta *Neogobius melanostomus* i *Ponticola kessleri* te daje dublji uvid u akustičku divergenciju vrsta pontokaspijskog područja koje su prijašnjim morfološkim istraživanjima bile svrstane u jedinstveni rod *Neogobius*. Istovremeno, rezultati publikacije **II** pokazuju kako su tonalni zvukovi pontokaspijskih glavoča izrazito stereotipni i specifični za ispitane dvije vrste, s određenim parametrima akustičkih signala koji bi potencijalno mogli biti zaslužni za njihovu divergenciju. Nekoliko je različitih skupina prepoznato akustičkim analizama, na temelju akustičkih parametara, u sklopu publikacije **II**, a dvije srodne vrste (*N. melansotomus* i *N. fluviatilis*) dijele određene zajedničke karakteristike koje ih odvajaju od dotad dokumentiranih vrsta. Publikacija **III** izravno je povezana s trećim ciljem te pripada drugoj hipotezi ove disertacije. Naime, publikacija **III** daje dublji uvid u akustičku komunikaciju rotana *P. glenii,* člana bazalne porodice Odontobutidae unutar podreda Gobioidei. Rezultati te publikacije istaknuli su šest etoloških kategorija tijekom kojih dolazi do produkcije zvukova kod rotana za vrijeme reprodukcije te utvrdili kako nisu samo pulsatilni zvukovi sinapomorfno svojstvo za glavoče u širem smislu (Gobioidei), već da, uz pulsatilne, i tonalni zvukovi predstavljaju ancestralne akustičke signale. Uz to, publikacija **III** navodi anatomske strukture, tj. kranio-pektoralni mišić oplećja i lubanje podijeljen u tri snopa (*m. levator pectoralis*: *pars lateralis superficialis, pars lateralis profundus* i *pars medialis*) kao potencijalni mehanizam za produkciju zvukova kod rotana. Na kraju, publikacija **IV** izravno odgovara na prvu i treću hipotezu, a povezana je s drugim i četvrtim ciljem. Publikacija **IV** objedinjuje i analizira sve podatke (zvukove s njihovim akustičkim parametrima) dugogodišnjih akustičkih istraživanja, kao i ove disertacije, radi ispitivanja korelacije između akustičke i genetičke divergencije kod devet atlantsko-mediteranskih i pontokaspijskih vokalnih glavoča. Rezultati publikacije **IV** pokazuju kako su zvukovi kod vokalnih glavoča linije *Gobius* šireg mediteranskog područja izrazito specifični za svaku vrstu budući da su akustička divergencija i filogenetska diferencijacija pozitivno korelirane kod istraživanih vokalnih vrsta. Također, rezultati akustičkih analiza publikacije **IV** pokazuju kako određeni parametri zvuka najvjerojatnije prenose filogenetski signal zaslužan za razdvajanje srodnih vrsta. Filogenetski odnosi između vokalnih vrsta linije *Gobius* rekonstruirani su na temelju četiri mitohondrijska (mtDNA) i jezgrena (nDNA) genetička biljega, a rezultati navode kako je rod *Padogobius* parafiletskog podrijetla zbog bliske (utvrđene) genetske srodnosti talijanskog *P. nigricans* s pontokaspijskim vrstama *Neogobius* roda, kao i sličnog tipa akustičkog signala koje ove vrste produciraju.

2. ZNANSTVENE PUBLIKACIJE

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Sound production in the Ponto-Caspian goby *Neogobius fluviatilis* and acoustic affinities within the *Gobius* lineage: implications for phylogeny

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The aim of this study was to describe the vocal repertoire of the Ponto-Caspian goby *Neogobius fluviatilis* and to compare the acoustic properties of this species with those of other soniferous Mediterranean gobies belonging to the *Gobius* lineage. Vocalizations and associated behaviours were recorded under controlled aquarium conditions in female and male *N. fluviatilis*. Sound emission was elicited by means of 'intruder tests', using an individual of the same or opposite sex as an intruder, and recording sounds using a hydrophone placed 20 cm from the shelter used as a nest for the resident fish. Five acoustic properties, including spectral and temporal properties, were measured from 13 individuals. The vocal repertoire of the species consisted of sequences of short vocalizations during both agonistic and reproductive intraspecific interactions. The wave form of each sound resolved in a pure sine wave composed of rapidly repeated pulses. Sounds lasted about 200 ms, showing an average fundamental frequency of about 80 Hz. Sound properties did not differ between reproductive and the aggressive contexts, and the general structure of sounds was highly stereotyped. The individual means of three acoustic independent traits characterizing the sounds of seven species of the *Gobius* lineage, including *N. fluviatilis*, were then entered in a discriminant function analysis to assess how well species could be differentiated on the basis of acoustics, and their degree of affinities. The results suggested that the pulse repetition rate of the sounds, i.e. the relative tonal/pulsatile nature of the sounds, was the most important property in differentiating the species, and that this trait may contain a high level of phylogenetic signal, as the species producing tonal sounds clustered together, in line with the results of recent molecular phylogenic studies. The results were discussed in light of the geological and phylogeographical events believed to have driven the diversification of European gobies. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **117**, 564–573.

ADDITIONAL KEYWORDS: acoustic properties – gobies – Messinian salinity crisis.

INTRODUCTION

Acoustic signals convey crucial information on species, sex and individual identity, individual motivation and quality (Bradbury & Vehrencamp, 1998). The degree of similarity among acoustic signals in groups of closely related species could be related to phylogenetic relationships, as shown for anurans, insects, birds and mammals (Robillard *et al.*, 2006; Tavares *et al.*, 2006; Cap *et al.*, 2008; Gingras *et al.*, 2013). Among the vertebrates, the largest diversity of sound-generating mechanisms for acoustic communication has evolved in fishes (Myrberg & Lugli, 2006; Fine & Parmentier, 2015). In comparison with tetrapods, fish have relatively simple central and peripheral vocal mechanisms and thus typically lack the ability to emit complex frequency-modulated calls (Rice & Bass, 2009). Sound production in a communicative context has been documented in over 800 fish species representing 109 families (Kasumyan, 2008), although phylogenetic reconstruction based on acoustics has seldom been attempted, and the state

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*Corresponding author. E-mail: mala@unive.it of knowledge is at an early stage as compared with amphibians or other animal groups. Nevertheless, as for anurans, the acoustic signals of teleosts could be useful for phylogenetic reconstruction, due to the instinctual and stereotyped nature of their vocalizations. Attempts have been made to study the species-specificity of acoustic signals and phylogenic relationships and similarities in sound structure in three teleost families in particular – batracoidids, pomacentrids and gobids (Malavasi, Collatuzzo & Torricelli, 2008; Parmentier *et al.*, 2009; Rice & Bass, 2009). In some cases, constraints related to body size seem to mask the species-specificity of the sounds, and sounds do not succeed in discriminating species, as was shown for clownfishes (Colleye & Parmentier, 2012).

Sound production has been documented in at least 21 gobioid species belonging to ten different genera (Bass & McKibben, 2003; Polgar *et al.*, 2011). Within this large and diverse family, pulsatile and tonal sounds are emitted by the male as a part of the breeding and aggressive behavioural repertoire (Torricelli, Lugli & Pavan, 1990; Lugli *et al.*, 1997; Lugli & Torricelli, 1999; Malavasi *et al.*, 2003; Myrberg & Lugli, 2006; Amorim & Neves, 2007). Gobies are distributed worldwide, in marine, estuarine and freshwater habitats. Acoustic communication has been widely investigated, especially in Mediterranean gobies, such as in the genera *Gobius*, *Padogobius*, *Zosterisessor*, *Pomatoschistus* and *Knipowitschia* (Bass & McKibben, 2003; Myrberg & Lugli, 2006). According to existing molecular phylogenies, gobioid fishes (Gobiiformes, Gobioidei) can be subdivided into several lineages (Agorreta *et al.*, 2013), with some basal groups well resolved, such as Odontobutidae, Butidae and Eleotridae (Thacker, 2003, 2009; Neilson & Stepien, 2009; Agorreta *et al.*, 2013). With the exception of *Odontobutis obscura*, a basal gobioid whose vocal repertoire was documented by Takemura (1984), most of the documented soniferous species are concentrated in the derived family Gobiidae. Within this family, sound production is mostly documented in gobies occurring across the Mediterranean–Atlantic and Ponto-Caspian areas. These species can be subdivided into two main lineages according to the most recent molecular phylogenies (Table 1, Thacker & Roje, 2011; Agorreta *et al.*, 2013). The acoustic structure showed great variability, from pure tonal to pulsatile and complex sounds, within the *Gobius* lineage, while only pulsatile sounds were reported for the *Pomatoschistus* lineage (Ladich & Kratochvil, 1989; Rollo *et al.*, 2007; Malavasi *et al.*, 2008; Sebastianutto *et al.*, 2008; Polgar *et al.*, 2011; Amorim & Neves, 2007; Parmentier *et al.*, 2013; Table 1). A comparative analysis of the sound structure within Mediterranean–Atlantic gobies showed a clear distinction between these two lineages, suggesting a good degree of congruence between acoustic affinities and phylogenetic relationships (Malavasi *et al.*, 2008). Given that the phylogeny of this group of species was probably driven by complex geological events related to separation of the Tethys and Paratethys, the Messinian salinity crisis, and the subsequent re-flooding of the Atlantic Sea (Penzo *et al.*, 1998; Huyse, Van Houdt & Volckaert, 2004; Miller, 2004; Malavasi *et al.*, 2012; Vanhove *et al.*, 2012), the clarification of the phylogenetic relationships within these two lineages is of particular phylogeographical interest. Nevertheless, due to the systematic complexity of this group and the high number of species, a great deal of information is still required to obtain a reliable, complete picture. Furthermore, the sound production mechanism remains to be elucidated, at least with some

Table 1. Systematic position, geographical range and sound structure of the European soniferous gobies (Gobioidei, Gobiidae), according to the current literature (see text for references)

Sub-families	Lineages	Atlantic–Mediterranean species	Ponto-Caspian species	Sound structure
Gobiine-like	*Gobius*	*Gobius niger*, *G. paganellus*, *G. cobitis*, *G. cruentatus*, *Zosterisessor ophiocephalus*, *Padogobius bonelli*, *P. nigricans*	*Proterorhinus marmoratus*, *Neogobius melanostomus*	Tonal, pulsatile and complex (sounds composed of both pulsatile and tonal segments)
Gobionelline-like	*Pomatoschistus*	*Pomatoschistus marmoratus*, *P. pictus*, *P. minutus*, *P. microps*, *P. canestrinii*, *Knipowitschia panizzae* *K. punctatissima*		Pulsatile

degree of detail, despite recent insight provided by Parmentier *et al.* (2013), who suggested a cranio-pectoral mechanism in *Gobius paganellus*.

Recent molecular phylogenies reveal that Ponto-Caspian gobies form part of the *Gobius* lineage (Agorreta *et al.*, 2013), together with other Mediterranean species, mostly belonging to the genus *Gobius.* With the exception of few sound recordings reported for *Proterorhinus marmoratus* and *Neogobius melanostomus*, the vocal repertoire of these species remains to be described. Rollo *et al.* (2007) recorded a single sound emitted by *Neogobius melanostomus* in the field and then, using that single sound as a stimulus, recorded several other sounds in the laboratory. The aim of this study was to describe the vocal repertoire of *Neogobius fluviatilis* (Pallas, 1814) and to compare the acoustic properties of this species with those of the other soniferous species belonging to the '*Gobius* lineage' (*sensu* Agorreta *et al.*, 2013), using the data of the present paper and those provided by Malavasi *et al.* (2008) for Mediterranean species. The final objective was to explore the degree to which the affinities in acoustic signals could be related to phylogenetic relationships within this group of closely related species.

METHODS

STUDY SPECIES

Neogobius fluviatilis (Pallas, 1814), the monkey goby, belongs to a group of transverse gobiids that includes the euryhaline Mediterranean *Zosterisessor*, exclusively freshwater Mediterranean *Padogobius*, and four Ponto-Caspian groups, namely *Mesogobius*, *Neogobius*, *Proterorhinus* and *Chasar*, and the tadpole gobies. The Sarmatic taxa (*Neogobius*, *Mesogobius*, *Proterorhinus* and the tadpole gobies) together with the Mediterranean *Padogobius* are synapomorphic with the euryhaline Mediterranean *Zosterisessor ophiocephalus* in reduction or loss of free pectoral rays (Miller, 2004). The monkey goby is a euryhaline species, found on sandy bottoms, and distributed in rivers and estuaries in the Ponto-Caspian basin. Spawning commences at the end of April, when temperatures rise to about 13 °C, and reaches a peak in May, at temperatures of 18–19 °C. The majority of spawning is completed in June, with only limited occurrence in July (Miller, 2004).

FISH COLLECTION AND HOUSING

The experiment was conducted from the end of March until the beginning of June 2014, during the reproductive season of the studied species. Fish were caught during March and April 2014 in the artificial channel system Kupa-Kupčina in Croatia (GPS coordinates; *x*, 45:31:53.7; *y*, 15:47:18.5; *z*, 93.2 m), where this species has the status of an invasive species. Fish were collected using electrofishing (electric unit, power: 7.5 kW) and injury and fatalities were carefully avoided. After capture, the fish were transported in aerated containers from Croatia to the laboratory of the Ca Foscari University (Venice, Italy) where they were housed in suitable aquaria. Upon their arrival at the laboratory, fish were examined for sex on the basis of urogenital papilla (Miller, 1984). Larger fish used in the experiments were placed in smaller glass-tanks (capacity 120 L), each individually, while the smaller individuals were placed in larger glass-tanks (capacity 300 L). Shelters made of tiles were placed in each tank to serve as a nest. Tanks were sound-proofed from surrounding noise using foam rubber shims as a base. Each tank was provided with a filtration system, proper substrate of a 5- to 10-cm-thick layer of sand on the bottom, and aerators to maintain oxygen levels. Water temperature (range 19–21 °C) and salinity (0.05 PSU, obtained from tap water) were maintained at levels within the average values found in the natural environment at the same time of the year. Fishes were left to acclimate in the tanks for 1 week and were fed chironomid larvae and mytili *ad libitum*.

SOUND COLLECTION

Experiments were set up to elicit sounds in male–male or female–female aggressive interactions (aggressive behaviour) and male–female (courtship or spawning behaviour) reproductive interactions. According to the protocol used by Malavasi *et al.* (2008), isolated resident fish (either males or females) were placed in experimental tanks (120 L) and left to establish territorial behaviour for about 5–8 days before the beginning of recordings. Each isolated fish was provided with a tile shelter (10 × 20 cm). Before recordings, an intruder (either male or female) was placed in a metal cage in front of the shelter entrance in order to elicit behavioural and acoustic responses. As regards reproductive interactions, only male fish were used as the resident fish, assuming that only males are vocal during courtship, according to the literature data on soniferous gobies. The lights and all pumping devices were switched off 10–15 min before the beginning of the experiments to minimize external noise. After the experiments, water temperature was measured with a digital thermometer, and the body size of each tested individual was measured using callipers. Sounds were recorded with a custom-made hydrophone (Gulton Industries; sensibility: −164 dB, re

1 μPa, frequency response ± 3 dB from 30 Hz to 2 kHz) positioned about 20 cm from the entrance of the nest, according to Malavasi *et al.* (2008). The hydrophone was connected to a conditioning amplifier (B&K 2626) and sounds for analysis were recorded using a portable digital audio recorder (Tascam Linear PCM Recorder; wav 16/44.1 k mono), and stored on the recorder memory card, and were then imported to a personal PC. Kraun headphones were connected to the digital audio recorder for better sound quality. Overall, sounds were recorded from 13 individuals (eight males, five females). Of the eight males, four were tested with a male intruder (aggressive interactions) and four with a mature female intruder (reproductive interactions). Each recording lasted 30 min from the emission of the first sound.

SIGNAL PROCESSING AND SOUND ANALYSIS

All sounds were analysed in real time using Avisoft-SASLab Pro Software. Analog signals were digitalized at 1500 Hz sampling and were band-pass filtered (band: 50–500 Hz) to eliminate acoustic components different from those of fishes and which might disturb or distort the waveform of any fish sound. The temporal and spectral structures of sounds were investigated using the time signal and power spectrum features provided by the software. A spectrogram of each sound was obtained by setting the spectral parameters to achieve the best representation of signals in relation to their acoustic structure (window type: hamming; fast Fourier transform: 256; frame: 100; band-width: 8 Hz; resolution: 5 Hz; overlap: 93.75%).The following acoustic properties were measured following Malavasi *et al.* (2008): (1) duration (DUR; total length of the call, measured in milliseconds); (2) number of pulses (NP); (3) pulse repetition rate (PRR; obtained by dividing NP by DUR and expressed in Hz); (4) peak frequency (PF; obtained from the power spectrum function); and (5) frequency modulation (FM; calculated as the difference between final PRR and initial PRR and expressed in hertz).

STATISTICAL ANALYSIS

To assess the level of intraspecific variation in sound production, coefficients of variation [$CV = (SD/X) \times 1000$] were calculated for each property, on untransformed data, at each source of variation (within-individual and between-individual variation). Acoustic properties measured for each sound, individual means of acoustic properties and grand means calculated on the 13 individuals recorded were used to calculate within-individual (CVw) and between-individual (CVb) coefficients of variation. The CVb/CVw ratio was used as a measure of the relative variability among individuals. To test for the differences in the acoustic properties of males between reproductive and aggressive contexts, and between males and females within the aggressive contexts, a *t*-test was performed on log-transformed data. Individual means of acoustic properties were related to size and temperature using the Pearson correlation to check for the dependency of sound structure on these two physical characteristics. To assess the affinities of *Neogobius* sounds with the other soniferous species of the '*Gobius* lineage' (*sensu* Agorreta *et al.*, 2013), recordings were obtained from Malavasi *et al.* (2008) for six species belonging to the genera *Gobius*, *Padogobius* and *Zosterisessor*, and then analysed. To explore the affinities in terms of sound structure within this group of species, a multivariate statistical approach was used. First, the log-transformed individual means of the five acoustic properties (DUR, NP, PRR, PF, FM) recorded for the seven species investigated (with one species, *Padogobius bonelli*, being split in the pulsatile and tonal components of the complex sounds, according to Malavasi *et al.*, 2008) were tested for correlation using the Pearson correlation. Then, a discriminant function analysis (DFA) was performed on the log-transformed individual means of those acoustic properties that proved to be independent on the basis of the correlations (DUR, PRR, PF). The aim was to assess how well individuals can be classified into the correct species, which acoustic properties differentiate the species, and their relative distance and affinities based on acoustics. Significant differences among the species were examined by quantifying Mahalanobis distances between the group centroids.

RESULTS

SOUND PRODUCTION IN *NEOGOBIUS FLUVIATILIS* AND INTRASPECIFIC VARIATION

Neogobius fluviatilis emits sequences of short vocalizations during both agonistic and reproductive intraspecific interactions. The wave form of each sound resolves in a pure sine wave composed of rapidly repeated pulses, which represents a periodically repeated longitudinal sound wave (Fig. 1). The power spectrum revealed that most of the sound energy was concentrated within the narrow band of 60–100 Hz (Fig. 1), with an average peak frequency of about 80 Hz (Table 2). These properties revealed the tonal nature of the sound that can be assessed on the basis of spectrogram, power spectrum and wave form of each sound (Fig. 1). Each sound

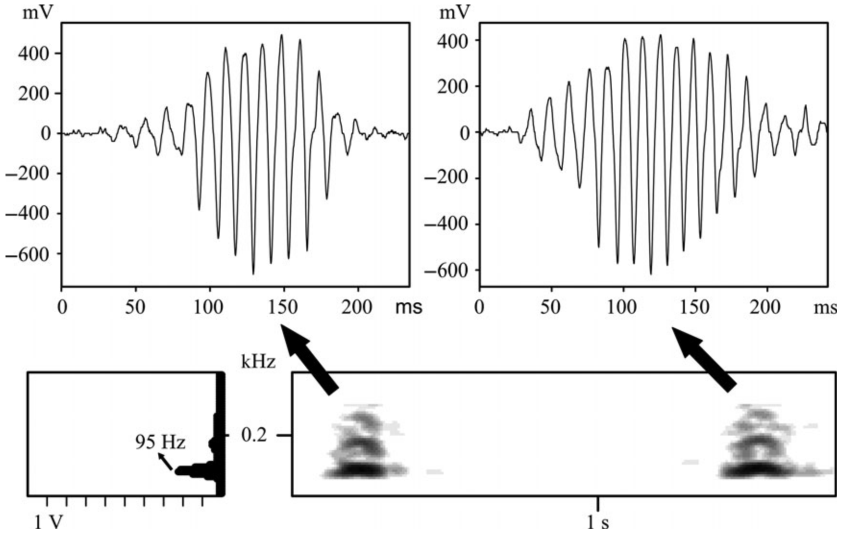

Figure 1. Structural details of two consecutive, representative sounds of *Neogobius fluviatilis*: below, spectrogram (right) and power spectrum (left); above, the wave form of both sounds.

Table 2. Individual means (± SD) of body size, water temperature of recordings, and the five measured acoustic properties in each sex and behavioural context

Behavioural context	Male (N = 8)	Female (N = 5)
Body size (mm)		
Aggressive	132.7 ± 8.3	116.2 ± 11.8
Reproductive	128.5 ± 11.2	–
Temperature (°C)		
Aggressive	20.2 ± 0.4	19.8 ± 0.4
Reproductive	20 ± 0	–
DUR (ms)		
Aggressive	179 ± 29.5	163.62 ± 20.5
Reproductive	161 ± 38.5	–
NP		
Aggressive	12.7 ± 1.3	12.2 ± 1.3
Reproductive	12 ± 2.9	–
PRR (Hz)		
Aggressive	72.6 ± 8.8	74.4 ± 2.4
Reproductive	74.8 ± 1.0	–
PF (Hz)		
Aggressive	77.2 ± 6.6	78.4 ± 2.4
Reproductive	78.8 ± 3.6	–
FM (Hz)		
Aggressive	9.4 ± 2.1	11.8 ± 3.5
Reproductive	8.3 ± 5.6	

DUR, duration; NP, number of pulses; PRR, pulse repetition rate; PF, peak frequency (fundamental frequency); FM, frequency modulation.

is resolved in a main single harmonic and in a sinusoidal wave form, with most of the energy of the sound concentrated at a single fundamental frequency slightly less than 100 Hz (Fig. 1). The single sound is part of the sequence in which the intra-sound period (period between the completion of one sound and the onset of the next) lasted from 1.3 to 1.6 s (Fig. 1). Sounds lasted from 127 to 226 ms, with an average of 169.3 ms. The first two-thirds of the sound duration was marked by a moderate increase in amplitude, which then rapidly declined (Fig. 1). The average value of FM ranged from 2 to 13 Hz, indicating a slightly upward modulation. The number of pulses for each sound varied between eight and 16, with an average of 12.3 (Table 2). The pulse repetition rate ranged from 69 to 81 Hz, with an average of 73.6 Hz (Table 2), overlapping with the mean peak frequency of 77 Hz, and constituting the fundamental frequency of the sound, again revealing the harmonic nature of these vocalizations. The general structure of vocalizations was highly invariant both with respect to the behavioural context (reproductive vs. aggressive context) and between the sexes, and a single type of sound was recorded during all experimental trials, indicating the stereotyped nature of the sound production. Of the five acoustic properties of tonal segments, DUR, NP and FM (within reproductive interactions) and PRR (within aggressive interactions) had a CVb/CVw ratio > 1 (Table 3), suggesting a higher level of inter-than intra-individual variability. This confirms the stereotyped nature of vocalizations.

The fish produced sounds while swimming or while resting on the substrate. Sound emission of the sounds was preceded by a clearly visible upward thrusting of the head, during which a dorsolateral movement of the opercula was performed. Breeding

Table 3. Intra-individual and inter-individual CV and their ratio for the five acoustic properties measured, in each behavioural context

Acoustic property	CVw		CVb		CVb/CVw	
	Aggr.	Repr.	Aggr.	Repr.	Aggr.	Repr.
DUR (ms)	0.20	0.15	0.16	0.23	0.81	1.51
NP	0.21	0.17	0.11	0.24	0.54	1.41
PRR (Hz)	0.06	0.06	0.08	0.01	1.25	0.21
PF (Hz)	0.09	0.47	0.06	0.34	0.63	0.71
FM	0.91	0.23	0.37	0.87	0.40	3.69

DUR, duration; NP, number of pulses; PRR, pulse repetition rate; PF, peak frequency (fundamental frequency); FM, frequency modulation (absolute numbers, as in this case the value could be negative).

males significantly changed their coloration, becoming gradually darker, and eventually black. In contrast to breeding males, females retained their semi-transparent, very pale coloration. Although individuals were not free to interact with one another due to the cage, the change in coloration allowed for discrimination between aggressive and courtship interactions. An intense change of males towards darker coloration was observed only during male–female interactions, making this a reliable indicator of courtship motivation.

There were no statistically significant differences in any of the behavioural properties, either between behavioural contexts or between sexes (*t*-test, $p > 0.05$). There were no statistically significant correlations between any of the acoustic properties with temperature or with body size (Pearson correlation, $N = 13$, $P > 0.05$).

ACOUSTIC AFFINITIES WITHIN THE *GOBIUS* LINEAGE

Results of the correlations showed that NP was significantly related to PRR and DUR, and PF and FM were also in turn significantly associated. Therefore, only PRR, DUR and PF were used in the DFA (Table 4). Using species as the grouping variable in the DFA, individuals were well classified into the correct species, with an average correct species classification rate of 88.46%. The first discriminant function accounted for 65.4% of the variation and distinguished three groups of species (Fig. 2): (1) *Gobius paganellus*, the tonal component of *Padogobius bonelli*, *Neogobius fluviatilis* and *Padogobius nigricans*, on the positive side of the axis; (2) *Gobius cobitis*, the pulsatile component of *Padogobius bonelli* and *Gobius niger* in the central zone of the diagram,; and (3) *Zosterisessor ophiocephalus* to the left of the axis, characterized by pulsatile sounds. The first discriminant function, as revealed by the standard structure coefficients of DFA (Table 5), differentiated species with a high PRR, and low DUR and PF (right side of the axis, tonal nature of the sounds, Fig. 2), from those with low PRR and high DUR and PF (left side of the diagram, pulsatile sounds, Fig. 2). The second discriminant function accounted for 30.1% of the variation, and further supported the separation of the tonal vs. pulsatile components, with DUR, PRR and PF giving a negative contribution on this axis, and again with the major contribution given by PRR, followed by PF and DUR (Table 5). *Neogobius fluviatilis* clustered into the top right quadrant of the chart; i.e. in comparison with the remaining six species, this species is characterized by a high PRR and low PF.

Table 4. Pearson correlation coefficients of the relationships between the individual means of the five acoustic properties of the goby species belonging to the *Gobius* lineage ($N = 52$)

	NP	PRR	PF	FM
DUR	0.54 *	−0.15	0.12	0.22
NP		0.74 *	0.04	0.33 *
PRR			−0.04	0.22
PF				0.29 *

DUR, duration; NP, number of pulses; PRR, pulse repetition rate; PF, peak frequency (fundamental frequency); FM, frequency modulation.
*$P < 0.05$.

DISCUSSION

The results of the present study showed that intra-male and intra-female aggression and male courtship are associated with sound production in the Ponto-Caspian goby species *Neogobius fluviatilis*. Sound production consisted of short (about 200 ms), low-frequency (about 70–80 Hz) vocalizations, with a pure tonal structure and highly stereotyped nature. Sounds were invariant, in their structure and mean properties, in respect to both sex and behavioural context. Temperature did not affect any acoustic property, probably due to the restricted range of water temperature values recorded during the trials (19–20 °C).

Sound production has been also documented in *Neogobius melanostomus*, with some calls recorded from individuals of the invading populations in the United States (Rollo *et al.*, 2007). These authors designated these calls as ‘pulse series’, although the

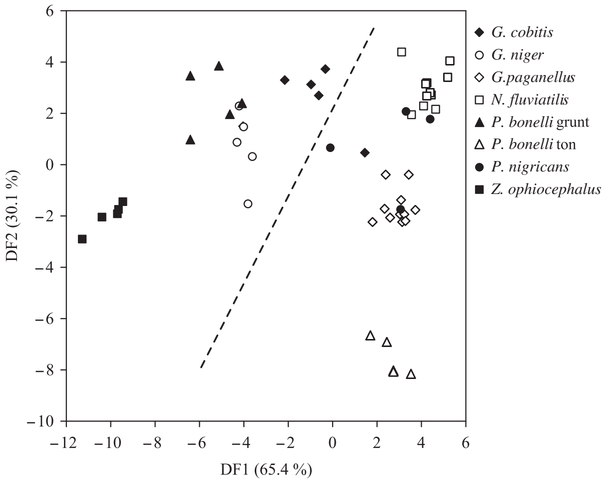

Figure 2. Bivariate comparison between discriminant functions DF1 and DF2 where individuals are delineated by species (different symbols represent different species). The dashed line separates species producing sounds with a high pulse repetition rate (tonal sounds, right side) from species producing sounds with a low pulse repetition rate (pulsatile sounds, left side).

Table 5. Results of the discriminant function analysis with species as the grouping variable and the three independent acoustic properties as dependent variables

	Standardized coefficients		Factor structure coefficients	
Acoustic property	DF1	DF2	DF1	DF2
DUR	−0.39	−0.62	−0.11	−0.15
PRR	0.90	−0.55	−0.67	0.20
PF	−0.89	−0.77	−0.38	0.64

The results shown are the standardized canonical coefficients and the factor structure coefficients. Factor structure coefficients are the bivariate correlation between a species/individual value for a dependent variable and the individual's discriminant function score and are thus important to characterize the contribution of each dependent variable.

wave forms of these sounds were not expanded and we strongly suspect that these authors did not perceive the tonal nature of these calls. If this is the case, the sounds of *N. melanostomus* would be similar to the con-generic species recorded by the present study. Regardless, the vocal repertoire of *N. melanostomus* requires more detailed study, considering the importance of this species as an alien invader. Furthermore, sounds recorded from the Ponto-Caspian goby *Proterorhinus marmoratus* (Ladich & Kratochvil, 1989) appeared to have a very similar structure to those described here for *N. fluviatilis*. In that species, both males and females produced tonal sounds during aggression, while males also emitted the same type of sounds during courtship. These affinities are expected, considering that both *Neogobius* and *Proterorhinus* share the Ponto-Caspian geographical distribution area, and belong to the same clade (Simonovic, 1999; Medvedev *et al.*, 2013). More surprising is the overlap in the structure and the acoustic properties of *N. fluviatilis* sounds with those documented for the Arno goby *Padogobius nigricans* (Lugli *et al.*, 1995; Malavasi *et al.*, 2008), which is an endemism of the Tuscan–Latium freshwater fish fauna of central Italy (Miller, 2004). The frequency, duration, general structure and stereotype of sound production and associated behaviours are strongly similar (Lugli *et al.*, 1995), as the wave form and the power spectrum mostly overlapped, despite slight differences in the mean values of the acoustic properties (present paper; Lugli *et al.*, 1995). This high degree of affinity is consistent with the morphological affinities between the two species, noted by Miller (2004). According to Miller, the Ponto-Caspian group is synapomorphic with the Italian freshwater *Padogobius* in possessing a higher number of vertebrae (29–35), but also having a reduced or atrophied swimming bladder, and reduced scapula. The most recent molecular phylogenies agree in clustering the Ponto-Caspian gobies with other Mediterranean gobies belonging to the genera *Gobius*, *Padogobius*, *Zosterisessor* and *Zebrus* (Thacker & Roje, 2011; Agorreta *et al.*, 2013; Fig. 3). The results of the present study support these phylogenies on an acoustic basis, as *Neogobius* clustered with both the *Padogobius* species and *Gobius paganellus*, i.e. species producing vocalizations with a high pulse repetition rate, that result in an acoustic tonal structure, and the main separation among these species, primarily the tonal component of *Padogobius bonelli*, was due to differences in peak frequency. In contrast, the comparative analysis conducted here revealed that *Zosterisessor ophiocephalus* and *Gobius niger* formed a separate cluster on an acoustic basis, due to the lower pulse repetition rate and the consequent pulsatile nature of their sounds. This acoustic affinity parallels the molecular affinities revealed by recent phylogenic studies, as these two species clustered together as a sister group (Huyse *et al.*, 2004; Thacker & Roje, 2011; Agorreta *et al.*, 2013; Fig. 3). Thus, our results suggest strongly that the pulse repetition rate, i.e. the degree to which pulses are stacked together in producing a continuum from pulsatile to tonal sounds, is the most important property in species discrimination, and that this acoustic property could contain a certain degree of a phylogenetic signal. A robust phylogenetic analysis based on goby acoustics was beyond the scope of the present

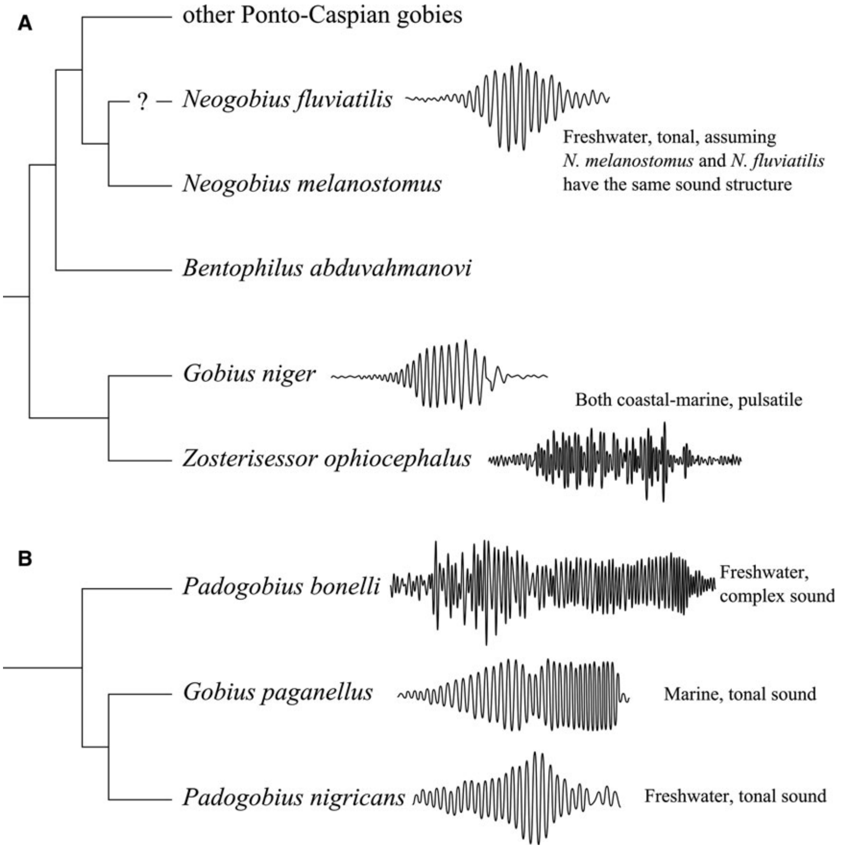

Figure 3. Two clusters extracted and adapted from Thacker & Roje (2011) (A) and Huyse *et al.* (2004) (B) showing the relationships between certain soniferous gobies, and the wave forms of their typical sounds.

analysis, due to the lack of intervening species in the available molecular phylogenies, and an exact parallelism between documented acoustic production and resolved systematic position of the different species.

Nevertheless, both the present study and the analysis by Malavasi *et al.* (2008), conducted at a higher phylogenetic scale in comparing the *Gobius* and the *Pomatoschistus* lineages (*sensu* Agorreta *et al.*, 2013) on an acoustic basis, seem to indicate that affinities in sound production, specifically in the pulse repetition rate, correlate with phylogenetic relationships. Alternative hypotheses are related to convergent evolution or rapid evolution due to sexual selection, as observed for vocalizing groups of tetrapods, especially anurans (Robillard *et al.*, 2006; Tavares *et al.*, 2006; Cap *et al.*, 2008; Gingras *et al.*, 2013). To discriminate the contribution of phylogenetic signal from that of evolutionary convergence, analyses relating the molecular affinities and acoustic affinities in the same group of species are needed, as well as a full clarification of the emission mechanisms in the different species. The only experimental study on a sound emission mechanism available for a goby species is that recently provided by Parmentier *et al.* (2013), conducted on *Gobius paganellus*, which suggested that sound might be generated by the periodic contraction of the *levator pectoralis* muscle. This study, together with the observations reported by Lugli & Fine (2003), suggests that the swimbladder is not involved in sound emission. Note that both *N. fluviatilis* and *Padogobius nigricans*, i.e. gobies producing pure tonal sounds, lack a swimbladder. By contrast, many other goby species have retained the swimbladder, which poses an interesting question about the evolutionary pattern of swimbladder loss or retention in this group. In light of these considerations, a comparative analysis of the sound emission mechanism in gobies is urgently needed to depict the phylogeny of acoustic communication in this fish group. Assuming that the acoustic affinities relate to phylogeny in the *Gobius* lineage, certain preliminary hypotheses on the natural history of this lineage can be presented.

The acoustic clustering of *N. fluviatilis* with other species producing tonal sounds, i.e. the *Padogobius* complex and *Gobius paganellus*, in turn clustered together according to the molecular phylogenies provided by Penzo *et al.* (1998) and Huyse *et al.* (2004), suggest a common ancestor for this group of species. According to Penzo *et al.* (1998), a possible hypothesis is a Ponto-Caspian ancestor that migrated through the Pannonian channel to the Mediterranean basin in the early/middle Miocene (24–15 Mya). According to this scenario, during the Messinian salinity crisis (5.5 Mya), when virtually the entire Mediterranean Sea was desiccated, several hyper- and hypo-saline lakes appeared (Huyse *et al.*, 2004). Those hypo-saline lakes became refuges for the euryhaline species within them. Those species had to adapt to the new environmental conditions, i.e. a freshwater lifestyle. Such isolation probably gave rise to the freshwater endemism existing today in the Mediterranean (Miller, 1990). With the opening of the Strait of Gibraltar (5.33 Mya), and subsequent re-flooding of the Mediterranean basin, the Mediterranean again became a marine habitat. Due to its distribution, it is possible that this common ancestor had to adapt then to the new environment, i.e. a marine lifestyle. Adaptation to the new and free ecological niches probably led to radiation resulting in the present-day goby fauna. A similar evolutionary Messinian and post-Messinian scenario with an ancestral freshwater life style and a derived marine life style was proposed by Malavasi *et al.* (2012) for a group of Mediterranean sand gobies on the basis of behavioural phylogeny. Although preliminary and, to certain degree still speculative, these scenarios indicate that acoustic properties should not be neglected when reconstructing phylogenetic pathways.

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Comparative analysis of sound production between the bighead goby *Ponticola kessleri* and the round goby *Neogobius melanostomus*: Implications for phylogeny and systematics

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Abstract Divergence in acoustic traits between closely related species can be explained by phylogenetic history. In gobies, phylogenies reconstructed with acoustic signals primarily overlap with studies based on morphological or molecular data. Here, sound production of the two Ponto-Caspian gobies, *Neogobius melanostomus* and *Ponticola kessleri*, was recorded in controlled conditions and compared to determine the degree of interspecific acoustic variation across benthophilin gobies. Both species produced tonal-like sounds characterized by unique temporal and spectral properties during agonistic and reproductive intraspecific interactions, while the acoustic comparison revealed that the vocalizations of these two species differ in almost every acoustic property. *N. melanostomus* vocal structure was characterised by short (c. 100 ms), low-frequency (< 100 Hz) tonal sounds repeated at a relatively faster rate, while *P. kessleri* sounds appeared as a broadband, downward frequency modulated longer calls (c. 450 ms). Some acoustic features (sound rate and duration) proved to be stereotyped and could be considered a species-specific trait that could potentially be utilized to discriminate males or used by females for mate assessment. The tonal sounds appear to have a deeper origin within the Benthophilinae subfamily, as all acoustically investigated species to date have been able to produce this sound type. The recorded vocal repertoire represents a baseline for future comparative acoustic studies among the benthophilin gobies, aiming to gain additional information on the evolution of acoustic communication within Ponto-Caspian gobies and highlighting their importance in reconstructing phylogenetic relationships.

Keywords Ponto-Caspian gobies · Acoustics · Tonal · Phylogeny

Introduction

Determining species identity can be an important task for related species in sympatry (Zeyl et al. 2016). Species identity may be encoded by acoustic signals (Myrberg et al. 1978; Spanier, 1979; Crawford 1997; Lobel 1998) and several examples demonstrating divergence in acoustic traits between closely related species can be found in different vocal taxa (Robillard et al. 2006; Cap et al. 2008; Gingras et al. 2013; Hamao et al. 2015). In many different groups of vocal animals, phylogenies reconstructed with acoustic signals are congruent with studies based on morphological and molecular data (Cocroft and Ryan 1995; Laiolo and Rolando 2003;

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Robillard and Desutter-Grandcolas 2004; Malavasi et al. 2008; Rice and Bass 2009; Horvatić et al. 2015). In fishes, the auditory system plays a crucial role in their life history, enabling them to interpret information from the acoustic environment, mainly for predator and prey detection (Remage-Healey and Bass 2006), and for inter- and intraspecific communication (Bradbury and Vehrencamp 1998; Malavasi et al. 2008; Colleye et al. 2011; Amorim et al. 2013; Mélotte et al. 2016). Due to the instinctual and stereotyped nature of their vocalizations, teleost sounds are useful for phylogenetic reconstructions (Malavasi et al. 2008; Amorim et al. 2011). Within gobies (Gobiiformes; Gobiidae), drumming, stridulatory and tonal sounds are emitted by the male as a part of the breeding and aggressive behavioural repertoire (Torricelli et al. 1990; Lugli et al. 1997; Lugli and Torricelli 1999; Malavasi et al. 2003; Myrberg and Lugli 2006; Amorim and Neves 2007). In recent decades, the vocal repertoire has been described for at least 23 species of gobies, primarily belonging to the Atlantic and Mediterranean genera *Gobius*, *Padogobis*, *Zosterisessor*, *Knipowitschia* and *Pomatoschistus* (Lugli et al. 1997; Lugli et al. 2003; Malavasi et al. 2008; Malavasi et al. 2009; Lugli 2010; Zeyl et al. 2016). Within the Ponto-Caspian lineage, however, few species have been acoustically tested (*Neogobius melanostomus, N. fluviatilis and Proterorhinus marmoratus*) (Ladich and Kratochvil 1989; Rollo et al. 2007; Horvatić et al. 2015), and the vocal repertoires of most species remain unknown. In addition, the systematic position and phylogenetic relationships of Ponto-Caspian gobies have been disputed and show certain unresolved aspects. Berg (1949) separated the relict Sarmatian gobies from the genus *Gobius* into a single genus *Neogobius*. Over the years, the genus *Neogobius* proved to be paraphyletic, and recently, Neilson and Stepien (2009) grouped all Ponto-Caspian gobies into a monophyletic lineage within the subfamily Benthophilinae, further dividing them into three well-supported tribes: Neogobiini (genus *Neogobius*), Ponticolini (genera *Babka*, *Mesogobius*, *Ponticola*, and *Proterorhinus*), and Benthophilini (genera *Benthophilus* and *Caspisoma*). Furthermore, Thacker and Roje (2011) examined the molecular interrelationships between many gobiid lineages and recognized all the proposed tribes of benthophilin species from Neilson and Stepien (2009), though with a slightly different position of the tribe Neogobiini, which they placed as a sister group to the Ponticolini tribe. Later, Agorreta et al. (2013) used multiple nuclear and mitochondrial genes to indicate that Ponto-Caspian gobies are deeply nested within the "*Gobius*" lineage, together with other Mediterranean genera, supporting their close relationship. Therefore, while the systematic position and phylogenetic relationships of Ponto-Caspian gobies await clearer resolution, the goal of this study was to investigate the phylogenetic relationships within Benthophilinae species based on the structure of their acoustic signals. The main objective was to record the sound production of two Ponto-Caspian gobies, the bighead goby *Ponticola kessleri* (Günther 1861) and the round goby *Neogobius melanostomus* (Pallas, 1814) under controlled aquarium conditions, and to quantitatively describe the spectral and temporal properties of their acoustic signals. Although round goby sounds have previously been recorded (Rollo et al. 2007), no detailed vocal repertoire of this species was available in the literature. A second goal was to compare the acoustic signals of these two species and to determine the degree of interspecific variation across benthophilin gobies, based on acoustic data. The vocal repertioire of these two species may represent a first insight into future phylogenetic comparisons within the subfamily, and could provide additional information on the evolution of acoustic communication within the Ponto-Caspian gobies.

Materials and methods

Fish collection

Fifteen specimens of *Ponticola kessleri* (140–180, L_T, mm, seven males and eight females) and 15 specimens of *Neogobius melanostomus* (120–170, L_T, mm, nine males and six females) were collected from the Drava River near Osijek, (GPS coordinates: 45°33′45.73’ N, 18°42′1.37″E) and from the Sava River downstream of Zagreb (GPS coordinates: 45°44′19.28”N, 16°13′48.40″E), respectively. Individuals were caught using electrofishing (electric unit, power: 7.5 kW) in spring 2016 and 2017 from artificial riprap systems.

Fish housing and experimental procedure

Upon arriving to laboratory, fish were sexed on the basis of urogenital papilla (Miller 1984) and housed in suitable aquaria, either in experimental tanks or in the community tank. All tank temperatures ranged from 16 to 22 °C throughout the observation period. Each tank was equipped with a filtration system, 5–10 cm thick sand layer and aerators to maintain proper oxygen levels. Artificial shelters made of tiles (10 × 20 cm) were placed in each tank to serve as a nest. Experimental tanks (90 L) were sound-proofed from surrounding noise using a thick layer of acoustically isolating material (foam rubber shims). The experiments were carried out during the reproductive season of both investigated species, from March until August 2016 and 2017, and outside the reproductive season following the protocol proposed by Malavasi et al. (2008). The "Intruder test" was set up to elicit sounds in male–male, female–female or female–male aggressive interactions (aggressive behaviour) and male–female (courtship or spawning behaviour) reproductive interactions. For the experiment, the tested resident fish (either male or female) was first measured (L_T) with a ruler and then placed indivdually into experimental tanks (90 L) and left to establish territorial behaviour for about three days before the beginning of recordings. The remaining individuals from both species were separated by sex, and placed in four larger community glass-tanks (capacity 300 L) to be used as the intruder during the experiments. Lights and all pumping devices were switched off 10–15 min before the beginning of experiments to minimize external noise. Prior to the start of recording, an intruder (either of the same or opposite sex, depending on the interaction being tested) was placed in a metal cage and lowered into the tank and positioned in front of the shelter entrance in order to elicit behavioural and acoustic responses. Each audio recording lasted 30 min from the emission of the first sound and during the experiments, the acoustic signals were noted. After the experiments, water temperature was measured with a digital thermometer and the intruder fish was placed back in the community tank, and the process was repeated with two other individuals during one recording session. Once the resident had fought against all the other intruders, it was replaced in the experimental tank by another fish. Each individual was tested acoustically at least twice, though in inverted roles (resident vs intruder), waiting at least five days between interactions, to avoid the effects of the previous outcome (Torricelli et al. 1988; Sebastianutto et al. 2008).

Acoustic methods

Sounds were recorded using a High Tech Inc. hydrophone with built-in preamplifier (HTI 94 SSQ, sensitivity: −165 re 1 VμPa^{-1}, frequency response from 2 Hz to 30 kHz) positioned about 10 cm from the entrance of the nest to avoid signal attenuation and alteration due to tank reflections and reverberation, according to Akamatsu et al. (2002). Acoustic signals for analysis were recorded using a portable digital audio recorder (ZOOM H4n; wav 16/44.1 k mono), stored on the recorder memory card and monitored on headphones. Analog signals were digitalized at 4000 Hz (16 bit) and analysed using Avisoft-SASLab Pro 5.2 Software (512-point fast fourrier transform FFT, Hamming window). All sounds were band-pass filtered (band: 50–500 Hz) to eliminate background noise and + 10 dB amplification was applied for better auditory and visual inspection of the audio tracks. Only sounds with a higher signal to noise ratio were analysed. Temporal features were measured from the oscillogram while frequency variables were obtained from the power spectra (filter bandwidth 300 Hz, 256 points FFT, frame size 100%, time overlap 96.87% and a Hamming window). The following acoustic properties were measured: (1) sound rate (SR; sounds min^{-1}); (2) duration (DUR; milliseconds); (3) number of pulses (NP); (4) pulse repetition rate (PRR; obtained by dividing NP by DUR and expressed in Hz); (5) peak frequency (PF; Hz); (6) frequency modulation (FM; after the sound has been divided on three sections, frequency modulation was calculated as the difference between final PRR and initial PRR and expressed in Hz).

Data analysis

We calculated the mean and standard deviation (x ± SD) for each of the six acoustic variables emitted by individual fish of both species and tested them for inter- and intraspecific differences. Sound features were examined using from five to ten sounds with high sound quality/noise ratio for each subject. Since the assumption of normality was not met for all acoustic parameters (Kolmogorov-Smirnov test, $P < 0.05$), non-parametric Kruskal-Wallis ANOVA by Ranks followed by subsequent post hoc multiple comparison test was used to investigate variation of acoustic properties between individuals of each species (intraspecific variation). Differences in the mean acoustic properties of vocal subjects between different behaviour contexts ("reproductive" vs. "aggressive) and species (*N. melanostomus* v. *P. kessleri*) were tested using the Mann-Whitney U test on untransformed data. Since the reproductive and aggressive sounds produced by *N. melanostomus* did not differ

(Mann-Whitney *U* test, $P > 0.05$), the mean values were pooled together and used as a single set of acoustic variables for further comparative analysis. In addition, individual means of the six acoustic properties (SR, DUR, NP, PRR, PF, FM,) were tested against each other for correlation using the Spearman rank correlations, and then subsequently related to size (L_T) and temperature to check for the dependency of sound structure on these two physical characteristics. To examine individual acoustic differences (stereotypy) among individuals of both species, the *CVb/CVw* ratio was examined. We calculated the mean within-individual (*CVw*), and between-individual (*CVb*) coefficients of variation (CV=SD/x) × 1000; on untransformed data), utilizing overall means and SDs for each acoustic variable using the previously calculated mean values for each subject. A ratio of >1.0 suggests that the acoustic parameter is more variable between individuals relative to its variability within individuals, and could be used as a cue for individual discrimination and stereotypy (Christie et al. 2004). All statistical analyses were carried out with STATISTICA 13 (Statsoft Inc., Tulsa, OK, USA). Results were considered significant at $P < 0.05$.

Results

Neogobius melanostomus

All seven resident males of *Neogobius melanostomus* produced short vocalizations during both reproductive and agonistic intraspecific interactions. No females produced sounds during the acoustic experiments, neither in the resident nor intruder role, while males in the intruder role also did not produce sounds. Three males were soniferous during only one context (two reproductive, one aggressive), while the remaining four individuals produced sounds during both reproductive and aggressive interactions (Table 1). Each sound is part of a long sequence and characterized by intra-sound amplitude variation, which progressively increased throughout first two-thirds of the sound duration, and then rapidly dampened (Fig. 1). Vocalizations were short and lasted from 50.5 to 217.1 ms (mean ± S.D.) (114.8 ± 10.4 ms) composed from five to 15 pulses (9.2 ± 0.4) (Table 2). Spectrographically, most of the sound energy appeared as a single tone band within the range from 50 to 200 Hz (Hz), with a maximum amplitude represented at the peak frequency, ranging from 52 to 128 Hz (86 ± 6.5 Hz) (Table 2). Moreover, pulse repetition rate corresponded to peak frequency and ranged from 55.9 to 124.8 Hz (87.2 ± 5.9 Hz) (Table 2), suggesting that these sounds are purely tonal. Frequency modulation ranged from −49.1 to 56.3 Hz (3.5 ± 5.9 Hz), indicating slight upward modulation of the sounds. Sound rate ranged from one to 46 sounds per minute (10.1 ± 4.4), and it would speed up as the intruder approached the nest entrance (Table 2). The comparison between individual means of acoustic signals revealed that all sound features differed significantly between *N. melanostomus* males (Kruskal-Wallis test: d.f. = 6 $n = 88$, all tests $P < 0.05$). From the six acoustic features, DUR was highly and positively correlated with NP ($r_s = 0.8$, $P < 0.05$) and negatively with PRR ($r_s = -0.8$, $P < 0.05$) and with PF ($r_s = -0.8$, $P < 0.05$). In addition, PF was positively correlated with PRR ($r_s = 0.9$, $P < 0.05$). There were no statistically significant correlations between acoustic properties and water temperature or body size, due likely to the restricted range of values of these two physical parameters (Spearman correlation, $P > 0.05$). Strong stereotypy was found for SR, DUR, and NP, which were more variable among- than within-individuals (*CVb/CVw* > 1.0, Table 3).

Ponticola kessleri

All *Ponticola kessleri* males emitted sounds during the territory defence interactions, in an agonistic context, while no females produced any sounds during trials. The same males were retested for sound production within a reproductive context, with a female intruder, but none were vocal during courtship or pre-spawning trials. Acoustic signals were produced by both resident or intruder fish and vocalizations were recorded from nine vocal males in total (Table 1). Sounds were produced singly, or more commonly, in short sequences at a relatively low rate, with the sound rate ranging from one to five (mean ± S.D.) (2.5 ± 0.7) (Table 2). Signals were relatively long in duration, which ranged from 189.1 to 879.8 ms (457.9 ± 54.4 ms) and composed from 17 to 72 pulses (44.9 ± 4.4), yielding a continuous tone-like waveform (Fig. 2, Table 2). Energy ranged from 80 to 400 Hz (Hz), exhibiting broadband nature of the sound with a strong first harmonic. Peak frequency was, in most cases, the fundamental frequency, ranging from 81 to 209.9 Hz (104.9 ± 11.2 Hz) (Table 2), though in some cases, the spectrogram displayed two or three additional spectral harmonic components (< 400 Hz). Pulse repetition rate corresponded to peak frequency and ranged from 64.1 to

Table 1 Total number of individuals acoustically tested and sounds analysed for each species with behavioural context (aggressive and reproductive) and experimental role (resident and intruder)

Male, TL (mm)	Experimental role (resident/intruder)	Sound production context	n. of sounds recorded	Temp. °C
Neogobius melanostomus				
#1, 161	Resident	Reproductive	60	22.1
#2, 144	Resident	Aggressive	6	22.0
		Reproductive	13	22.2
#3, 125	Resident	Aggressive	5	22.1
		Reproductive	5	21.7
#4, 155	Resident	Aggressive	27	21.8
		Reproductive	14	21.2
#5, 145	Resident	Aggressive	17	21.5
		Reproductive	18	21.1
#6, 135	Resident	Reproductive	43	19.1
#7, 154	Resident	Aggressive	52	18.0
Ponticola kessleri				
#1, 146	Resident	Aggressive	27	17.5
#2, 149	Resident	Aggressive	26	19.3
#3, 147	Resident	Aggressive	50	19.6
#4, 176	Intruder	Aggressive	32	19.6
#5, 170	Resident	Aggressive	34	20.9
	Intruder	Aggressive	13	
#6, 171	Resident	Aggressive	46	17.9
#7, 166	Intruder	Aggressive	2	19.3
#8, 140	Intruder	Aggressive	6	17.7
#9, 154	Resident	Aggressive	12	22.3
	Intruder	Aggressive	27	

Values of both water temperature (mean, °C) and emitter's size (total length, mm) are reported. For each individual, from five to ten sounds, exhibiting good signal to noise (S/N) ratio, were analyzed and subsequently used in further inter- and intraspecific analysis. Individual #7 was subsequently excluded from inter- and intraspecific analysis due to small sample size, i.e. only two sounds recorded

128.8 Hz (99.9 ± 5.6 Hz), while the frequency modulation indicated downward modulation of sounds and ranged from −39.2 to 38.3 Hz (−9.1 ± 6.8 Hz) (Table 2). At the intraspecific level, all investigated sound properties differed significantly across *P. kessleri* individuals (Kruskal-Wallis test: d.f. = 7, $n = 75$, all $P < 0.05$) except for SR (Kruskal-Wallis test: d.f. = 7, n = 75, all $P > 0.05$). DUR was positively correlated with NP ($r_s = 0.7$, $P < 0.05$) while negatively with water temperature ($r_s = -0.6$, $P < 0.05$). NP was also negatively correlated with water temperature ($r_s = -0.7$, $P < 0.05$). Lastly, PRR was positively correlated with PF ($r_s = 0.7$, $P < 0.05$). Of the six acoustic properties of tonal segments, SR, DUR, PF and FM were more variable between- than within-individuals, as all *CVb/CVw* ratios >1.0, indicating a higher level of inter-individual variability (Table 3).

N. melanostomus vs. *P. messleri* sounds

The mean acoustic properties were compared between the species, and SR, DUR, NP, PF and FM were found to differ significantly between species (Mann-Whitney *U* test, $N_1 = 7$, $N_2 = 8$, $P < 0.05$ all tests) while PRR showed no statistically significant differences (Mann-Whitney *U* test, $P > 0.05$). Generally, *P. kessleri* sounds were longer in DUR (Mann-Whitney *U* test: $N_1 = 7$, $N_2 = 8$, U = 0.0, $P < 0.05$) and with higher NP (Mann-Whitney *U* test: $N_1 = 7$, $N_2 = 8$, U = 0.0, $P < 0.05$) and PF (Mann-Whitney U test: $N_1 = 7$, $N_2 = 8$, $U = 11.0$, $P < 0.05$; Fig. 3). Although not significant, *P. kessleri* vocalizations exhibited greater PRR mean values compared to *N. melanostomus* (Fig. 3), which in turn, had greater FM values than *P. kessleri*

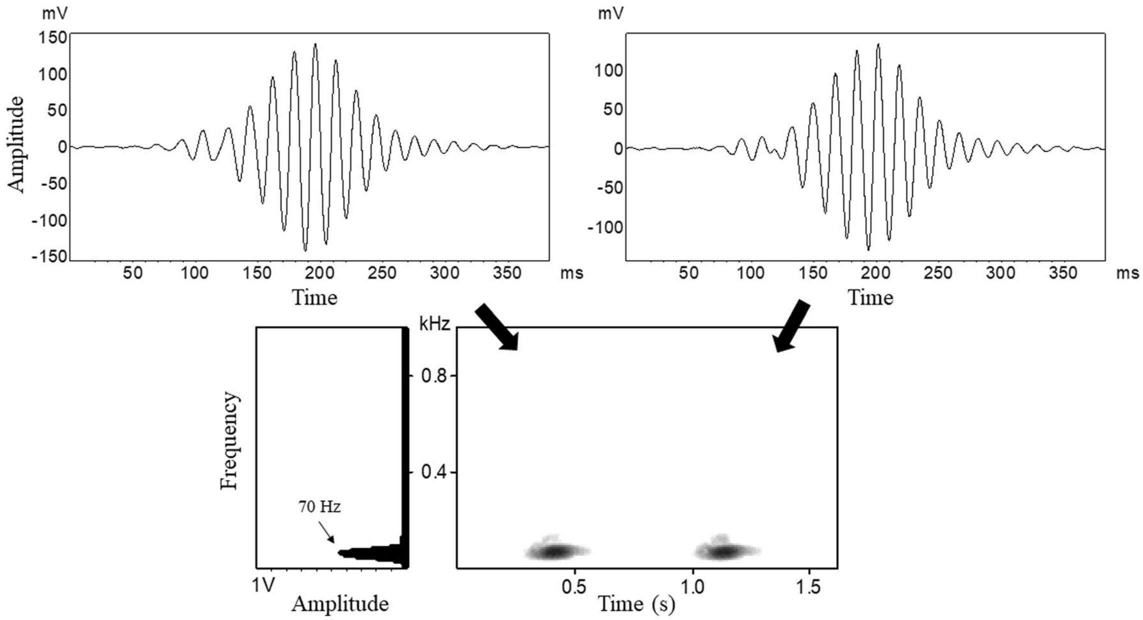


Fig. 1 Temporal and spectral details of two consecutive vocalizations produced during reproductive interactions by resident *Neogobius melanostomus* (male #2, 144 mm L_T). Above: oscillograms (left and right): below: spectrogram (right) and power spectrum (left) (window type: Hamming; FFT: 512; frame: 100; bandwidth: 10 Hz; resolution: 8 Hz; overlap: 93.75%; filter band: 50–500 Hz). Arrow on the power spectrum refers to the peak frequency of produced sounds sequence

(Mann-Whitney *U* test: $N_1 = 7$, $N_2 = 8$, $U = 5.0$, $P < 0.05$). In addition, SR was also higher in *N. melanostomus* and differed significantly between the two species (Mann-Whitney *U* test: $N_1 = 7$, $N_2 = 8$, $U = 0.0$, $P < 0.05$; Fig. 3), highlighting once again the short duration of *N. melanostomus* sounds.

Discussion

The present study showed that the two investigated Ponto-Caspian species previously placed in the same genus *Neogobius* sensu (Berg, 1949), *Neogobius melanostomus* and *Ponticola kessleri*, produced distinct

Table 2 Descriptive statistics of the tonal sounds recorded from round goby *Neogobius melanostomus* and bighead goby *Ponticla kessleri* males

Acoustic variable	*Neogobius melanostomus*			*Ponticola kessleri*			
	x ± SD	Range	n	x ± SD	Range	n	*P* value
Sound rate (sounds per min.)	10.1 ± 4.4	1–46	7	2.5 ± 0.8	1–5	8	**< 0.05**
Duration (ms)	114.8 ± 10.4	50.5–217.1	7	457.9 ± 54.5	189.1–879.8	8	**< 0.05**
Number of pulses	9.2 ± 0.4	5–15	7	44.9 ± 4.5	17–72	8	**< 0.05**
Pulse repetition rate (Hz)	87.2 ± 5.9	55.9–124.8	7	99.9 ± 5.6	64.2–128.9	8	> 0.05
Peak frequency (Hz)	86 ± 6.5	52–128	7	105 ± 11.2	81–209.9	8	**< 0.05**
Frequency modulation (Hz)	3.5 ± 13.5	−49.1 – (+56.3)	7	−9.2 ± 6.9	−39.2 – (+38.3)	8	**< 0.05**

Individual means of the acoustic features of both species were compared with the Mann–Whitney *U* test

P values refer to the results of the non-parametric Mann-Whitney *U* test, n-number of individuals acoustically tested. Sounds from *N. melanostomus* produced within reproductive and aggressive context were first compared against each other to test for differences, and since there were none based on all acoustic properties (Mann-Whitney *U* test, $P > 0.05$), they were pooled and compared with *P. kessleri* vocalizations

Bold values indicate the sound properties that differed significantly between two investigated species except for PRR, emphasizing the acoustic differences in produced vocalizations

Table 3 Within male variability (*CVw*) and between-male variability (*CVb*) for the six acoustic variables analysed for seven *Neogobius melanostomus* males and eight *Ponticola kessleri* males

Acoustic variable	*Neogobius melanostomus*			*Ponticola kessleri*		
	CVw	*CVb*	*CVb/CVw*	*CVw*	*CVb*	*CVb/CVw*
Sound rate (sounds per min)	0.32	0.33	**1.05**	0.29	0.36	**1.23**
Duration (ms)	0.17	0.17	**1.03**	0.23	0.23	**1.01**
Number of pulses	0.14	0.15	**1.01**	0.21	0.20	0.97
Pulse repetition rate (Hz)	0.11	0.11	0.95	0.09	0.09	0.99
Peak frequency (Hz)	0.16	0.15	0.95	0.11	0.11	**1.06**
Frequency modulation (Hz)	−1.13	3.80	−3.34	−1.07	−1.25	**1.17**

From five to ten sounds were analysed for each male of both species. Since no significant difference was found between aggressive and reproductive sounds produced by *N. melanostomus* (Man-Whitney *U* test; $P > 0.05$), the data were pooled and analysed together for the overall mean and standard deviation, and subsequently for CV coefficients and *CVb/CVw* ratio

Bold features represent the acoustic variables which are more variable among- than within-males (CVb/CVw > 1), indicating that these features could potentially discriminate individuals

vocalizations during intraspecific aggressive and reproductive interactions, showing well distinguishable species-specific sound structures. The *Neogobius* structure is characterised by short, low-frequency tonal sounds, while the *Ponticola* sounds appear as a broadband, frequency modulated longer call. According to Amorim et al. (2013), stereotyped features (*CVb/CVw* > 1.0) could potentially discriminate males and/or be used by females for mate assessment. In this study, there was strong stereotypy in the tonal sounds produced by both *N. melanostomus* and *P. kessleri*, since certain temporal acoustic features (mainly DUR and SR) showed larger between- than within-individual variation, and therefore presented a potential role for acoustic communication

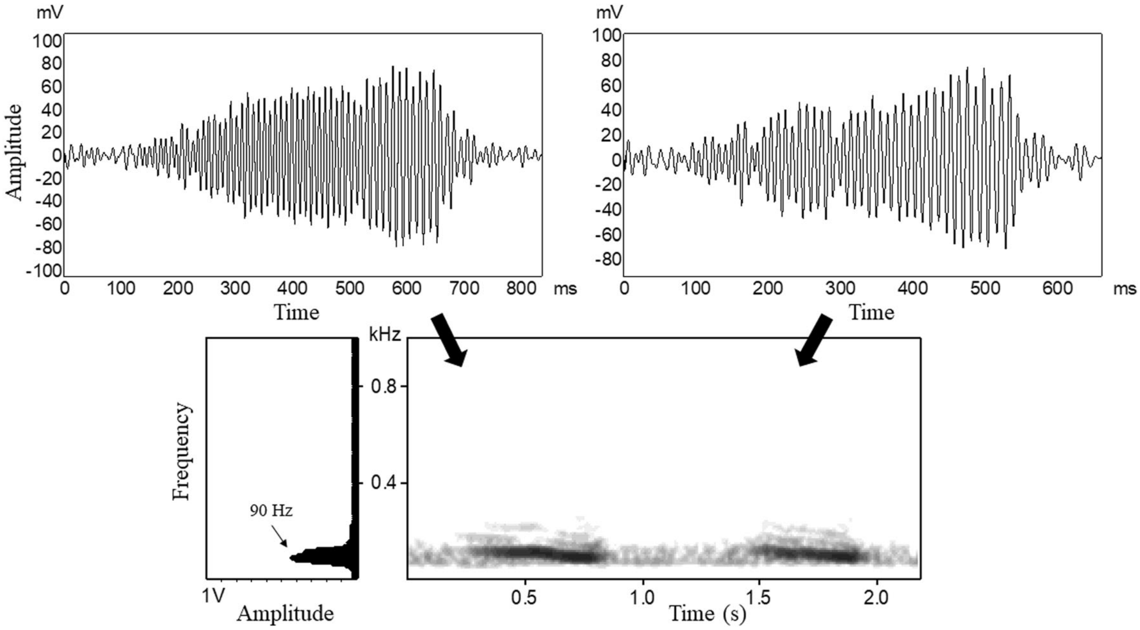


Fig. 2 Temporal and spectral details of two consecutive vocalizations produced during aggressive interactions by resident *Ponticola kessleri* (male #6, 171 mm L_T). Above: oscillograms (left and right): below: spectrogram (right) and power spectrum (left) (window type: Hamming; FFT: 512; frame: 100; bandwidth: 10 Hz; resolution: 8 Hz; overlap: 93.75%; filter band: 50–500 Hz). Arrow on the power spectrum refers to the peak frequency of produced sounds sequence. Grey area on the spectrogram indicates low-frequency ambient noise (50–100 Hz) captured with the hydrphone during aggressive trials

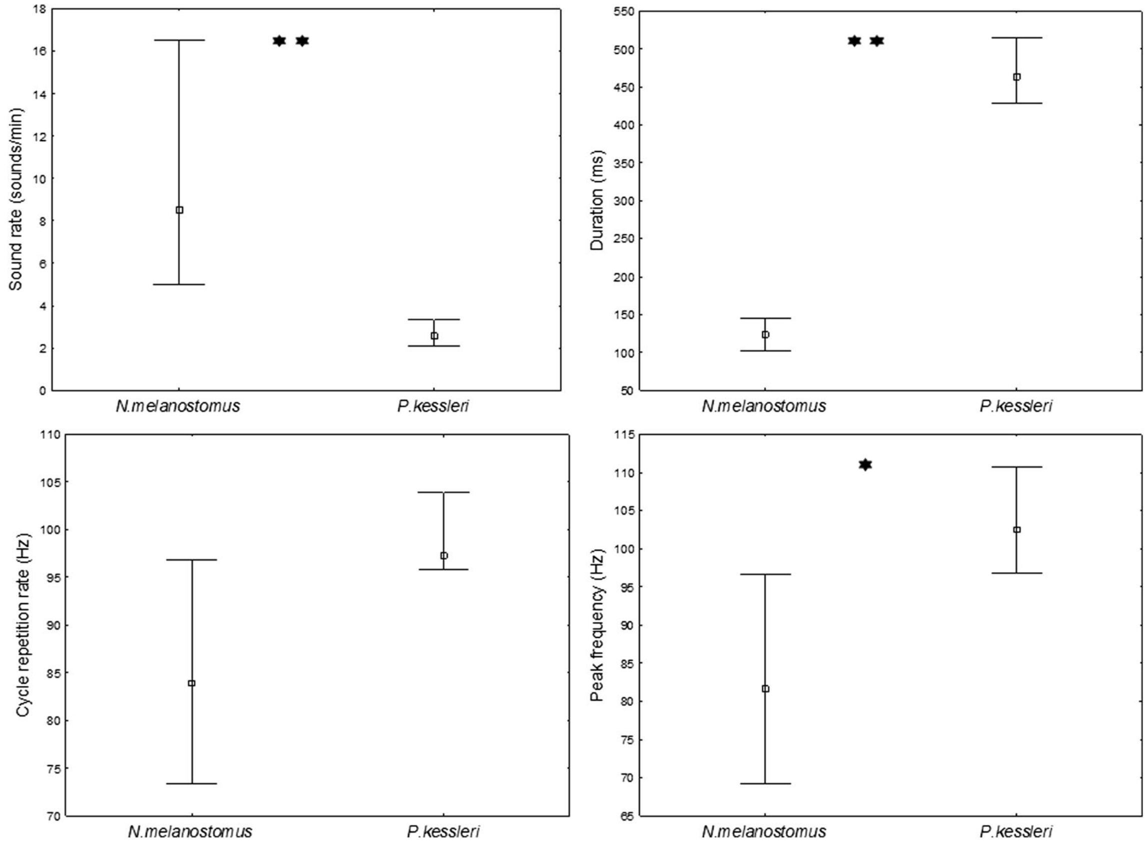
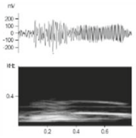
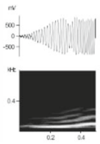
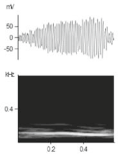
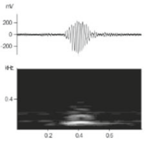
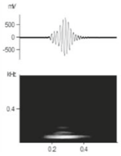
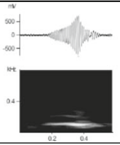


Fig. 3 Comparison between *Neogobius melanostomus* and *Ponticola kessleri* for sound rate, sound duration, cycle repetition rate and peak frequency. * and ** indicate significant difference (Mann-Whitney *U* test, $*P < 0.05$; $**P < 0.01$) Medians (point) and percentiles (whisker value) are depicted

and individual discrimination. At the interspecific level, communicative vocalizations differed markedly according to acoustic parameters and sound structures, indicating that each species possess fine-tuned temporal and spectral characteristics enabling them to be differentiated between conspecifics. In this sense, *N. melanostomus* calls can be recognized as short tonal sounds organised in long series and intensively repeated (high sound rate), while *P. kessleri* produces longer calls, which are downward frequency modulated and rarely produced in long trains, more frequently singly (low sound rate). The higher sound rate of *N. melanostomus* sounds seems to indicate that these shorter sounds could be produced more easily than longer frequency modulated calls, without great energy consumption. However, future studies dealing with the metabolic cost of sound production are needed to clarify this hypothesis. In addition, we suggest that sound rate, showing a stereotyped nature, could be an informative acoustic trait for *N. melanostomus*, since the only other acoustically tested congeneric species, *Neogobius fluviatilis*, produces the same sound type but with less repetition within the same time frame (Horvatić et al. 2015), again confirming that congeneric species can be differentiated according to acoustic signals. In *P. kessleri*, sounds are longer and scarcer, and this study indicates their information content is contained within the frequency modulation, which also proved to be stereotyped, rather than in a series of repeated calls. It is worth mentioning that the sound emission rate could be a reliable indicator of male size, condition and motivation (Bass and McKibben 2003; Amorim et al. 2013), while Mitchell et al. (2008) proposed that calling rate may be limited by sonic muscle fatigue. In addition, long duration tonal sounds are expected to generate fatigue more quickly, as supported in the boatwhistles of Gulf toadfish, *Opsanus beta* (Thorson and Fine 2002). While physiological studies remain to be conducted, in this study, *N. melanostomus* emitted calls during both aggressive and reproductive contexts, which could partially explain

Table 4 Sound structure (oscillogram-above; spectrogram-below), systematic position, phylogenetic relationship and distribution of soniferous Ponto-Caspian and some Mediterranean gobies

Species	Systematic position and phylogeny	Distribution	Sound structure	Acoustic properties	References
Padogobius bonelli (Bonaparte, 1846)	Gobiidae[a]; *Gobius* – lineage[b]; proposed Ponto-Caspian origin[c]	Northern Adriatic basin: (Po River basin, Italy) and (Istrian Peninsula; Zrmanja and Krka River basins, Croatia); introduced into rivers of the west coast of Italy		Long duration, downward frequency-modulated calls repeated with high intensity	Lugli et al. (1997), Lugli et al. (2003), and Malavasi et al. (2008) (acoustics); Penzo et al. (1998), Huyse et al. (2004) (genetics)
Gobius paganellus Linnaeus, 1758	Gobiidae[a]; *Gobius* – lineage[b]; Mediterranean and North-Eastern Atlantic gobies[c]	Eastern Atlantic Ocean, Mediterranean Sea and Black Sea		Long duration, upward frequency-modulated calls repeated with high intensity	Malavasi et al. (2008) and Parmentier et al. (2013) (acoustics); Penzo et al. (1998), Huyse et al. (2004) (genetics)
Ponticola kessleri (Günther, 1861)	Gobiidae[a]; *Gobius* – lineage[b]; Ponto-Caspian gobies[c]; Ponticolini[d]	Black Sea basin; invasive in western and central Europe		Long duration, downward frequency-modulated calls repeated with low intensity	This study (acoustics); Neilson & Stepien (2009), Medvedev et al. (2013) (genetics)
Proterorhinus marmoratus (Pallas, 1814)	Gobiidae[a]; *Gobius* – lineage[b]; Ponto-Caspian gobies[c]; Ponticolini[d]	Black Sea basin; invasive in Danube basin since the 1970s	Not available	Medium long, probably repeated in series of moderate intensity	Ladich & Kratochvil (1989) (acoustics); Stepien & Tumeo (2006), Neilson & Stepien (2009) (genetics)
Neogobius fluvitilis (Pallas, 1814)	Gobiidae[a]; *Gobius* – lineage[b]; Ponto-Caspian gobies[c]; Neogobiini[d]	Azov and Black Sea basin; invasive in western and central Europe		Short duration, upward frequency-modulated calls repeated with moderate intensity	Horvatić et al. (2015) (acoustics); Neilson & Stepien (2009), Medvedev et al. (2013), Šanda & Kovačić & Šanda (2016) (genetics)
Neogobius melanostomus (Pallas, 1814)	Gobiidae[a]; *Gobius* – lineage[b]; Ponto-Caspian gobies[c]; Neogobiini[d]	Azov, Black and Caspian Sea basins; invasive in eastern, western and central Europe and Great Lakes (North America)		Short duration, upward frequency-modulated calls repeated with high intensity	This study (acoustics); Neilson & Stepien (2009), Medvedev et al. (2013), Šanda & Kovačić & Šanda (2016) (genetics)
Padogobius nigricans (Canestrini, 1867)	Gobiidae[a]; *Gobius* – lineage[b]; proposed Ponto-Caspian origin[c]	Central west Italy, rivers Arno, Ombrone, Tiber, Amaseno		Medium long, upward frequency modulated calls repeated with high intensity	Lugli et al. (1996) (acoustics); Penzo et al. (1998), Huyse et al. (2004) (genetics)

[a] According to Thacker (2011)

[b] According to Agorreta et al. (2013). Ponto-Caspian gobies were deeply nested within the Gobius-lineage, together with other large Atlantic-Mediterranean gobies

[c] According to Thacker and Roje (2011). Authors subdivided the European gobies into three groups: Ponto-Caspian gobies, Mediterranean and North-Eastern Atlantic gobies

[d] According to Neilson and Stepien (2009). Authors subdivided subfamily Benthophilinae on three well supported tribes: Ponticolini, Neogobiini and Benthohpilini

the higher sound rate compared to *P. kessleri* sounds, since it is well know that sound rate increases when the emitter is courting or facing a potential female intruder (Mann and Lobel 1995; Amorim 2006). Regardless of inter- or intrasexual interactions, the general pattern of interspecific differences remains the same, taking into consideration the sound complexity and sound structure. Our results are consistent with Malavasi et al. (2008), who suggested that the temporal patterning of sounds, i.e. DUR and PRR, are the acoustical properties responsible for discriminating between the Mediterranean gobies. Acoustic differences from this study seem also to be consistent with previous phylogenetic studies, which separated species from the former *Neogobius* genus into additional tribes, namely Neogobini (*Neogobius melanostomus*, *N. fluviatilis* and *N. caspius*), Benthophilini (tadpole gobies) and Ponticolini (genera *Mesogobius*, *Proterorhinus*, *Babka*, and *Ponticola*) (Neilson and Stepien, 2009; Medvedev et al. 2013). In addition, our results indicate that there is a strong similarity of *N. melanostomus* sounds with those documented from another vocal con-generic species, i.e. the monkey goby *N. fluvitalis* (Horvatić et al. 2015). From a comparative perspective, the two *Neogobius* species, sharing a close genetic and morphological relationship (Miller 2003; Neilson and Stepien 2009; Medvedev et al. 2013; Kovačić and Šanda 2016), were able to produce same sound type, with a strong overlap in the general structure and acoustic behaviour. While this resemblance in acoustic features is not so surprising for con-generic species, more striking is the similarity of acoustic signals for the two *Neogobius* species and another vocal Mediterranean species, the Arno goby, *Padogobius nigricans*. *P. nigricans* is an endemic Italian freshwater fish, geographically well separated from rest of the Ponto-Caspian basin (Miller 2004). The oscillogram and the power spectrum of *P. nigricans* and *Neogobius* spp. sounds mostly overlap, with mean acoustic values varying slightly between species (Lugli et al. 1996, 1997). According to morphological studies, a close relationship between the genus *Padogobius* and the Ponto-Caspian clade was previously suggested on the basis of morphological traits (Bianco and Miller 1990; Simonovic et al., 1996; Miller 2003, 2004), indicating that Ponto-Caspian group is synapomorphic with the Italian freshwater *Padogobius* in possessing a higher number of vertebrae, but also having a reduced or atrophied swim bladder. Interestingly, both *Neogobius* species and *P. nigricans* lack the swim bladder (Lugli et al. 1996; Miller 2004). In contrast, according to literature, many other European gobies sharing a close affinity with *P. nigricans* (i.e., *Padogobius bonelli* and *Gobius paganellus*) have retained the swim bladder through all life stages, which poses an interesting question about the evolutionary pattern of swim bladder loss or retention in this group of species, and possible relationships with sound structure and emission mechanisms. In rocky goby (*G. paganellus*), cranial-pectoral muscles (*m. levator pectoralis*) contractions were responsible for the production of long, frequency modulated tonal sounds (Parmentier et al. 2013) similar to *P. kessleri* vocalizations. Further investigations on sound emission mechanisms should clarify whether this cranio-pectoral system is common to all goby acoustic systems or, alternatively, whether different mechanisms are used by different groups of species with different sound structures, such as the "neogobini" group. These observations raise the question about the systematic position of *P. nigricans*, and its relative *P. bonelli*, another Italian goby endemism, since many authors have previously mentioned that this species corresponds to different genera and that they could be more closely related to Ponto-Caspian lineage than to European gobies (Lugli et al. 1996; Penzo et al. 1998; Huyse et al. 2004; Miller 2004; Kottelat and Freyhof 2007). Observed acoustic similarities parallels the molecular affinities revealed by recent phylogenetic studies, which suggested that Ponto-Caspian and Mediterranean gobies share close phylogenetic relationships (Thacker and Roje 2011; Agorreta et al. 2013; Thacker 2015). In *Proterorhinus marmoratus*, another soniferous Ponto-Caspian species, the vocal repertoire is strictly tonal and both sexes emit short duration (around 250 ms), low frequency sounds, (Ladich and Kratochvil 1989) which appear very similar in structure to vocalizations described for *Neogobius* spp., with minor acoustic differences. According to Neilson and Stepien (2009) and Thacker and Roje (2011), *P. marmoratus* is positioned as a basal member within the tribe Ponticolini, and regarding its acoustic traits, it could represent a transitional taxon between the former tribe and Neogobiini. On the other hand, *P. kessleri* produces vocalizations that on oscillogram mostly resemble the sounds emitted by two vocal Mediterranean species, *G. paganellus* and *P. bonelli* (tonal signals), with minor differences in mean acoustic values (Lugli et al. 1995, 1997; Malavasi et al. 2008; Parmentier et al. 2013). Tonal-like sounds produced by these Mediterranean species are characterized by a high pulse repetition rate (around 100 Hz) of long duration (> 300 ms) (Zeyl et al. 2016), and overlapping in values with the *P. kessleri* vocalizations. Penzo et al. (1998) and Huyse et al. (2004) documented the close affinity between *G. paganellus* and *P. nigricans*, emphasizing that the genus *Padogobius* could be paraphyletic. Considering the present observations, *G. paganellus* acoustically could be more closely related to *P. bonelli* than to *P. nigricans*, again supporting the complex explanation of this phylogenetic relationship. From the results of this study and the existing literature on vocal European gobies, two groups of species producing tonal-like sounds can be recognized (Table 4). Ponto-Caspian *N. fluviatilis, N. melanostomus*, *P. marmoratus* and the Arno goby *P. nigricans* form the first group, while *P. kessleri*, *P. bonelli* and *G. paganellus* constitute the second group of soniferous gobies, differentiated according to their signal properties. By exploring the freshwater lifestyle history of Mediterranean gobies, Penzo et al. (1998) proposed that *Padogobius* spp. and *G. paganellus* originated from the same ancestor, while the former genus retained its freshwater distribution, further subdivided during the Messinian salinity crisis (MSC; 5.9–5.3 Mya), while the latter secondarily recolonized marine habitats and nowadays inhabits the Eastern Atlantic, Mediterranean and Ponto-Caspian area (Kovačić and Patzner 2011). According to this scenario, the Ponto-Caspian ancestor migrated through the Pannonian channel to the Proto-Mediterranean (24–15 Mya) and consequently adapted to new ecological niches during the later stage of MSC known as “Lago Mare” (5.5 Mya), during which habitats gradually alternated from freshwater and brackish (< 5.3 Mya) to exclusively marine (> 5.3 Mya). Although speculative, the relationship between *G. paganellus* and the Ponto-Caspian gobies remains unknown and future phylogeographical studies are needed to clarify this hypothesis and the patterns of goby diversification throughout the Mediterranean basin. In addition, this grouping emphasizes that there is a certain degree of variability between species within the same genus (i.e. *Padogobius*) and that detailed phylogenetic analysis is urgently needed to fully resolve their systematic position. Based on our results, the presence of sound production within the benthophilin gobies showed that acoustic communication is a synapomorphy for this subfamily and that acoustic signals play a crucial role in their life history. Also, tonal sounds seems to have a deeper origin within Benthophilinae subfamily, since all acoustically investigated species were able to produce this sound type (present study; Ladich and Kratochvil 1989; Horvatić et al. 2015). In conclusion, it is important to note that different soniferous European gobies produce similar types of vocalizations, exemplifying the continuum from pulsatile to tonal sounds, though each species quantitatively modifies its vocal repertoire, allowing specific identification. The present study suggests that the two investigated species belonging to the subfamily Benthophilinae, *Neogobius melanostomus* and *Ponticola kessleri*, were able to produce frequency modulated tonal sounds, but each taxon modified their acoustical features and developed vocalizations differently according to their phylogenetic history. From the phylogenetic context, vocalizations produced by gobies from Benthophilinae subfamily diverge according to existing molecular studies (Neilson and Stepien 2009; Thacker and Roje 2011; Medvedev et al. 2013) and proposed tribes can be well recognized according to sound structure and acoustic traits. Species from the Neogobiini tribe sensu Neilson and Stepien (2009) are characterized by producing intensive, repeated, short, tonal sounds, while the only “real” Ponticolini species acoustically tested, *P. kessleri*, produce longer, downward frequency modulated tonal calls. These scenarios emphasize that acoustic properties should not be neglected when reconstructing phylogenetic relationships, especially for the Ponto-Caspian group, which have proved to contain vocal taxa with well-developed sound production abilities. The recorded vocal repertoire present a baseline for the future comparative acoustic studies within the subfamily Benthophilinae, aiming to gain additional information on the evolution of acoustic communication within Ponto-Caspian gobies and highlighting their importance in reconstructing phylogenetic relationships.

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Acoustic communication during reproduction in the basal gobioid Amur sleeper and the putative sound production mechanism

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Keywords
Perccottus glenii; vocal fish; tonal sounds; sound production; acoustic behaviour; levator pectoralis muscle; acoustic communication; gobioids.

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Abstract

Gobioids (Gobiiformes: Gobioidei) are a large group of vocal fishes with four sound types documented during aggressive or reproductive interactions in 23 species. Most attention has been dedicated to sound production in Gobiidae and Gobionellidae, while acoustic communications in other phylogenetically distant gobioid groups have been neglected. Odontobutidae, a basal family within the gobioids, is a poorly studied fish assemblage, with sounds documented in only a single species. The goal of this study was to record and describe the acoustic signals produced by *Perccottus glenii* (Odontobutidae) under laboratory conditions, with particular focus on the reproductive phase (courtship and pre-spawning), and to provide insight into the anatomical basis of the sound emission mechanism. We recorded two acoustically different call types, thumps and tonal sounds. Thumps were low-frequency sounds (~95 Hz) with an irregular waveform, produced by males during both the courtship and pre-spawning phases. Thumps were frequently organized in long trains, a thump burst, composed from approximately five thumps and lasting over 10 s. Tonal sounds were short vocalizations (~90 ms) produced only during courtship interactions, characterized by a sinusoidal oscillogram and a single frequency peak (~120 Hz). Additionally, anatomical examination focusing on the pectoral girdle identified the muscles that could be responsible for sound emission. The levator pectoralis muscle, originating on the neurocranium and attaching to the cleithral bone, is separated into three bundles: a pars lateralis superficialis, a pars lateralis profundus and a pars medialis. These results expand the knowledge about gobioid vocal behaviour and underline the importance of acoustic communication within this group of fish. Odontobutidae is a sister group to rest of the gobioids, and therefore, our results have significant impact for future comparative studies dealing with sound production.

Introduction

Although numerous fishes can produce different sounds, their production for social communication is documented in a restricted number of species from around 110 families (Parmentier & Fine, 2016). Gobioid fishes (phylogeny according to Thacker, 2009) form one of the most investigated Teleost groups in terms of vocal repertoire. Within this group, acoustic communication has been documented in 23 species (Horvatić *et al*., 2015; Zeyl *et al*., 2016). In general, gobioid sound emission occurs during agonistic and reproductive contexts. Acoustically, the reproductive sequence can be further subdivided into the courtship, pre-spawning and spawning phases (Lugli *et al*., 1997; Myrberg & Lugli, 2006; Amorim & Neves, 2007; Malavasi, Valerio & Torricelli, 2009; Blom *et al*., 2016; Zeyl *et al*., 2016). Courtship behaviour usually commences from the moment the female enters the male territory, while the pre-spawning phase is characterized with the ripe female entering the male's nest. When the female inverts in the nest to release eggs onto the ceiling, spawning has started.

Four major sound types can be recognized in gobioids: pulsed sounds consisting of pulse trains repeated at a slow rate, tonal sounds characterized by faster pulse repetition rates and a sinusoidal-like waveform, and complex sounds that are a combination of the two (Lugli *et al*., 1997; Zeyl *et al*., 2016). Lastly, thump sounds are short low-frequency pulsed signals with sound energy under 1 kHz (Amorim & Neves, 2007; Malavasi, Collatuzzo & Torricelli, 2008; Zeyl *et al*., 2016). Briefly, in the most investigated species, pulse trains dominate during both agonistic and reproductive (pre-spawning) interactions, while thumps and tonal sounds are involved in courtship activities in many gobioids (Ladich & Kratochvil, 1989; Lugli *et al*., 1996; Lindström & Lugli, 2000; Amorim & Neves, 2007, 2008; Malavasi *et al*., 2008, 2009; Horvatić *et al*., 2015; Zeyl *et al*., 2016). Complex sounds, reported in only three gobioids to date, were recorded during aggressive circumstances (*Gobius cruentatus* and *Periophthalmodon septemradiatus*) or as a part of spawning acoustic repertoire in *Padogobius bonelli* (Lugli *et al*., 1995, 1997; Sebastianutto *et al*., 2008; Polgar *et al*., 2011; Zeyl *et al*., 2016).

Recently, molecular studies of the relationships between the gobioids strongly support that Odontobutidae is a sister group of all other lineages (Thacker, 2009; Agorreta *et al*., 2013). This group contains 22 species, with acoustic communication studied in just one species: *Odontobutis obscura* (Takemura, 1984). The acoustic feature of its recorded calls resembles the pulsatile sounds previously documented in many other gobies (see Zeyl *et al*., 2016). These results support the claim that sound production based on pulsed repertoires could be a synapomorphic trait within the gobioids (Malavasi *et al*., 2008; Zeyl *et al*., 2016). However, additional acoustic research is required to understand the ancestral state of sound communication in gobioid fishes.

The diversity of sonic mechanisms in fishes is so great that it has not been possible to group them in appropriate categories (Ladich & Fine, 2006). In gobioids, different mechanisms have been proposed; however, these hypotheses should be considered with caution as they have rarely been tested, and deeper multidisciplinary investigations are needed to fully understand sound production in the gobioids. Parmentier *et al*. (2013, 2017a) experimentally tested the hypothesis of contraction of the pectoral girdle muscles in two European gobies, *Gobius paganellus* and *Pomatoschistus pictus* (Gobiidae and Gobionellidae, respectively). Multidisciplinary investigation (sound analysis, electromyography and electron microscopy), together with morphological and high-speed videos, has suggested that drumming sounds might be generated by the periodic contraction of the levator pectoralis muscle (Parmentier *et al*., 2013, 2017a). These findings proposed that the pectoral girdle mechanism could be common to the gobies and may have possibly evolved in other gobioid groups as an exaptation (Parmentier, Diogo & Fine, 2017b), likely from locomotory movements.

Perccottus glenii Dybowski, 1877 is a freshwater gobioid fish, widespread in the lentic waters, ponds and marshes of Europe and Central Asia (Kottelat & Freyhof, 2007; Freyhof, 2011). It belongs to the family Odontobutidae, an early branching lineage of the Gobiiformes, native to East Asia (Kottelat & Freyhof, 2007; Thacker, 2009), and as such offers a great opportunity to expand the knowledge on the vocal repertoire in Odontobutidae and to better clarify the ancestral acoustic structure within the gobioid fishes. The spawning habits of *P. glenii* are similar to the general goby pattern, and during reproduction, which starts in May or July, *P. glenii* males occupy and defend nests that are used as spawning sites and exhibit territorial behaviour (Miller, 1986; Kottelat & Freyhof, 2007).

The aim of the present study was to assess the patterns of acoustic communication in *P. glenii* during courtship while also providing insight into the anatomical basis of the sound emission mechanism. More specifically, the goals were to: (1) document sound production during reproduction in the male *P. glenii* and quantitatively describe the sound structure; (2) explore the association between reproductive behaviours and sound production; and (3) investigate the morphological components of the pectoral girdle and associated muscles possibly related to sound production in the odontobutid *P. glenii*.

Materials and methods

The study was carried out from April until the end of June 2018, which corresponds with the breeding season of *P. glenii*. Fish were caught in October 2017 from a well-vegetated tributary to the main Danube River channel, near Veliko Gradište (Serbia). Individuals (25 individuals; 10 males and 15 females) were collected using electrofishing and transported to the laboratory where they were sexed according to urogenital papillae (conical in males, wider and fimbriose in females; Miller, 2003) and separated by sex into two stock glass tanks (each 120 L). Six males exhibiting pronounced breeding behaviour with obvious dimorphic traits (swollen nape and dark coloration) were used as residents in acoustic tests and placed individually in 90-L glass tanks. Each tank was supplied with a tile shelter to serve as a nest, aerators to ensure optimal oxygen levels and a gravel or fine sand substrate layer on the bottom. To avoid sound disturbance and minimize the surrounding noise during audio recordings, each tank was placed on foam-rubber shims. Photoperiod and water temperature followed natural environmental reign, and in situations when water temperature dropped below 15°C, water heater (ATMAN brand; AT 1008 type; temp. range: 20–34°C) was used to maintain optimal water temperatures for reproduction (Miller, 2003). Inside each tank, water temperature (range: 15.7–20.8°C) was monitored with the thermometer (AQUATERRA, Garešnica, Croatia).

Experimental procedure

Sounds were recorded from six males (size range: 84–96 mm L_S), during the reproductive interactions (courtship and prespawning phases). Experiments were set up according to the protocol provided by Horvatić *et al*. (2015) to record reproductive (male–female) or aggressive interactions (male–male). We used an acoustic intruder test, during which the fish was left in the observational tank for acclimatization (10–15 days) to establish territoriality. An intruder (i.e. a female showing an enlarged belly and nuptial coloration, or aggressive male) was selected from the stock tank. The intruder fish (size range: 73–

89 mm L_S) was placed in metal cage in front of the nest in the observational tank to elicit a behavioural or acoustic response from the resident male. Each session lasted *c.* 30 min. All pump devices, aerators and lights were switched off 10 min prior to recordings to minimize ambient noise.

Sound collection

Sound production was recorded at various times during the day with an omnidirectional H2A-XLR hydrophone (Aquarian Audio & Scientific, Anacortes, WA, USA; sensitivity: -180 dB re: 1 V μ^{-1} Pa; frequency range ±4 dB from 0.01 to 100 kHz) positioned less than 10 cm from the nest entrance according to Akamatsu *et al.* (2002). The hydrophone was connected to an IRIG PRE preamplifier (Aquarian Audio & Scientific), and sounds were recorded using a ZOOM H4n portable digital audio recorder (wav 16/44.1 k mono; ZOOM, Tokyo, Japan), stored on the recorder memory card and monitored on headphones.

Behavioural recording

The courtship and pre-spawning phases of reproductive behaviour were video-recorded and analysed. Courtship behaviour began when the females entered into the male territory, while the pre-spawning phase was observed when the ripe female entered the male's nest. No sounds were produced during actual spawning. Video recordings of acoustic behaviour were performed using a camcorder (Canon Legria FS200, 2000× digital zoom, Tokyo, Japan) positioned *c.* 30 cm from the front of the experimental glass tanks. Audio recordings, as described above, were conducted simultaneously, enabling sounds to be associated with specific behaviours. Male behaviours and the associated sound emissions were observed during 17 recording sessions (2.8 per male) and analysed using Windows Movie Maker (size: 1004 bytes; Microsoft, Redmond, WA, USA). Behavioural categories expressed by the males were classified and scored according to our observations and the existing behavioural literature for gobioids (Takemura, 1984; Amorim & Neves, 2007; Malavasi *et al.*, 2009, 2012).

Signal processing and sound analysis

Analog sounds were digitalized at 4000 sampling rate (128 accuracy, 16 bit resolution) and high-pass filtered (0.06 kHz) to eliminate background noise. Additionally, +10 dB amplification was applied for better auditory and visual inspection of the audio tracks. Digitalized sounds were analysed using Avisoft-SASLab Pro 5.2 Software (512-point fast Fourier transform FFT, Hamming window; Avisoft Bioacoustics, Berlin, Germany). Only sounds with a good signal to noise ratio (SNR) were analysed. Temporal features were measured from the oscillogram, while frequency variables were obtained from the logarithmic power spectra (Hamming window, 512-points FFT, resolution 7 Hz). More rarely, thumps were produced in sequences of several sounds, that is thump bursts, for which the sound parameters were also calculated. Visual inspection in the software allowed us to separate two different thump bursts according to a time interval longer than 2-s, which separated the last thump in one and the first thump in an additional burst. All thumps in the sequence with an interval shorter than 2-s were considered one thump burst. The following acoustic properties were measured for thumps and thump bursts, respectively: (1) thump rate (number of thumps emitted in 1 min; sound min^{-1}); (2) thump duration (total length of the call, measured in milliseconds); (3) number of cycles; (4) peak frequency (obtained from the power spectrum function); (5) burst duration (total length of the thump burst, measured in seconds); (6) number of thumps in the burst; and (7) thump interval (time interval within the burst measured from the end of one thump to the start of the following one, measured in seconds). For tonal sounds, we measured the following: (1) sound rate (tonal sounds emitted in 1 min; sound min^{-1}); (2) duration (ms); (3) number of cycles; (4) cycle repetition rate (Hz); (5) frequency modulation (after the sound has been divided into three sections, frequency modulation was calculated as the difference between the final and initial cycle repetition rate and expressed in Hz); and (6) peak frequency (Hz).

Statistical analysis

The mean, standard deviation ($x \pm \text{SD}$) and range for each acoustic variable emitted by individual fish were calculated. Sound features were examined using from 10 to 20 thumps, 10 thump bursts and 5 to 10 tonal sounds per individual, when possible. To investigate the behavioural context of sound production, two analyses were performed independently on the same dataset. The first was the chi-square test (at 5% level of significance) used to study the association of sound production with particular behavioural displays, in both thumps and tonal sounds. Males courted females by displaying various behavioural displays: behaviours expressed by the males were quantified as the frequency of events, or the number of observed behavioural acts occurring in a given time (n min^{-1}). Six behavioural categories were identified from the videos and analysed: leading, chase, nest display, frontal display, upside-down and pre-spawning. Sounds from vocal individuals were pooled, and the number of times each behavioural category was performed with or without sound emissions was counted. Adjusted residuals from the chi-square test were then used to assess which behavioural categories were positively or negatively associated with the emission of sounds; that is, residuals indicated whether the frequency of the cell was respectively overrepresented or underrepresented in the sample compared to the expected frequency (Sebastianutto *et al.*, 2008). The second analyses involved dividing the reproductive sequence into two phases (courtship and pre-spawning), and the number of sounds recorded per phase was noted, separately for each male. As previously mentioned, courtship and pre-spawning phases were recognized according to female absence or presence in the nest, respectively. For the comparison between the frequency of calls produced by each male during the courtship or pre-spawning phase, the *t*-test was applied for dependent samples (5% level of significance) after logarithmic transformation of data. In addition, individual mean values of sound parameters were tested for correlation using the Pearson correlation to investigate their mutual relationships and relationship with physical characteristics (size and water temperature). Data were analysed with STATISTICA 13 (Statsoft Inc., Tulsa, OK, USA).

Morphology

Eight males (six vocal and two randomly selected from stock aquaria, size range: 84–96 mm L_S) were removed from tanks using hand nets. All fish were measured, euthanized with an overdose of MS-222 (tricaine methane sulphonate; Pharmaq, Overhalla, Norway), and stored for 1 week in 7% formaldehyde fixative solution and then transferred to 70% ethanol. Dissections of specimens were performed at the University of Liège (Belgium). Fish were dissected and examined with a Wild M10 binocular microscope (Leica Camera, Leica, Wetzlar, Germany) equipped with a camera lucida. Dissections concern mainly muscles in relation to the pectoral girdle because of previous work implicating the pectoral fin motion in sound production. The nomenclature used to designate parts of the musculature is based on previous studies (Winterbottom, 1974; Adriaens, Decleyre & Verraes, 1993).

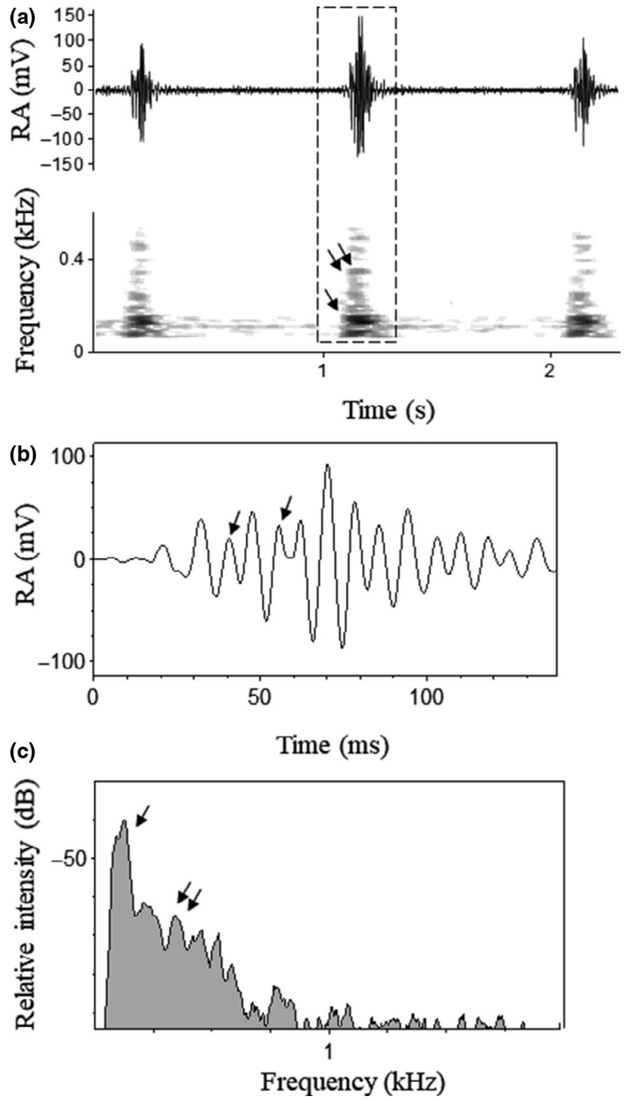

Figure 1 a) Oscillogram and spectrogram of the thump burst, b) enlarged oscillogram of an individual thump and c) power spectrum of the isolated thump produced during reproductive interactions of *Perccottus glenii.* On the spectrogram, the marked area represents an energy band (reaching up to 500 Hz) with the arrows on the power spectrum indicating two frequency peaks (at 120 and 340 Hz). Marked lines on the lower oscillogram represent sound duration, i.e. the onset and the end of one thump. On the oscillogram, arrows indicate extra-peaks. RA – relative amplitude.

Results

Acoustic properties of reproductive calls

Sound production was documented in six *P. glenii* males during reproductive interactions (courtship and pre-spawning). Males were acoustically active in the presence of females, mostly when they approached the nest entrance. A total of 734 sounds were documented, and 171 were analysed (mean ± SD = 15.3 ± 6.8 thumps per male; 8.6 ± 3.2 thump bursts per male; and 9.0 ± 1.0 tonal sounds per male). Thump sounds and thump bursts (long trains of thumps) were produced by all males though tonal sounds were produced by three males only. During male–male aggressive interactions, all males were mute and no sounds were documented.

Thumps are low-frequency sounds (~100 Hz) composed of irregularly repeated sound cycles of a relatively short duration (~95 ms; Fig. 1a). On the waveform, cycles varied in amplitude but the highest peak was the one in the first two-thirds of the sound. The first individual cycle had one peak, while the following cycles exhibited smaller extra-peaks, which again disappeared as the sound ceased (Fig. 1b). On the power spectrum, fundamental frequency was always the dominant frequency representing the peak with greatest energy, but sometimes, additional harmonics were observed. Call energy was decreased by *c.* −30 dB (relative intensity) by 600 Hz and did not exceed 1 kHz (Fig. 1c, Table 1). During reproductive interactions, males produced a mean of 9 thumps min^{-1}, increasing vocal activity up to 19 thumps min^{-1} when the female approached the nest. Thumps were organized in long trains, that is sound bursts, in which thumps were spaced with a time interval of *c.* 1.2 s when the female hesitated on the nest entrance or was inspecting the cavity (Fig. 1a, Table 1). Bursts were composed from 2 to 11 thumps and in some cases lasted more than 10 s. Within the burst, thumps had the same structure and temporal organization as when produced as individual thumps; that is, they were short duration and low-frequency signals. The number of thumps within the burst was positively correlated with burst duration (Pearson $r^2 = 0.97$, $P < 0.05$; Fig. 2). Positive and significant correlations were detected between water temperature and three thump burst parameters: number of cycles (Pearson $r = 0.44$, $N = 6$, $P < 0.05$), cycle repetition rate (Pearson $r = 0.30$, $N = 6$, $P < 0.05$) and peak frequency (Pearson $r = 0.40$, $N = 6$, $P < 0.05$).

Perccottus glenii males also produced short tonal sounds. These were produced inside the nest or from its opening during courtship in the presence of the ripe female. These sounds were never produced when the female entered the nest during the pre-spawning phase. Tonal sounds were short sinusoidal vocalizations (~90 ms) composed of about 10 sound cycles, whose repetition rate ranged from 91 to 125 Hz (Fig. 3a,b). Peak frequency was low (~120 Hz) and appeared as a narrow peak on the spectrogram, strongly overlapping with the values of the cycle repetition rate (Fig. 3c, Table 2). In all cases, peak frequency was the fundamental frequency. Energy ranged up to 300 Hz and did not exceed 500 Hz. Frequency modulation was slightly upward, with values averaging around 3 Hz. Sound rate was lower when the female was further from the nest, but increased as the female approached the nest entrance. In these situations, tonal sounds were organized in series, averaging six repeated sounds per minute (Fig. 3a, Table 2). Sound variables, such as sound rate and frequency modulation, correlated positively with water temperature (Pearson $r = 0.80$ and $r = 0.40$, respectively; both $N = 3$, $P < 0.05$).

Behavioural context of sound production

During reproductive interactions, the male courted the female by performing a series of breeding displays separated in the six behavioural categories (Fig. 4), while swimming or while supported on the substrate by the pelvic fins. Body and head movements observed during the sound emission in vocal fishes were similar for both thump and tonal calls, without any obvious differences in terms of motions. During sound production, fish exhibited pectoral fin abduction, rapid thrust of the throat region, and fin erection and body undulation. There was no sound production during actual spawning (egg laying).

During the 17 recording sessions (2.8 per male), 304 behavioural acts were identified and separated into the six behavioural categories. In total, 85 behaviours (42%) were associated with sound production. Generally, the most frequently observed behavioural categories (irrespective of sound type) were as follows: Nest display (33%, $n = 99$), Frontal display (22%, $n = 68$) and Leading (21%, $n = 66$). Tonal sounds were most commonly recorded during Frontal display (75%, $n = 28$), while 62% of documented categories were associated with sound production (23 of 37 behaviours; Fig. 5). Thumps were most frequently observed during Nest display (35%, $n = 94$) and Leading (24%, $n = 65$). Only 23% of behavioural categories were associated with thumps (62 of 267 behaviours; Fig. 5).

Sound production differed in its association with the behavioural categories. The emission of thumps was positively associated with Frontal display and Pre-spawning ($\chi^2(6) = 804.2$, $P < 0.001$). A positive association was also found between tonal sounds and Frontal display ($\chi^2(6) = 62.3$, $P < 0.001$), while fewer tonal sounds than statistically expected were observed in other categories ($\chi^2(6) = 62.3$, $P < 0.001$). In addition, there was a significant difference between courtship

Table 1 Sound parameters of recorded thumps and thump burst during reproductive interactions in six *Perccottus glenii* males

Sound parameters	Mean	SD	Range
Thumps			
Thump rate (sounds min^{-1})	9.3	3.2	2–18.5
Duration (ms)	95.5	7.3	64.0–148.0
Number of cycles	8.7	0.9	6.3–13.1
Peak frequency (Hz)	97.9	4.4	74–121.1
Thump bursts			
Thump duration (ms)	99.9	32.8	66.1–157.0
Thump number of cycles	7.6	1.1	5.5–9.2
Thump peak frequency (Hz)	95.0	9.2	81.6–108.9
Burst duration (s)	4.4	3.8	1.3–13.2
Number of thumps	4.4	3.1	2–11.5
Thump interval (s)	1.2	0.2	0.9–1.5

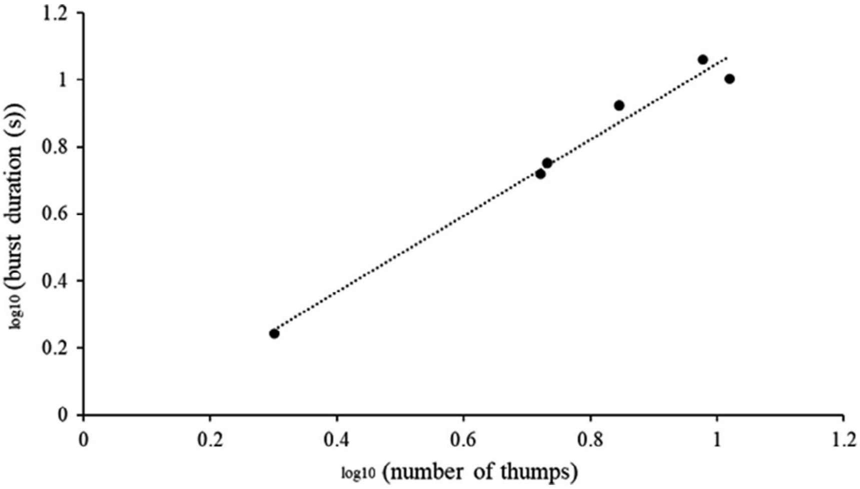

Figure 2 Relationship between burst duration and the number of thumps in *Perccottus glenii*. The curve was fitted by: $y = 1.1354x - 0.0861$ ($r_2 = 0.97$, $P < 0.05$).

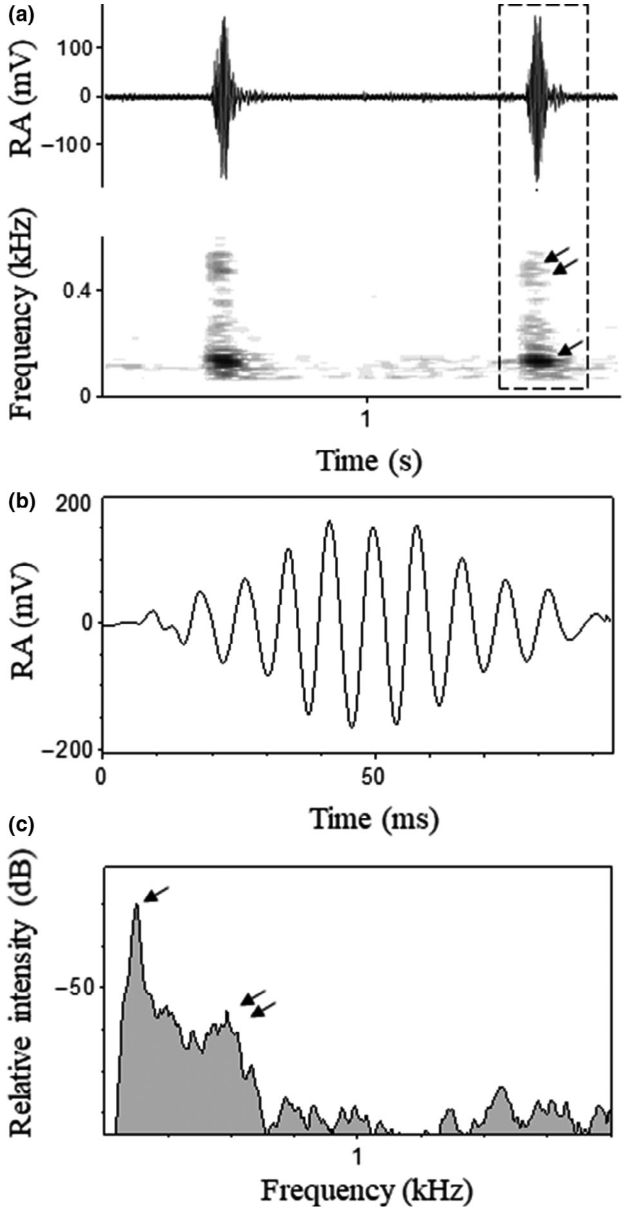

Figure 3 a) Oscillogram and spectrogram of the sinusoidal tonal sounds, b) enlarged oscillogram of an individual tonal sound and c) power spectrum of the isolated tonal sound produced during reproductive interactions (courtship) of *Perccottus glenii.* On the spectrogram and power spectrum, the marked area represents the energy band (reaching up to 500 Hz) with the arrows on the power spectrum indicating important frequency peaks (at 120 and 470 Hz). Marked lines on the lower oscillogram represent sound duration, i.e. the onset and the end of one tonal sound. RA – relative amplitude.

and pre-spawning in the frequency of recorded sounds only in the case of the tonal calls, where sounds were produced more frequently during courtship than pre-spawning (mean ± SD; courtship = 9 ± 1 vs. pre-spawning = 1 ± 0, dependent *t*-test = 34.02, d.f. = 2, $P < 0.001$). For thumps, there was no significant difference between the courtship or pre-spawning phases and the frequency of produced calls (courtship = 63.5 ± 47.9 vs. pre-spawning = 54.6 ± 73.0, dependent *t*-test = 1.29, d.f. = 5, $P > 0.05$).

Anatomical findings

In previous studies on the sound-producing mechanisms in Gobiidae and Gobionellidae (Parmentier *et al*., 2013, 2017a), anatomical studies mainly concerned the pectoral girdles and associated muscles, that is levator pectoralis muscles, whose contractions were thought to be responsible for pectoral girdle motion during sound emission. In *P. glenii*, the pectoral girdle is articulated on the skull by the posttemporal bone. It consists of a basal plate with two processes that form a fork, preventing forward displacement of the posttemporal bone (Fig. 6). The dorsal process articulates dorsally on the skull at the level of the epiotic. The ventral process is situated beneath the dorsal process and extends rostrally into a ligament attached to the intercalar bone. The supracleithrum connects the posttemporal to the cleithral bone. It is anteriorly covered by the posttemporal, but is caudally lateral to the dorsal process of the cleithrum. Baudelot’s ligament inserts on the medial face of the supracleithrum, bypasses caudally the anterior process of the cleithrum and inserts laterally on the basioccipital. Muscles described as sound-producing muscles were found in this part of the body.

The levator pectoralis muscle is easily distinguishable and is separated into three bundles: a pars lateralis superficialis, a pars lateralis profundus and a pars medialis (Fig. 6). The musculus levator pectoralis pars lateralis superficialis originates from the dorsal caudal part of the pterotic bone of the neurocranium and inserts on the medial face of the posttemporal, at the level of the basal plate. Some fibres also insert on the ventral process of the posttemporal. The musculus levator pectoralis pars lateralis profundus originates on the caudal margin of the pterotic bone, rides ventrally along the ventral process of the posttemporal and inserts on the rostral margin of the cleithral bone. The musculus levator pectoralis pars medialis originates more medially, on the exoccipital bone, and is attached to the rostral side of the cleithral bone. On the cleithral bone, the insertion of the pars medialis is dorsal to pars lateralis.

Discussion

The present study provides a qualitative and quantitative assessment of the acoustic behaviour in the basal gobioid *P. glenii* during reproduction and some insights into the putative sound emission mechanism. *Perccottus glenii* males produced two different sound types, thumps and tonal sounds, during courtship and pre-spawning interactions. We did not record sounds during male intrasexual aggressive interactions, what is interesting result considering high territoriality and breeding competition observed in *P. glenii* males and gobioids in general (Takemura, 1984; Lugli *et al*., 1997; Amorim & Neves, 2008). Thumps were the most frequent sound type and

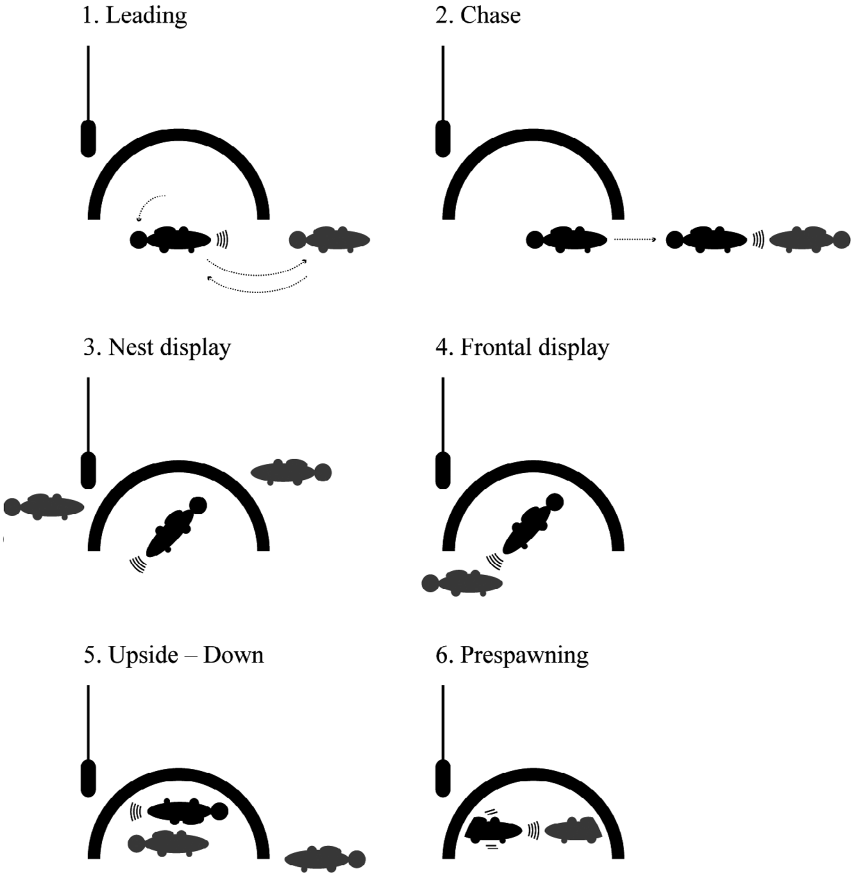

Figure 4 Behaviours associated with the sound production during reproductive interactions in Perccottus glenii. Descriptions: 1. Leading: a courtship behaviour where the male completely leaves the nest, approaches the female and then rapidly returns to the reproductive site; 2. Chase: male vigorously chases the female from the nest over a short (> 5 cm) or long (< 5 cm) distances while constantly biting her and producing sounds; 3. Nest display: male is completely inside the nest while the female swims around and away from the entrance, i.e. there is no visual contact; 4. Frontal display: male is orientated toward the female from the nest entrance (there is visual contact), and the female is positioned less than five centimetres from the nest opening, but not inside; 5. Upside-down: male turns upside down in the nest so that its genital papilla comes in contact with the nest ceiling; 6. Pre-spawning: while the female is completely inside the nest, the male undulates the body and waves the pectoral fins with erected dorsal fins. Resident males are black and intruder females grey. Nest and hydrophone size not to scale.

were produced during both courtship and pre-spawning, while the female was outside the nest or within the cavity. Acoustically, thumps were short duration (<200 ms) and low-frequency (<500 Hz) sounds, and exhibited an irregular waveform structure. Similar to sand gobies (Lindström & Lugli, 2000; Amorim & Neves, 2007; Malavasi *et al.*, 2009), sounds of *P. glenii* were organized in long trains (sound bursts) composed from 2 to 12 thump sounds, whose mean acoustic parameters were similar to those of individually produced thumps. This study provides the first record of thump production outside the Gobionellidae, highlighting the complexity of acoustic signals within gobioids.

Table 2 Sound parameters of recorded tonal sounds during reproductive interactions in three *Perccottus glenii* males

Sound parameters	Mean	SD	Range
Tonal sounds			
Sound rate (sounds min^{-1})	6.4	1.2	4.7 to 7.7
Duration (ms)	89.7	16.8	70.4 to 119.5
Number of cycles	9.7	1.4	8.3 to 12.3
Cycle repetition rate (Hz)	111.1	10.4	91.7 to 123.7
Frequency modulation (Hz)	2.4	15.0	–25.0 to 20.4
Peak frequency (Hz)	117.1	4.5	112.3 to 124

We also recorded tonal-like sounds, which were only previously observed in a small proportion of European gobiids from the *Gobius* lineage and *Periophthalmodon septemradiatus* (Ladich & Kratochvil, 1989; Lugli *et al.*, 1996, 1997; Malavasi *et al.*, 2008; Sebastianutto *et al.*, 2008; Polgar *et al.*, 2011; Agorreta *et al.*, 2013; Horvatić *et al.*, 2015). In the case of *P. glenii*, tonal sounds on the spectrogram were almost flat non-modulated calls of short duration (<100 ms) with one important frequency peak (~120 Hz) corresponding to the cycle repetition rate, indicating its sinusoidal quality.

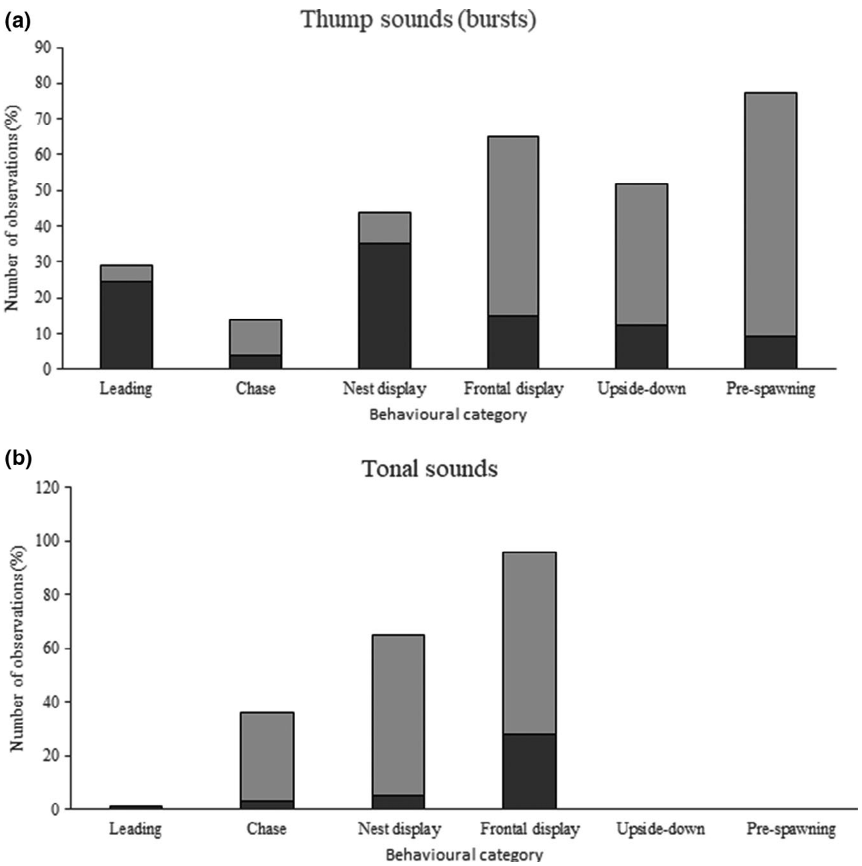

Figure 5 Percent of documented courtship behavioural acts (dark grey; total number of recorded acts; for thumps (n = 267) and tonal (n = 37) sounds) and the percentage of acts accompanied with the production of a) thump or b) tonal sounds (light grey) during courtship behaviour of *Perccottus glenii* males.

Perccottus glenii tonal calls show many common features with previously documented short tonal sounds described in certain gobiids, that is *Neogobius fluviatilis* and *Padogobius nigricans* (Lugli *et al*., 1996; Horvatić *et al*., 2015), highlighting the spectro-temporal acoustic complexity (short, non-modulated vs. long, frequency-modulated vocalizations) within gobioids, even in the case of tonal sounds. In *P. glenii*, short tonal sounds were frequent during the courtship phase, that is, when females were inspecting the male territory. Though the small sample size hinders us from drawing deeper conclusions, the occurrence of tonal sounds during the initial phase of the reproductive interactions (i.e. courtship) has been well documented in previous investigations of territorial nest-holding gobies (Ladich & Kratochvil, 1989; Lugli *et al*., 1996; Horvatić *et al*., 2015). Our results emphasize the complex situation about reproductive sequence-specific sound types in gobies. We propose that, in the case of *P. glenii*, tonal sounds could serve for female attraction (i.e. when the female is outside the nest), while thump sounds are utilized in pre-spawning interactions, once the female has entered the nest hollow.

In this study, a relationship was found between water temperature and acoustic parameters, in both sound types. Sound characteristics are expected to change with temperature since it influences muscle contraction properties (Feher, Waybright & Fine, 1998), and in our case, thumps tended to have a higher number of cycles, cycle repetition rate and peak frequency in relation to higher temperatures within the burst. Tonal sounds were more frequency modulated and produced with a higher emission rate with increasing temperature. Although preliminary, our findings are consistent with the hypothesis about temperature-dependent sound-producing muscles in fishes (Bennett, 1985; Feher *et al*., 1998; Rome & Lindstedt, 1998). However, detailed study is needed in order to understand how temperature variation affects acoustic features in *P. glenii*.

Male Padanian goby *Padogobius bonelli* produce tonal-like calls separately from drumming sounds, or combine them to form complex sounds (Lugli *et al*., 1995, 1997). Though the drumming and tonal components of complex sound never overlap, these components differ slightly in their acoustic parameters compared to the mean values of isolated sounds (shorter and with a lower number of pulses). Therefore, it was suggested that a single sonic mechanism could be responsible for sound production in *P. bonelli* (Lugli *et al*., 1995). Variation rates of the same muscles could generate either discrete pulses (drums) or rapidly repeated pulses yielding tone-like sounds. Our study demonstrates that *P. glenii* males were also

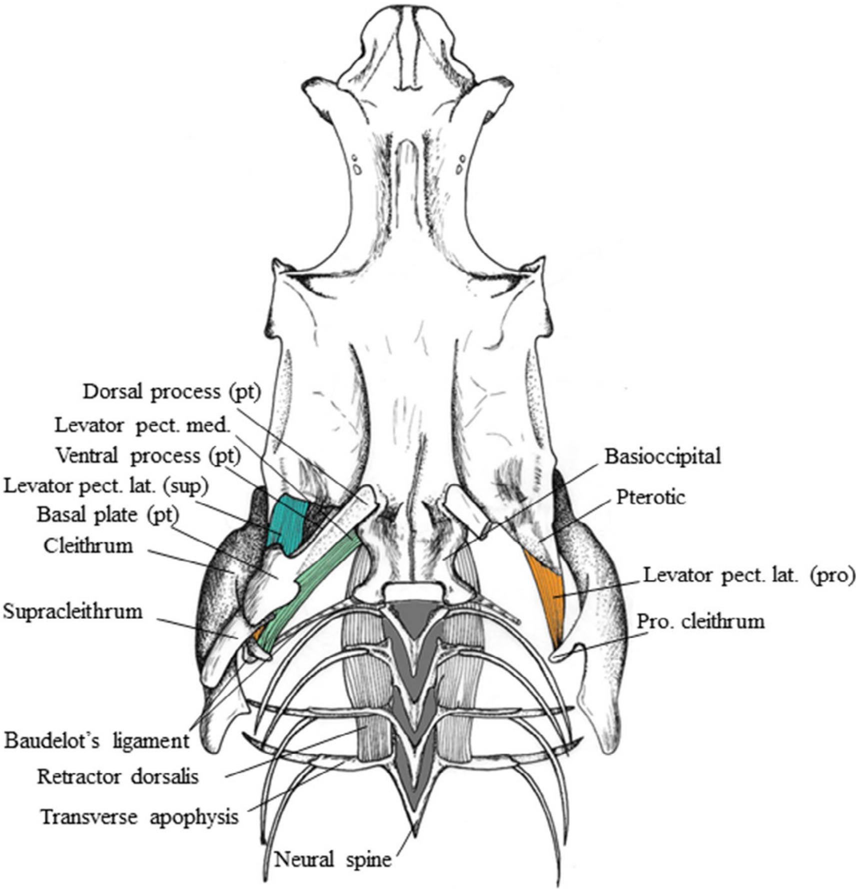

Figure 6 Dorsal view of the putative sound-producing mechanism in *Perccottus glenii*. On the right side, the posttemporal, levator pectoralis lateralis superficialis and levator pectoralis medialis muscles were removed to see the levator pectoralis lateralis profundus muscle. [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com]

able to produce two acoustically different sound types. However, the question remains as to how *P. glenii* thumps and tonal sounds are produced. No clear differences were observed in the cranio-pectoral movements associated with thump or tonal sound production. Further, we did not observe nodding behaviour or forward girdle displacement, as previously suggested for some cottids and gobies (Colleye *et al*., 2013; Zeyl *et al*., 2016). Therefore, it is possible that the basal odontobutid *P. glenii* possesses a single mechanism that is responsible for the production of both sound types. Detailed high-speed recordings and multidisciplinary research (muscle histology and electromyography) are needed to fully explain the sound emission process in *P. glenii*.

Fine *et al*. (2001) demonstrated that muscle contraction rate dictates the fundamental frequency of calls of the male oyster toadfish (*Opsanus tau*), with values overlapping peak sound amplitude. In our study, the comparison of oscillograms and power spectra between thump and tonal sounds provides interesting information. Dominant frequencies in both thump and tonal sounds mostly overlapped but thumps showed additional spectral components at frequencies above 200 Hz (Figs 1c and 3c). These additional components in thumps reflect the less regular sinusoidal shape of the oscillogram (Fig. 1b). Contrary to this, the oscillogram shape of tonal sounds is more constant, providing less additional spectral components (Fig. 3c). In this case, tonal sounds could correspond to fully completed thumps. In other words, thumps and tonal sound could be produced by the same sound-producing mechanism involving the levator pectoralis contractions, though these contractions provide more regular cycles in tonal sounds.

According to Malavasi *et al*. (2008), interspecific comparison of acoustic signals across eleven Mediterranean gobies suggested that the pulsatile sound could represent an ancestral state within the gobies. In respect to this hypothesis, the present results broaden the evolutionary scenario regarding acoustic communication among the gobioids. We suggest that pulsatile (thump-like) and short tonal-like sounds could constitute the fundamental acoustic unit within the gobioids, since they are clearly documented in basal odontobutids (present study and Takemura, 1984). Although speculative, it is possible that these 'stem' sounds could have evolved in terms of temporal complexity (i.e. temporal spacing of pulse repetition) and frequency variation (modulation), presenting a diversity of long, frequency-modulated tonal and pulsatile/drumming signals reported currently for gobiids and gobionellids. By producing these fundamental units and additionally combining them, some gobiids (i.e. *P. bonelli* and *Gobius cruentatus*) have the ability to emit complex calls possessing both tonal and drumming components within the same burst.

Conclusions

This study supports the hypothesis that sound production and acoustic communication evolved early within the gobioid lineage. Thumps and short tonal-like sounds reported for the odontobutid *P. glenii* could be considered a synapomorphic trait for gobioid fishes. However, additional experimental study is required to determine whether sounds are produced in other basal gobioid families (mainly Eleotridae and Butidae) and to be able to compare them with other taxa. In addition, we propose that muscles originating on the skull and attaching to the pectoral girdle have an important role in the sound emission of *P. glenii* and therefore present a putative mechanism involved in their acoustic communication. In summary, this study expands the knowledge about gobioid vocal behaviour and underlines the importance of acoustic communication within this group of fish.

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Conflict of interest

None.

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Correlation between acoustic divergence and phylogenetic distance in vocal European gobiids (Gobiidae; *Gobius* lineage)

Relationship between sound variability and genetic structure in gobies

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Abstract

In fish, identity can be encoded by sounds, which have been thoroughly investigated in European gobiids (Gobiidae, *Gobius* lineage). Recent evolutionary studies suggested that deterministic and/or stochastic forces could generate acoustic differences among related animal species, but this was never investigated in any Teleost group up to date. In the present comparative study, we analysed the sounds from nine vocal gobiids and quantitatively assessed their acoustic variability. Overall, we analysed the means of 616 sounds from 67 males. Our interspecific acoustic study, incorporating for the first time the acoustic signals from the majority of vocal gobiids, suggested that their sounds are truly species-specific (92% of sounds were correctly classified into exact species) and that each taxon possesses a unique set of spectro-temporal variables. In addition, we reconstructed phylogenetic relationships from a concatenated genetic dataset consisting of multiple genetic markers to track the evolution of acoustic communication in vocal gobiids. The results of this study indicated that the genus *Padogobius* is polyphyletic, since *P. nigricans* was nested within the Ponto-Caspian clade, while the congeneric *P. bonelli* turned out to be a sister taxon to the remaining vocal species. Lastly, by extracting the acoustic and genetic distance matrices, sound variability and genetic distance were correlated for the first time to assess whether sound evolution follows the similar phylogenetic pattern. Significance of sounds in goby evolution, invoked by many previous studies, was empirically corroborated by the positive correlation between the sound variability and genetic distance, emphasizing that some acoustic features could carry the phylogenetic signal important for accurate species-discrimination in vocal gobiids. Positive correlation supported the hypothesis that sound variability in nine European gobiids is under the influence of stochastic forces. Our study was the first attempt to evaluate the mutual relationship between acoustic variation and genetic divergence in any Teleost fish.

Introduction

Many animal species use sounds, together with other communication signals, to express their behaviour, and by actively changing their acoustic properties, they can control the information content of these signals [1,2]. Interspecifically, sounds can encode the identity of the signalling individual [3,4,5] and for related species in sympatry, this is an important discrimination trait during reproductive interactions [6,7,8]. Given the significant role of sound production in the species recognition process, it is believed that divergence in acoustic signals could drive speciation [9-12].

One of the central questions in bioacoustics, when it comes to divergence caused by variations in acoustic signals, is to determine which evolutionary forces have generated prominent interspecific differences among animal taxa. Even though a signal evolution is rarely explained by a single evolutionary force, most of the studies invoke two common processes or forces which are generally thought to be responsible for acoustic divergence: deterministic and stochastic. Deterministic (or 'adaptive') processes, such as habitat adaptation [13,14], divergence in morphology [15,16] or selection for species recognition ("reproductive character displacement" or sometimes even the sexual selection) [17,18,19] act to amplify the signal variations already present among species. These processes generate straightforward predictions about the direction of evolution [20]. In these circumstances, an absence of association between genotype and acoustics highlights the importance of deterministic factors and other selective pressures in shaping acoustic traits. On the other hand, stochastic (or 'neutral') processes, such as sexual or social selection [21,22] or more commonly, genetic drift and mutation [13,23,24,25] could be the driving forces for signal divergence. These mechanisms make signal divergence a highly stochastic and unpredictable process [20], where a positive correlation is usually observed between call divergence and genotype. Likewise, some studies indicate that the two processes sometimes interact and act mutually, causing the signal divergence [for references, see 20].

Accordingly, studies on animal sounds, supporting both the association between acoustic variation and genetic divergence [20,25-30] and the lack of association between the two patterns [13,31,32].

The vocal repertoire among Teleosts has been thoroughly investigated in the gobioids (Gobiiformes; Gobioidei). Soniferous species produce different types of acoustic signals, presenting a rich repertoire composed from pulsatile to tonal sounds. The acoustic structure shows great variability at both the inter- and intraspecific levels, with four different sound types [thump, pulsatile (drum), tonal, and complex] recorded to date, emitted mainly by males as part of the breeding and aggressive behavioural repertoire. Specifically, since sounds are produced during reproduction for inter- and intrasexual interactions like in, for example, painted goby (*Pomatoschistus pictus* (Malm, 1865)) and monkey goby (*Neogobius fluviatilis* (Pallas, 1814)) [33-35], they are important for evolutionary studies examining the processes leading to species radiation. In addition, due to the morphological similarities and the lack of sonic specializations in currently investigated species [36,37], it can be expected that gobies utilize similar acoustic components for vocal communication which could reveal a certain phylogenetic pattern.

Taxonomically, the suborder Gobioidei is one of the largest vertebrate groups, including several families [38-40]. The European gobies belong to two of these families, Gobiidae and Gobionellidae [41]. Within the European gobies, three lineages have been recognized, with the *Gobius*- and *Aphia*-lineage as part of the family Gobiidae (gobiine-like clade in [42]), while the *Pomatoschistus*-lineage was nested within the family Gobionellidae (gobionelline-like clade in [42]). Traditionally, molecular studies have strongly emphasized that the European gobies from the *Gobius*-lineage form a monophyletic group [40,42,43], whereas the genus *Gobius* (including *Zosterisessor*; [40,42,44,45] and endemic goby species from the Ponto-Caspian region (e.g. genera *Babka*, *Benthophilus*, *Mesogobius*, *Neogobius Proterorhinus*, *Ponticola*) [40,42,46] are by far the most species-rich groups. Up to date, sound production was most commonly documented within *Gobius* lineage, for which 11 species proved to be soniferous during the experimental trials (8).

From the total number of vocal gobiids (Gobiidae), this makes around 73% of all soniferous taxa and highlights the fact that the sound production is a common phenotypic trait for *Gobius* lineage. On the contrary, only eight gobionellids (Gobionellidae) were vocally active during the bioacoustic experiments, and the majority of taxa, i.e. six species, belonged to *Pomatoschistus* lineage (8). Lastly, only two species of odontobutids (Odontobutidae) were tested for sound production (47,48), emphasizing that the acoustic communication is rarely investigated outside Gobiidae and Gobionellidae. In *Gobius* lineage, interspecific sound diversification is thought to be highly important, since closely related species show less overlap of their call features and have even developed different kinds of calls. It was proposed that those differences follow the phylogenetic pattern to certain degree [34,48,49,50], however, these studies were empirically limited, since no one has compared acoustic signals with genetic data to corroborate possible concordance. Furthermore, these studies have supported findings from other fish groups [51-56], showing that some acoustic features could be a reliable species or individual identifier. Likewise, recently documented acoustic communication in Amur sleeper *Perccottus glenii* (Odontobutidae) suggests a deeper sound production ancestry within the gobioids [48]. Since gobies are widely distributed in European waters [57-59] and many species live in sympatry with at least one other species, communication signals (including sounds) likely play a significant role in mating recognition and prevention of hybridization. Therefore, the observed vocal diversity and sound utilization during reproduction indicate that acoustic communication could have a prominent role in the evolution and speciation of the European gobies. To date, no robust, comparative or in-depth studies incorporating a correlation between acoustic signals and genetic markers have been conducted on the acoustic communication between closely related species in Gobiidae. Therefore, the object of this study was to give a quantitative evaluation of the relationship between interspecific acoustic variation and genetic divergence in vocal *Gobius* lineage gobies and, according to the observed association, discuss about the potential evolutionary forces promoting the sound divergence.

By combining quantitative bioacoustics and multiple genetic markers, this study examines previously documented but never comprehensively analysed acoustic diversity of the sounds produced by nine gobiid species (Gobiidae, *Gobius* lineage) and explores the degree to which the affinities in acoustic signals between closely related species could be related to their phylogenetic relationships. Specifically, this study aimed to: *i.* explore the interspecific acoustic variation among nine *Gobius* lineage gobiids and assess the acoustic variables responsible for the species differentiation; *ii*. investigate the phylogenetic relationship between vocal *Gobius* lineage gobiids; *iii*. examine the correlation between interspecific acoustic divergence and genetic distance based on multilocus data (two mtDNA and two nDNA markers) to explore the phylogenetic significance of acoustic signals in species diversity; *iv*. obtain insight into the evolutionary forces driving acoustic divergence in these taxa, and *v*. construct phylogenetic hypotheses on the evolution of acoustic communication in soniferous gobioids.

Material and methods

Study species

This study analysed acoustic signals and compared the vocal communication of nine vocal gobiids (Gobiidae, *Gobius* lineage) belonging to five genera (*Gobius*, *Padogobius*, *Zosterisessor*, *Neogobius* & *Ponticola*). The sounds were previously recorded and described by the authors of the present study (see ‘Sound recording and bioacoustic analyses’) but were never assembled into single comprehensive phylogenetic framework. Therefore, our species composition was based on the availability of audio tracks for interspecific acoustic analysis. Amur sleeper *Perccottus glenii* is a vocal Eurasian gobioid belonging to the family Odontobutidae or the “sleepers”. Due to the sister phylogenetic position of sleepers to the rest of the gobies including Gobiidae [41,42], *P. glenii* served as an outgroup in the analyses. The nine investigated goby species belong to the family Gobiidae [41-43], more specifically, to the *Gobius* lineage *sensu* [42]. Traditionally, these nine gobiids have been split into two groups, Atlantic-Mediterranean (AM) and Ponto-Caspian (PC) [59]. In the study, the AM gobies include the genera *Gobius, Zosterisessor* and *Padogobius*. Among them, black goby *Gobius niger* Linnaeus, 1758*,* giant goby *G. cobitis* Pallas, 1814, rock goby *G. paganellus* Linnaeus, 1758 and grass goby *Zosterisessor ophiocephalus* (Pallas, 1814) are marine/brackish inhabitants. *Gobius niger* usually occupies similar muddy habitat as *Z. ophiocephalus*, while the two *Gobius* species (*G. paganellus* and *G. cobitis*) appear sympatric in rocky habitats (pers. obs.). Two *Padogobius* species (Padanian goby *Padogobius bonelli* (Bonaparte, 1846) and Arno goby *P. nigricans* (Canestrini, 1867) are pure freshwater Italian endemics, while *P. bonelli* also occurs in Croatian rivers. *Gobius paganellus, G. cobitis* and two *Padogobius* species usually occupy stony/pebble substrates, while *Gobius niger* and *Zosterisessor ophiocephalus* can be found on sandy/muddy bottoms [59,60]. The PC species are mostly brackish to freshwater residents; bighead goby *Ponticola kessleri* (Günther, 1861) occupies stony or gravel habitat, similarly to monkey goby *Neogobius fluviatilis* (Pallas, 1814) which is common on gravel or sandy substrates. Round goby *Neogobius melanostomus* (Pallas, 1814) is common on a wide range of substrates [57,58,61]. In several Croatian watercourses, PC gobies live sympatrically and occupy similar bottom type (pers. obs.). The data regarding life-history traits (number of vertebrae and swim bladder presence) were adopted from the available literature (57,58,62) or from our field observations (habitat).

Genomic sampling and phylogenetic analyses

DNA was extracted from fin clips preserved in 96% ethanol using a Geneaid® DNA Isolation Kit. Samples were amplified for mitochondrial genes cytochrome b (*cytb*) and cytochrome c oxidase subunit I (*cox1*), and for nuclear genes Recombination activating gene 1 (*rag1*) and Rhodopsin (*rho*). *Cytb* and *rag1* were amplified according to the protocol described in

[63] using either primers AJG and H5 [64] or GluF and ThrR [65] for *cytb* and RAG1F1 and RAG1R1 [66] for *rag1*. *Cox1* was amplified with primers FishF1 and FishR1 [67] according to the PCR protocol of [68]. *Rho* was amplified with primers RhodF and RhodR [69]. The PCR protocol was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation, annealing and elongation (94°C for 45 s, 53°C for 1 min and 72°C for 2 min), and the final elongation at 72°C for 10 min. PCR products were purified with ExoSAP-IT and sequencing was performed by Macrogen Europe (Netherlands) using amplification primers. The remaining sequences were downloaded from GenBank [42,46,70] (Table 1). Sequences [S2] were visually checked in Chromas v2.6.4 and aligned in Bioedit v7.2.6.1 [71]. New sequences were deposited in GenBank (Table 1). The phylogenetic reconstruction analyses were conducted on a concatenated dataset of all genes. Concatenation was recently evaluated as an appropriate method [72,73]. Prior to analysing the sequence data, the best fitting model of nucleotide substitution for each marker and subset of positions inside the codons was determined by PartitionFinder 2 [74], according to Bayesian information criterion (BIC) and under the all models option. The selected partitioning scheme is listed in the Table S1. Bayesian Inference (BI) and Maximum Likelihood (ML) approaches were used to estimate the phylogenetic relationships between the species. BI was conducted in MrBayes v3.2.2 [75] with four independent MCMC runs for 2 million generations, applying the partitioning scheme assessed by PartitionFinder 2. Trees were sampled every 1000 generations. The convergence of runs was analysed and visualised in TRACER v1.7.0. The first 25% of sampled trees were discarded as burn-in. The remaining trees were used to construct a 50% majority-rule consensus tree. Randomized Axelerated Maximum Likelihood [RAxML 8.2.12, 76] was used to assess ML, using Science Gateway portal CIPRES [77]. Partitioning scheme assessed by PartitionFinder 2 was applied. Support of nodes was estimated by applying 1000 nonparametric bootstrap replicates. Genetic distances (uncorrected p-distances) were assessed with MEGA 6 [78]. Nucleotide composition homogeneity within genes was tested with PAUP* 4.0b10 [79].

Table 1. Location, geographic position and GenBank accession numbers of gobioids used in the phylogenetic analyses.

Taxon	Location	Geographic position		GenBank accession no.			
		Latitude	Longitude	***cytb***	***cox1***	***rag1***	***rho***
Perccottus glenii	Danube River, Veliko Gradište, SRB	44.763586	21.431633	this study	AY722171	KF415837	KX224234
Gobius cobitis	Adriatic Sea, Seline, CRO	45.265168	15.479553	this study	KR914767	this study	this study
Gobius paganellus	Adriatic Sea, Karinsko ždrilo, CRO	45.170532	15.598772	this study	KR914777	this study	this study
Gobius niger	/	/	/	KF415583	KR914775	FJ526891	this study
Zosterissesor ophiocephalus	/	/	/	EU444670	this study	FJ526851	this study
Padogobius nigricans	Topino River, Umbria, IT	43.098344	12.765068	this study	KJ554001	this study	this study
Padogobius bonelii	Zrmanja River, Muškovci, CRO	44.208541	15.739067	this study	KJ554527	this study	this study
Neogobius fluviatilis	Kupa-Kupa Channel, Donja Kupčina, CRO	45.531749	15.789373	this study	FJ526807	EU444718	this study
Neogobius melanostomus	Sava River, Mačkovac, CRO	45.748060	16.229190	this study	FJ526801	FJ526857	JF261593
Ponticola kessleri	/	/	/	FJ526770	FJ526825	FJ526879	this study

Abbreviations: SRB, Serbia, CRO, Croatia, IT, Italy.

Sound recordings and bioacoustic analyses

For all investigated taxa, audio recordings were obtained from laboratory studies [34,49,50,48,80,81,82], and the recording protocols and acoustic terminology were adopted as closely as possible to allow for interspecific comparison. Our acoustic dataset consists of 67 vocal *Gobius* lineage individuals (for nine species) for which at least ten calls were recorded per individual and the individual means for each variable were calculated (mean ± SD = 87.0 ± 33.7 sounds analysed per species). Briefly, sounds were recorded from males under laboratory conditions during the mating season (all gobies spawn from early spring to late summer), using different audio equipment consisting of a hydrophone (Gulton Industries, HTI 94 SSQ or H2A-XLR) with preamplifiers (B&K 2626 or IRIG PRE) connected to a portable audio recorder (ZOOM H4n, Sony D7 or Tascam Linear PCM). Sounds were monitored and recorded during the “intruder test”, where one individual exhibiting highly territorial behaviour after one week of acclimatization to laboratory conditions was marked as the resident fish occupying the shelter, in order to elicit inter- (male-female) or intrasexual (male-male) interactions. After recording, sounds were digitized (.wav format) and analysed using AVISOFT SASLab Pro Software (version 5.2.14., Berlin, Germany) which allowed for calculation of the acoustic variables important for further interspecific acoustic comparison. In addition, the spectrogram, oscillogram and power spectrum were prepared using AVISOFT (Hamming window, 512-points FFT, resolution 7 Hz). Only sounds with a good signal-to-noise ratio were used in the analysis. Most investigated species produce only one type of sound, while some gobies (*P. glenii* and *G. paganellus*) produce two sounds or even possess an elaborate vocal repertoire composed of three different calls (*P. bonelli*). However, the criteria used in this study for all species implies only one representative sound type per species for further comparative analysis. The representative sound type for each species was selected based on the overall number of calls observed in the audio recordings, i.e. the sound type that was most frequently registered and recorded during the behavioural investigations. To investigate interspecific affinities, audio recordings obtained from previous studies were re-analysed [34,49,50,48,80,81,82] and the six acoustic properties describing the temporal and spectral structure of gobiid sounds were calculated. Temporal parameters were sound rate (SR, sounds/min), number of pulses (NP), duration (DUR, milliseconds), and pulse repetition rate (PRR, dividing number of pulses with duration, in hertz). Peak frequency (PF, highest peak in power spectrum; hertz) and frequency modulation (FM) calculated as the difference between final PRR and initial PRR and expressed in hertz, were spectral variables in our analyses. The main purpose of the acoustic analysis was to construct a robust acoustic dataset ready for pairwise comparison with genetic divergence.

Comparison between acoustic and genetic data

To assess whether the acoustic interspecific differences in gobiids were related to phylogenetic relationships, we investigated the association between call divergence and genetic distance using the Mantel test as a prior choice [83,84]. For the correlation, we used acoustic distance matrix constructed from Cluster analysis (Joining tree analysis) in STATISTICA® (version 13.6.0., TIBCO, USA), in which clustering was performed with nine Gobiidae species as a grouping variable and six acoustic features as the analysis variables (i.e. dimensions). For the amalgamation (aggregation) rule, we used unweighted pair-group average (UPGMA) linkage, while the distance matrix was computed from the means of all sound variables for each species and built using the City-block (Manhattan) distance metric procedure. Genetic distance matrix was assessed using the uncorrected p-distance method in MEGA (version 10.0.5., USA), based on the concatenated data set for all used markers, and separately for mtDNA (*cytb* and *cox1*) and nDNA markers (*rag1* and *rho*). We used the bootstrap variance estimation method with 1,000 replications and p-distance as a substitution model for constructing the pairwise distance between the taxa. The Mantel test was conducted in *PASSaGE* v.2 [85] on 10x10 distance matrices with 10,000 permutations. Likewise, two additional Mantel tests were performed between the obtained acoustic distance matrix and genetic distances based on 1) mtDNA (*cytb* and *cox1*) and 2) nDNA (*rag1* and *rho*) markers. These correlations were performed to investigate the relationship between call divergence and genetic distance using DNA markers with different rates of mutation or evolution and therefore, they could reveal different aspects of the speciation history of the examined taxa.

Statistical analyses

Descriptive statistics were calculated for each temporal (SR, NP, DUR, PRR) and spectral (PF and FM) property of the acoustic signal produced by each species. For the preliminary explorations, we considered all sound variables except in the case of *P. glenii*, where it was not possible to calculate the FM of the thump sounds (i.e. those sounds were not modulated in frequency), so this parameter was excluded from the comparative analyses. We transformed the overall acoustic dataset and tested it for the distribution fitting. Firstly, continuous variables were

$\log_{10}$-transformed (DUR, PRR, FM and PF), while discrete (SR and NP) were square root-transformed. We then tested the variables for normal distribution by using Shapiro-Wilks W test with a level of significance $P < 0.05$. Afterwards, to investigate the sound variation among the *Gobius* lineage gobies, species were used as grouping variable. Since the assumption of normality was not met, for interspecific comparison we used the non-parametric Kruskal-Wallis H test followed by Dunn's multiple comparison test (level of significance $P < 0.001$) to investigate the variation of individual means for each sound variable across species. Individual mean values of sound variables were tested for correlation using the non-parametric *Spearman* correlation (level of significance $P < 0.001$) to investigate their mutual relationships. Furthermore, to quantify acoustic variability among the species, we applied multivariate exploratory techniques. Individual means of all sound variables of nine gobiids were compared to test for the overall signal similarity using Principal component analysis (PCA). PCA (in our case based on the correlation matrix) creates a factor space for a set of variables, and therefore we used it specifically to identify the acoustic parameters that explain the most variance among the taxa in the obtained factor space. For the interpretation of PCA results, we used as many factors as the number of eigenvalues > 1.0. In order to discriminate the species according to the acoustic parameters, a forward stepwise Discriminant function analysis (sDFA) was also carried out on the individual mean acoustic variables, with the specific aim to determine which parameters are responsible for species differentiation. In addition, sDFA was also used to assess the probability (classification rate %) at which individual sounds will be classified into the correct taxa. Specifically, sDFA enters variables into the discriminant function model one by one, always choosing the variable that makes the most significant contribution to the discrimination model. Factor structure coefficients were chosen to indicate the correlations between the variables and the discriminant functions. Partial Wilks' Lambda was chosen to indicate the contribution of each variable to the overall discrimination between species. The selection criterion for an acoustic parameter to be entered was F = 1.0, while F = 0.0 ($P = 0.01$) was the exclusion criteria for removal from the analysis. All statistical analyses were performed in STATISTICA® (version 13.6.0., TIBCO, USA) software.

Authorisations

Since all the acoustic data were already published in previous papers, no experimental acoustic work was conducted within the present study. However, all the previous experiments described in this article were compliant with the current laws for animal experimentation in Croatia (Bioethics and Animal Welfare Committee, Faculty of Science, University of Zagreb; permit #251-58-10617-18-14) and with the Venice Declaration (Italy). In addition, the licences 525-13/0545-18-2 and 525-1311855-19-2 (Ministry of Agriculture) permitted the field sampling of a Croatian ichthyofauna and licenses released by Regione Veneto (Italy) for scientific fishery of Italian species. As regarding *P. nigricans*, sampling protocols have been established in compliance with the ethical standards, as approved by the Italian regulations and by local permitting authorities (Umbria Region), who provided the sampling authorizations (Resolution of the Regional Council (DGR) N. 19, session of 16/01/2017). All the experiments were performed in accordance with standard ethological and bioacoustics procedures (avoiding suffering or damaging of fish body parts), meaning that all tested fish, after the laboratory analyses, were returned safely and unharmed to their natural habitat.

Results

Interspecific acoustic variation and sound properties

Perccottus glenii produces thumps sounds, with an irregular waveform and a lack of frequency modulation. However, the nine soniferous gobiids share certain common characteristics of their vocal repertoire, allowing for interspecific comparison (Table S2). All acoustic variables differed significantly among the species (Kruskall-Wallis test: SR: H = 46.16, P < 0.001; DUR: H = 60.41, P < 0.001; NP: H = 64.64, P < 0.001; PRR: H = 55.22, P < 0.001; PF: H = 48.05, P < 0.001; FM: H = 60.95, P < 0.001; d.f. = 9, n = 73 for each sound property), with at least one species differing from the remaining taxa according to the acoustic variables (Fig 1). Correlation analysis performed on individual mean values of acoustic variables indicated that DUR and NP were mutually and significantly associated (*Spearman* r = 0.88, n = 67, P < 0.001; Table 2), meaning that as sounds become longer, more pulses are stacked together. On the other hand, the two spectral variables PF and FM were negatively correlated (*Spearman* r = -0.43, n = 67, P < 0.001; Table 2). In the principle component analysis (PCA), the axes PC1 and PC2 accounted cumulatively for 63% of the variation, with the first two axes explaining 35.6 and 27.6% of the variance, respectively (Table S3). Temporal properties of the sound DUR, NP and PRR were positively associated with PC1, while spectral variables PF (positively) and FM (negatively) contributed to PC2 (Table S3). The PC1 *versus* PC2 scatterplot of the taxa illustrated the acoustic variation between gobiids according to the call properties (Fig S1).

Fig 1. Box plot of the six acoustic variables of the sounds produced by ten gobioid species. The midline represents the median, x marks the mean, box values highlights 25 and 75 percentiles while the whiskers indicate minimum and maximum values of the acoustic properties for each species. Abbreviations of the six acoustic variables: SR = sound rate, DUR = sound duration, NP = number of pulses, PRR = pulse repetition rate, PF = peak frequency, FM = frequency modulation.

Table 2. *Spearman* correlation coefficient of the relationships between the six acoustic properties. Correlation is based on the individual means of six acoustic properties per species (N = 9).

Variable	SR	DUR (ms)	NP	PRR (Hz)	PF (Hz)	FM (Hz)
SR (s/min)	1.000	0.037	0.102	0.035	0.050	0.318

DUR (ms)	0.037	1.000	0.886*	-0.006	0.178	-0.201
NP	0.102	0.886*	1.000	0.360	0.052	0.109
PRR (Hz)	0.035	-0.006	0.360	1.000	0.115	0.326
PF (Hz)	0.050	0.178	0.052	0.115	1.000	-0.435*
FM (Hz)	0.318	-0.201	0.109	0.326	-0.435*	1.000

Asterisk (*) marks significant correlation under $P < 0.001$ criterion.

Stepwise Discriminant function analysis (sDFA) differentiate the gobies according to their sound properties (Wilks' Lambda = 0.0002, $F_{28, 264} = 25.28$, n = 67, $P < 0.001$). The first two discriminant functions (DF1 and DF2) cumulatively explained 64.4 and 27.5% of the variation, with the DF1 significantly loaded with call properties PRR and PF, while DF2 showed a positive correlation with NP and DUR (Table 3). In addition, the DFA indicated that individual sounds were correctly classified into corresponding species with an overall 92.5% correct classification rate (Table 4). Accuracy of the classification rate varied among the species, with *P. bonelli, G. paganellus, G. niger, P. kessleri* and *Z. ophiocephalus* classified with an accuracy of 100%, while *G. cobitis* had the lowest fidelity (66.6%), indicating that sounds could be more variable in this species compared to others. Partial Wilks' Lambda (for all variables < 0.5, $P < 0.001$) indicated that acoustic variables SR, DUR, NP, PRR and PF contributed, in the most significant order, to the overall discrimination contrary to FM which did not contribute (Partial Wilks' Lambda > 0.5, $P = 0.10$). The DFA differentiated several groups of species (Fig 2). Accordingly, *P. bonelli, P. kessleri* and *G. paganellus* were clustered on the right side of the diagram, though *P. bonelli* was slightly more distant along the negative side of DF1 in comparison with the remaining two species. This group was separated from the others due to high PRR and NP values, which contributed significantly to DF1 and DF2. *Neogobius fluviatilis, N. melanostomus* and *P. nigricans* were situated on the positive DF1 and negative DF2 axes, mainly because their sounds are characterized by a short duration with low NP and high PRR. Lastly, *G. niger, G. cobitis* and *Z. ophiocephalus* are situated on the lower-left part of the diagram, where *G. niger, G. cobitis* are clustered along the negative DF1 and DF2 sides. *Zosterisessor ophiocephalus* occupies a separate position from the two mentioned *Gobius* species along the negative DF1 axis (Fig 2). These species produce long sounds with a high NP but low PRR. By extracting the two most important temporal acoustic properties from DFA, DUR and PRR, a scatterplot was built to illustrate the acoustic structure in more details (Fig 3). Species producing short tonal sounds (< DUR, > PRR; *N. melanostomus, N. fluviatilis* and *P. nigricans*) clustered on the left-uppermost part of the diagram, while the species situated on the left-lowermost part are characterized by pulsatile sounds (*Z. ophiocephalus, G. niger* and *G. cobitis*; intermediate DUR, < PRR). Finally, the three species producing long tonal/complex sounds are positioned in the upper-middle part of the diagram (> DUR, > PRR; *P. kessleri, G. paganellus* and *P. bonelli*; see Fig 3).

Fig 2. Scatterplot of discriminant function 1 (DF1) *versus* discriminant function 2 (DF2) performed with individual means of the six acoustic properties. Each species, set as a grouping variable, is represented by a different symbol.

Fig 3. Categorized scatterplot of two temporal variables (DUR *versus* PRR) highlighting the acoustic variability between the nine gobiid species. For each species, the representative spectrogram in kilohertz is mapped, where brighter colours indicate higher energy intensity. Sounds were recorded at 44.1 kHz and 16-bit resolution while the spectrogram was prepared using AVISOFT software (Hamming window, 512-points FFT, resolution 7 Hz). On the scatterplot, each symbol represents the plot of a selected variable (species mean for DUR) against the value of another selected variable (species mean for PRR) broken down (i.e. categorized) by grouping variable (Species).

Table 3. Factor structure coefficients from the discriminant function analysis (DFA) representing the correlations between the six acoustic variables and the respective discriminant functions

(DF). In the DFA, species were set as a grouping variable and individual means of the six acoustic properties as the dependent variables.

Variable	**DF1**	**DF2**	**DF3**	**DF4**
SR (s/min)	0.05	0.06	0.84	0.17
DUR (ms)	-0.06	0.52	0.02	-0.79
NP	0.19	0.91	-0.07	-0.33
PRR (Hz)	0.44	0.37	0.02	0.60
PF (Hz)	-0.41	0.21	0.22	0.56
FM (Hz)	0.07	-0.05	0.24	0.11

Table 4. Stepwise classification matrix indicating the percent of cases (individual sounds) that are correctly classified in each group (species) by the classification functions and the cases that are misclassified in each group. Total classification rate is also indicated.

Taxon	**%**	***P. bonelli***	***P. nigricans***	***G. paganellus***	***G. cobitis***	***G. niger***	***Z. ophiocephalus***	***N. fluviatilis***	***N. melanostomus***	***P. kessleri***
P. bonelli	100	5	0	0	0	0	0	0	0	0
P. nigricans	75.0	0	3	1	0	0	0	0	0	0
G. paganellus	100	0	0	15	0	0	0	0	0	0
G. cobitis	66.7	0	0	1	4	1	0	0	0	0
G. niger	100	0	0	0	0	5	0	0	0	0
Z. ophiocephalus	100	0	0	0	0	0	8	0	0	0
N. fluviatilis	87.5	0	0	0	0	0	0	7	1	0
N. melanostomus	85.7	0	0	0	0	0	0	1	6	0
P. kessleri	100	0	0	0	0	0	0	0	0	9
Total	92.5	5	3	17	4	6	8	8	7	9

Phylogenetic affinities between vocal gobiids

The molecular analysis of a concatenated dataset, inferred from two nuclear (*rag1* & *rho*) and two mitochondrial (*cytb* & *cox1*) molecular markers, allowed us to reconstruct the phylogenetic relationships of nine vocal European gobiids and to build the genetic distance matrix for the pairwise comparison using acoustic data (Table 5). The matrix of 3961 base pairs (bp) contained 30% variable sites, of which 17% are parsimony informative. The sequence lengths of individual markers were: *cytb* 1113, *cox1* 650, *rag1* 1458 and *rho* 740. Phylogenies reconstructed based on the concatenated data using maximum likelihood (ML) and Bayesian inference (BI) method showed identical topologies. *Padogobius bonelli* is in a sister position to all remaining gobiids (Fig 4). The remaining species formed three well supported topological groups. Two marine gobies, *Gobius niger* and *Zosterisessor ophiocephalus,* grouped into one clade, *G. cobitis* and *G. paganellus* clustered into the second, while the third group was composed of gobiids distributed in the Ponto-Caspian region (genera *Neogobius* and *Ponticola*) and *Padogobius nigricans*, an Italian freshwater endemic species (Fig 4). In the third group, *P. kessleri* is a sister taxon to the remaining species, *P. nigricans* and *N. melanostomus* cluster as sister species, and *Neogobius fluviatilis* is a sister taxon in regards to these two species (Fig 4). These results support the monophyly of these vocal Ponto-Caspian species (including *Padogobius nigricans*) and suggest the polyphyly of the genus *Padogobius*.

Fig 4. Bayesian inference phylogenetic relationships between the studied goby species based on concatenated dataset of two mitochondrial (*cytb* and *cox1*) and two nuclear markers (*rag1* and *rho*). The numbers on nodes represent posterior probability (BI) and bootstrap support (%, ML) values. Nodes with values ≥0.95 for posterior probability and ≥75% for bootstrap support are considered well supported. For each taxon, a single representative sound waveform was mapped to underline the acoustic affinities between the investigated taxa. Species groups are distinctly shaded: dark grey - Atlantic-Mediterranean gobiids; medium grey - Ponto-Caspian taxa; light grey - odontobutid *Perccottus glenii*. For *P. glenii,* two different sounds are mapped in order to follow the evolutionary pattern of acoustic divergence. Sounds were recorded at 44.1 kHz and 16 bit. In addition, for each species, three life-history traits are indicated by □ (number of vertebrae: white - less than 28; grey - 27-31; black - more than 28), ○ (swim bladder: white - absent; black - present) and △ (habitat: white - freshwater; black - marine). Habitat refers to the water type from which individuals for the analysis were captured. Waveforms are not to scale.

Table 5. Genetic distance matrix estimated from the concatenated dataset (*cytb*, *cox1*, *rag1* and *rho*) using the p-distance method in MEGA.

Taxon	***G. cobitis***	***G. paganellus***	***G. niger***	***Z. ophiocephalus***	***P. nigricans***	***P. bonelli***	***N. fluviatilis***	***N. melanostomus***
G. paganellus	7.73							
G. niger	10.20	10.08						
Z. ophiocephalus	10.36	9.73	9.35					
P. nigricans	9.91	10.44	11.84	10.89				
P. bonelli	10.66	11.04	12.29	11.71	11.21			
N. fluviatilis	9.40	9.86	10.98	11.06	6.20	10.86		
N. melanostomus	9.61	9.94	11.67	11.07	6.05	11.24	5.75	
P. kessleri	10.93	11.54	12.52	12.49	10.04	12.04	9.50	9.94

Acoustic and genetic divergence comparison

We performed pairwise comparison between call divergence and genetic diversity to investigate their mutual relatedness in gobiids. Specifically, call divergence matrix was built from Cluster analysis (clustering performed by using six acoustic features), and genetic distance matrix, which was obtained by using the uncorrected p-distance method based on the concatenated data set for all used markers (*cytb*, *cox1*, *rag1* and *rho*), and separately for mtDNA (*cytb* and *cox1*) and nDNA markers (*rag1* and *rho*). A significant positive correlation was found between acoustic and genetic distance matrices (Mantel test r = 0.470, Z = 2298.756, $P_{two\text{-}tailed}$ = 0.01; Fig 5), even after performing the matrix permutation test (10,000 repetitions, $P_{two\text{-}tailed}$ = 0.03) indicating that in vocal gobiids from the *Gobius* lineage, interspecific divergence in sound follows the same phylogenetic pattern of diversification. In addition, when the acoustic distance was compared with genetic divergence based on nuclear (*rag1* and *rho*) or mitochondrial (*cytb* and *cox1*) markers, the results were similar to those with the concatenated dataset. There was a significant positive correlation between acoustic and nuclear distance (Mantel test for nDNA: r =0.485, Z = 793.852, $P_{two\text{-}tailed}$ = 0.005; Fig S2), and acoustic and mitochondrial distance (Mantel test for mtDNA: r = 0.450, Z = 4195.402, $P_{two\text{-}tailed}$ = 0.01; Fig S2), indicating once again that regardless the type of genetic marker and its evolutionary rate, sounds diverge in similar pattern to the phylogenetic affinities in gobiids.

Fig 5. Correlation between genetic distance and acoustic divergence in nine vocal *Gobius* lineage gobiids (Mantel test r = 0.47, $P_{\text{t.t.}}$ = 0.01). Genetic distance was estimated from the concatenated dataset (p-distance method), while acoustic distance was estimated from the standardized Manhattan distance metric procedure using species means of the six sound variables. The dashed patter shows linear trend line, while the scatterplot represents the relationship between species genetic differentiation and their acoustic distance.

Discussion

In comparison with the vocalizations of other vertebrates like frogs, birds or mammals, and given the possible absence of the confounding effects of learning [86,87], the relative simplicity and strong high stereotypy of teleost sounds make fish a useful group for studying the evolution of acoustic communication and its association with phylogeny. By correlating acoustic variability with genetic divergence, we sought to elucidate whether sounds in vocal gobiids (*Gobius* lineage) have a phylogenetic basis, and did stochastic evolutionary forces play a prominent role in signal divergence. No similar investigation has never been performed in gobiids or any other teleost group to date, and our results could be meaningful since speciation may be accelerated by the separation of signalling systems [20]. Since various communication signals, including acoustic signals reveal the identity of the signalling animal, they may be involved in species diversification [11].

This acoustic analysis allowed us to discriminate between nine gobiids according to the spectro-temporal properties of their sounds, indicating that each taxon produces species-specific calls characterized by a unique set of variables allowing for interspecific differentiation. Since most gobies live in natural sympatry with at least one other species [58-61], we believe that these sounds could act as accurate species-discrimination traits. From the acoustic analyses including PCA and sDFA, certain sound properties, especially temporal NP, DUR and PRR and spectral PF appear to be responsible for the observed interspecific call divergence. Since these sound properties accounted for most of the variation among species in both PCA and sDFA (variable-factor correlations ranged from 0.4 to 0.9), they can be considered the main acoustic components carrying the phylogenetic signal. However, since in the acoustic analyses, NP was one of the important sound features, but it was strongly correlated with DUR (Spearman r > 0.8), DUR can be considered an independent and phylogenetically more informative trait than NP. This is corroborated by the overall similarity in taxa composition comparing the sDFA scatterplot with the DUR *versus* PRR diagram, where both highlighted a similar pattern of acoustic divergence among the studied gobiids. Moreover, the sDFA emphasized that the individual sounds were accurately attributed to corresponding species with high classification fidelity (> 90%), meaning acoustic signals could indeed reflect phylogenetic taxon affiliation. The first comparative study on acoustic signals produced by Mediterranean gobies [49] proposed that relationships between Mediterranean gobies could be inferred using the signal structure as a reliable indicator of taxon affiliation, given the strong relationships between acoustic affinities and species traits. However, without genetic data, this was a long-standing hypothesis corroborated by this study.

Our phylogenetic analysis strongly indicated that *P. bonelli* is separated from the rest of the investigated taxa, occupying an isolated position on both the sDFA phenogram and concatenated phylogenetic tree. This is in agreement with the known vocal diversity of *P. bonelli*, with three different sound types reported to date [49,80]. Among the Atlantic-Mediterranean gobiids (*Gobius* and *Zosterisessor* genera), deeper phylogenetic relationships remain unresolved, though some interesting observations can be drawn from our results. For *Z. ophiocephalus* and *G.*

niger, an isolated group on the genetic tree, the observed phylogenetic relationship coincides with their similar acoustic structure of pulsatile sounds and substrate preferences [mud or silt; 60]. Likewise, their close phylogenetic affiliation has been confirmed using DNA sequences from both mitochondrial and nuclear markers [42,43,45], although these studies included a smaller number of species for phylogenetic analysis. In the PCA and sDFA, both species occupied the same acoustic space, even though *Z. ophiocephalus* had a slightly more isolated position in both PCA and sDFA scatterplot, due likely to the higher spectral sound properties, mostly PF. The observed acoustic and genetic relationship between *Z. ophiocephalus* and *G. niger* sheds light on the taxonomic position of *Z. ophiocephalus*, as some authors have suggested a close phylogenetic relationship with the genus *Gobius*. On the other hand, *G. cobitis* and *G. paganellus* are mostly found on rocky bottoms, and they share certain phenotypic traits [e.g. colouration pattern and sagittal otolith shape; 60,88]. The species inhabiting the Ponto-Caspian region (genera *Neogobius* and *Ponticola*), produce only one sound type [34,50], justifying their clustering into one well-supported clade [this study and 42,43,46,89]. However, from our results, a certain degree of acoustic variability is evident between the *Neogobius* group (*N. fluviatilis, N. melanostomus* and *Padogobius nigricans*), characterized by the production of short tonal sounds, and *Ponticola kessleri*, which was separated from the remaining Ponto-Caspian species in the PCA and sDFA diagrams, likely due to its long, frequency-modulated sounds, similar to *G. paganellus* vocalizations. This is interesting since both species (together with *P. bonelli*) share similar bottom preferences (rocks or coarse gravel), although *P. kessleri* is a freshwater resident. This observation might suggest that tonal sounds are more suitable for hard-bottom transmission, although this should be examined in future studies by investigating the ecological adaptations of gobies to certain habitat conditions. Recent studies have suggested that rocky or pebbly substrates, inhabited by the bottom-dwelling gobies producing tonal sounds (like *G. paganellus*, *P. kessleri* and *P. bonelli*), are unfavourable for sound emission, due to the low-frequency ambient noise and short-range transmission of sounds [90,91]. [92] proposed that tonal sounds could possess characteristics enabling longer-range transmission than pulsatile sounds, since the acoustic structure is simpler. In addition, according to [92], waveform differences between pressure and particle velocity spectra are less expressed in tonal than pulsatile sounds. In vertebrates, frequency modulated sounds (like the tonal sounds of gobiids) are generally long-range signals [93]. Furthermore, the close affinity of *P. nigricans*, an Italian endemic goby, with the Ponto-Caspian group in both the sDFA and the phylogenetic tree is phylogenetically interesting. According to [58], *P. nigricans* is similar to the Ponto-Caspian gobies due to the larger number of vertebrae (> 29), and the presence of a reduced swimming bladder and the presence of head canals, while *P. bonelli* has a swim bladder but lacks head canals [58]. Likewise, recent acoustic studies indicated that *Padogobius* could be polyphyletic [34,50], supporting previous molecular findings that emphasized that the two *Padogobius* species are of independent origin [94-96]. However, no studies have used robust sound data set to investigate acoustic diversity and to combined genetic data with sound variability to empirically confirm this hypothesis. Our comparative study strongly corroborated these hypotheses, indicating that the genus *Padogobius* is truly polyphyletic. Sounds, as shown here, proved to be a valuable species-specific trait in the European gobiids, and a suitable basis for future phylogenetic studies.

In young or emerging species, acoustic signals may serve as isolating mechanisms, leading to intraspecific acoustic variability. It has long been debated whether selection or drift have relative importance in the process of speciation [97,98]. To demonstrate that stochastic (also called ‘neutral’), and not deterministic (‘adaptive’) evolution is most important in driving acoustic differences, divergence in acoustic traits should be empirically confirmed to increase linearly with genetic distance, with little or no effect of selection [11,20]. Although we did not test the effects of selection, a positive linear correlation between acoustic distance and genetic divergence for all investigated species was obtained, using both a concatenated molecular dataset and individual mtDNA/nDNA markers. This indicates that sounds diverge in the same manner as DNA sequences, regardless the marker type and its evolutionary rate. In the evolutionary sense, these findings suggest that stochastic forces could be more responsible for shaping acoustic divergence than deterministic processes in European gobiids. In the presence of a positive correlation, most studies have emphasized that drift is the main driver behind differentiation in calls between species [11,99,100]. However, we propose that this should be verified by future studies exploring the intensity of sexual selection between closely related species. In addition, other factors such as social learning [101] and mutation-order processes [102] could drive acoustic differentiation. In learned vocal signals [e.g., bird song, 103; whale song, 104], cultural transmission and copying errors are major drivers of stochastic divergence. The ability of gobiids to learn sounds has never been investigated. Considering the recent findings of other teleosts, suggesting that the sounds in fish are innate [86,87], the effects of social selection as a driving force for the observed divergence can be excluded at this time. Mutation-order processes over time can cause the linear accumulation of acoustic differences [105,106], resulting in a highly complex interaction between such processes and drift. However, some gobiids hybridize [107], violating reproductive isolation as one of the main criteria for mutation-order speciation. Moreover, we have no precise knowledge whether acoustic divergence is associated with ecological or sexual trait divergence, and therefore their indirect influence on overall divergence cannot be excluded, since this divergence may result from a combination of selection and drift [20,108]. Geographic variation or ecological selection in gobiid vocalizations is still largely unexplored, with few reports of "dialects" in gobiids [37,50]. Therefore, comparing genetic distance and sound variability shaped by regional or habitat selection was not feasible in the present study [13,25,29]. We suggest that neutral evolution (i.e. stochastic processes) drives acoustic signal divergence in gobiids, although the influence of selection should not be neglected, since it likely has some impact on overall diversification. Sounds, including their acoustic features, carry important phylogenetic signals for species recognition, although deterministic processes such as sexual selection or habitat adaptations could have an indirect role in acoustic divergence. The exact degree of the phylogenetic signal carried by the acoustic features of gobiid sounds and their rate of evolution remains unclear. In their elaborate study, [106] tested whether the differences in male sexual signal (nuptial colour) were correlated with environmental, genetic or geographic distances in darters. From the observed correlation between overall male colour differences (i.e. scores for discrete colour categories) and genetic divergence, authors concluded that a single phenotypic trait, i.e. breeding coloration of the males (or in our case the sound), could possibly be a combination of various independent (continuous and discrete) characters, each operating under different selective regime. Having this in mind, we can expect that certain sound properties (as previously mentioned NP, DUR, PRR and PF) would carry different levels of phylogenetic information. The lower phylogenetic signal of the acoustic traits than morphological traits showed that sexual selection could be an important driver of diversification in bats and glass frogs [18,19]. If sexual selection was the main driver of acoustic divergence in gobiids, we would not expect a positive association between song similarity and genetic divergence, since acoustic signals would then diverge faster than genetic loci [11]. It is interesting that sympatric species in this study (i.e. gobies occurring together in the same region, but preferring different microhabitats; *Z. ophiocephalus* with *G. niger; G. paganllus* with *G. cobitis; P. kessleri* with *N. melanostomus* and *N. fluviatilis*) differed acoustically, which may be to avoid signal interference [109], since sympatric taxa compete for acoustic niches.

From our results and the existing literature, we were able to build the acoustic hypothesis explaining the evolution of sound communication in soniferous gobioids (Fig 6). Briefly, short tonal sound and thumps (i.e. short irregular pulses) noted in *P. glenii* (Odontobutidae) represent stem signals, i.e. the symplesiomorphic condition for gobioids. Observed phylogenetic pattern of sound production in gobioids suggests that acoustic signals probably evolved from multiple brief, rapidly repeated sounds (like thumps and tonal) to long and frequency modulated calls (pulsatile and complex) documented in vocal *Gobius* lineage gobiids. In these species (*P. bonelli* and genus *Gobius*), stem sound types gave the structural elements for the construction of complex calls, while the *Neogobius* group returned to the ancestral acoustic state or shares homoplasious trait with odontobutids (i.e. production of short tonal sounds).

Fig 6. Diagram depicting the divergence of acoustic signals between soniferous gobioids following the evolutionary hypothesis. Affinities between the gobiids follow the interspecific relationships obtained from the concatenated Bayesian inference (BI) phylogenetic tree from the present study. Abbreviations: DUR = duration (ms), PRR = pulse repetition rate, FM = frequency modulation (Hz), s., sounds.

Conclusion

In summary, our results corroborate previous evidence that interspecific acoustic signals are highly diverse among vocal European gobiids. Each species from our study is recognisable based on its sound structure and spectro-temporal features, making its vocalisations a highly species-specific trait. Since *P. nigricans* clustered in the same acoustic and genetic topology with *Neogobius* spp., we suggest that the genus *Padogobius* is polyphyletic and that *P. nigricans* is closely related to Ponto-Caspian gobies. This result is further supported by the morphological traits, i.e. vertebrate number and absence of a swimbladder. Furthermore, our comparative acoustic-genetic analyses suggest that signal divergence in European gobiids was likely caused by stochastic processes that shaped acoustic diversity. This is corroborated by the observed pattern of sound divergence, which correlated linearly with genetic distance. Therefore, we propose that certain acoustic properties of gobiid sounds carry a phylogenetic signal responsible for species recognition. Future studies should quantify the exact level of phylogenetic information present in these acoustic properties. However, these studies should not neglect the influence of deterministic process in the ecological and geographical divergence in gobiid sounds, and their roles in acoustic divergence should be determined. Phonotaxic experiments are necessary to test the influence of sexual selection in the sound differentiation process, highlighting sexually-selected acoustic traits. In conclusion, we strongly suggest that sounds in European gobiids are species-specific and evolutionarily based, presenting a promising phylogenetic tool for future comparative studies aiming to resolve their affinities and taxonomic status.

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Supporting information

Fig S1. Scatterplot from principle component analysis (PCA) performed with individuals means of the six acoustic variables. PC1 is loaded by temporal variables number of pulses and sound duration, while PC by spectral peak frequency and frequency modulation.

Fig S2. Correlation between genetic distance and acoustic divergence in nine vocal *Gobius* lineage gobiids. In A), correlation was achieved (Mantel test r = 0.48, $P_{\text{t.t.}}$ = 0.005) by using genetic distance obtained from p-distance method for nuclear markers (*rag1* and *rho*), while in B) correlation (Mantel test r = 0.45, $P_{\text{t.t.}}$ = 0.01) was inferred from mitochondrial *cytb* and *cox1* sequences while the divergence was obtained using p-distance method. The scatterplot represents the relationship between species genetic differentiation and their acoustic distance.

Table S1. Partitioning scheme for concatenated dataset assessed by PartitionFinder 2.

Table S2. Mean values and standard deviations of the total length and the six acoustic variables for the ten gobioid species. For *Perccottus glenii*, only thump sounds were used for the acoustic analysis.

Table S3. Percentage and cumulative percentage of variance explained by the first three axis of principal component analysis (PCA), with the loadings for these axes (i.e. factor coordinates) extracted from six acoustic variables. PC factor coordinates represent the correlations between the respective individual mean value of sound variable and each PC factor.

Figures

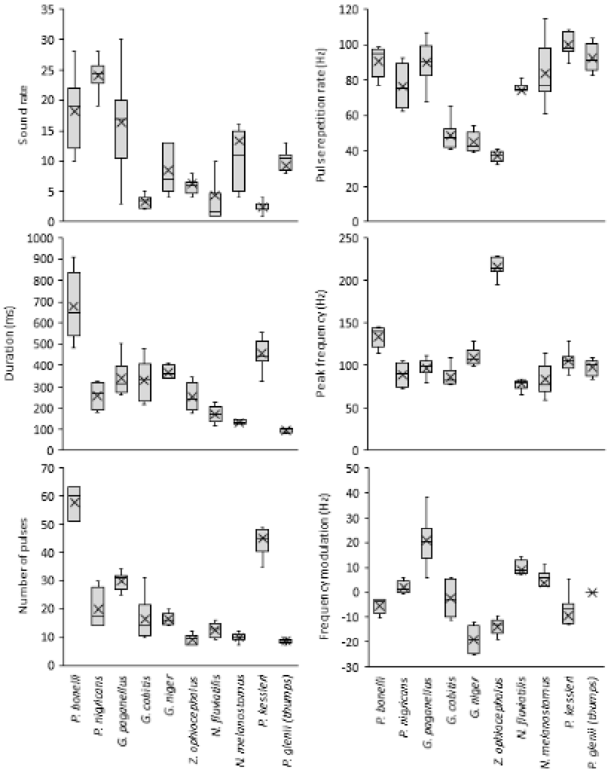


Fig. 1.

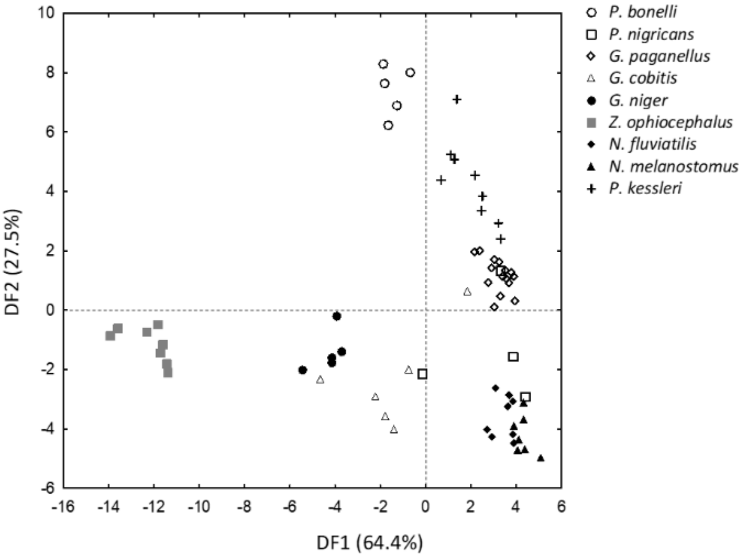


Fig. 2.

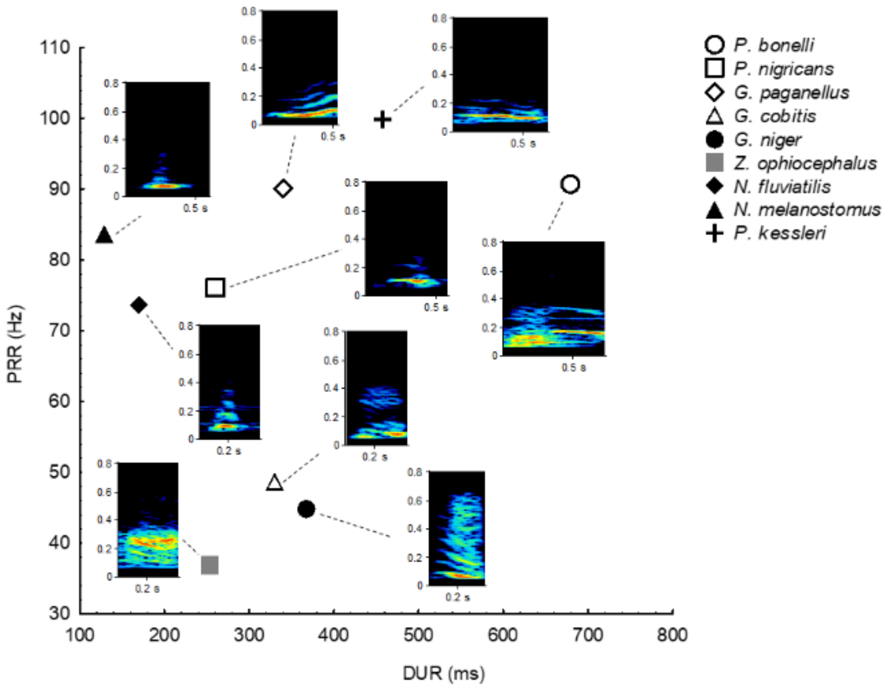
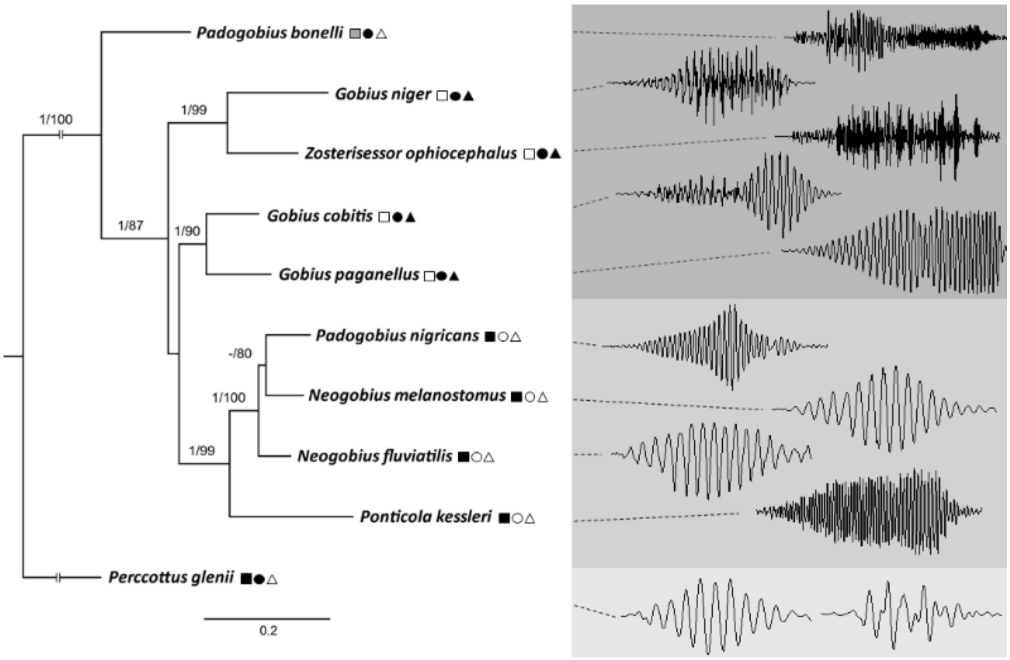

PRR (Hz)
DUR (ms)
110
100
90
80
70
60
50
40
30
100
200
300
400
500
600
700
800
0.8
0.6
0.4
0.2
0
0.5 s
0.2 s
○ P. bonelli
□ P. nigricans
◇ G. paganellus
△ G. cobitis
● G. niger
■ Z. ophiocephalus
◆ N. fluviatilis
▲ N. melanostomus
+ P. kessleri

Fig. 3.


Padogobius bonelli ▨●△
Gobius niger □●▲
Zosterisessor ophiocephalus □●▲
Gobius cobitis □●▲
Gobius paganellus □●▲
Padogobius nigricans ■○△
Neogobius melanostomus ■○△
Neogobius fluviatilis ■○△
Ponticola kessleri ■○△
Perccottus glenii ■●△
1/100
1/99
1/87
1/90
-/80
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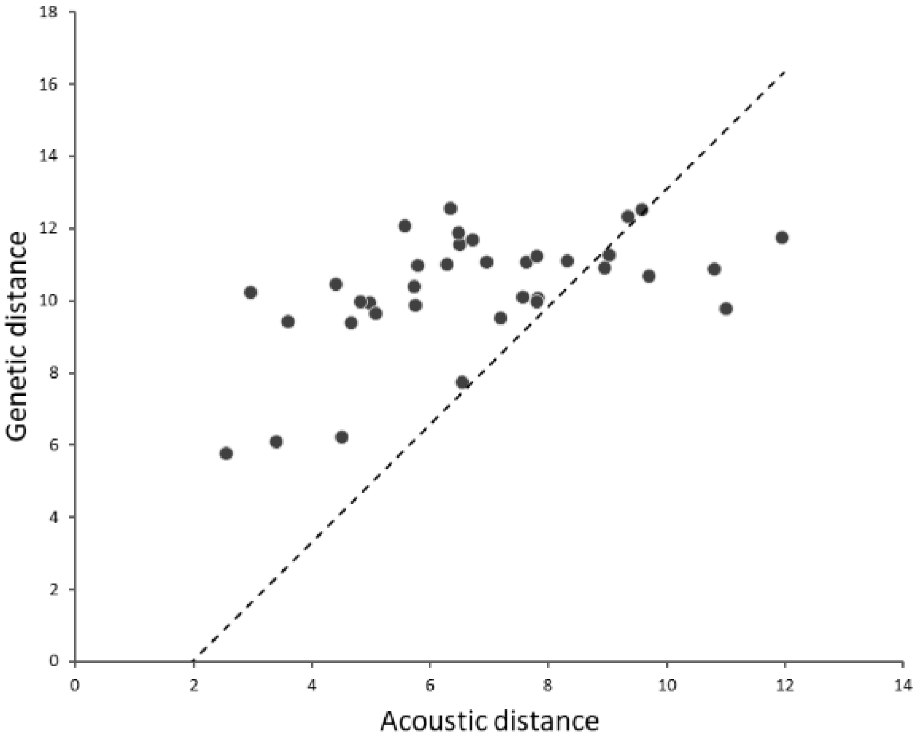
1/99
0.2

Fig. 4.


Genetic distance
Acoustic distance
0
2
4
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0
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14

Fig. 5.

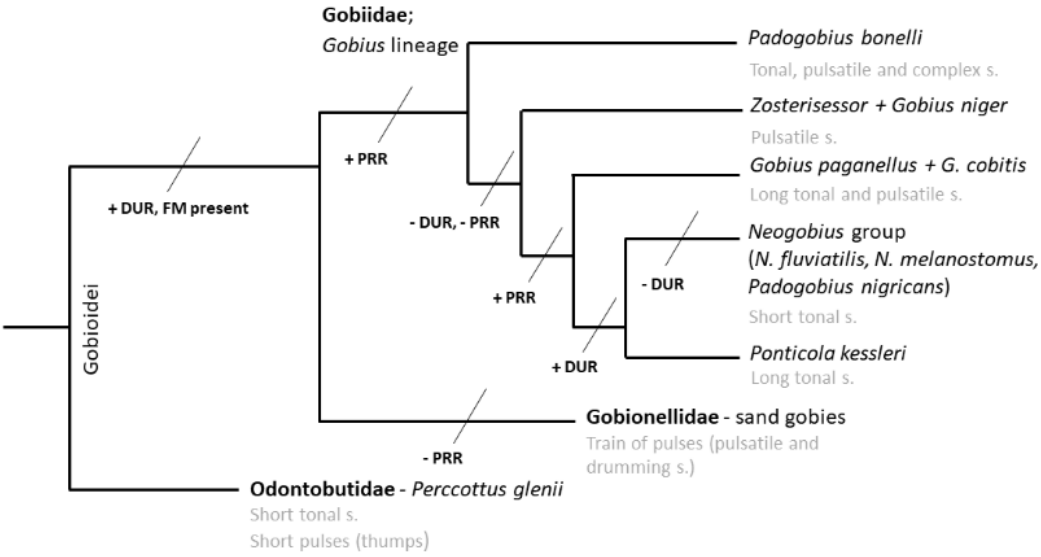
Gobiidae;
Gobius lineage
Gobioidei
+ DUR, FM present
+ PRR
- DUR, - PRR
+ PRR
- DUR
+ DUR
- PRR
Padogobius bonelli
Tonal, pulsatile and complex s.
Zosterisessor + Gobius niger
Pulsatile s.
Gobius paganellus + G. cobitis
Long tonal and pulsatile s.
Neogobius group
(*N. fluviatilis, N. melanostomus, Padogobius nigricans*)
Short tonal s.
Ponticola kessleri
Long tonal s.
Gobionellidae - sand gobies
Train of pulses (pulsatile and drumming s.)
Odontobutidae - *Perccottus glenii*
Short tonal s.
Short pulses (thumps)

Fig. 6.

Predano na recenziju u *Plos One*

Correlation between acoustic divergence and phylogenetic distance in vocal European gobiids (Gobiidae; Gobius lineage)

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SUPPLEMENTARY INFORMATION

Table S1. Partitioning scheme for concatenated dataset assessed by PartitionFinder 2.

Substitution model BI/RAxML	**Gene and codon position**
HKY+I / GTR+G	*cox1*-3, *rho*-1, *cytb*-1
TRN+G / GTR+G	*cytb*-2, *cox1*-1
K80+G / GTR+G	*cytb*-3
TRNEF+G / GTR+G	*cox1*-2
K81UF+I / GTR+G	*rag1*-1, *rho*-3
K80+G / GTR+G	*rag1*-2
TVM / GTR+G	*rho*-2, *rag1*-3

Table S2. Mean values and standard deviations of the total length and the six acoustic variables for the ten gobiod species. For *Perccottus glenii*, only thump sounds were used for the acoustic analysis.

Species	N	TL (mm)	SR (s/min)	DUR (ms)	NP	PRR (Hz)	PF (Hz)	FM (Hz)
P. bonelli	5	75.6±7.9	18.2±6.6	679.2±145.7	57.6±5.6	90.6±7.9	134.2±11.4	-5.3±2.7
P. nigricans	4	94.7±10.2	24.0±3.7	260.6±66.5	19.9±7.4	76.1±13.2	89.4±15.0	1.8±2.8
G. paganellus	15	117.2±23.0	16.3±7.6	340.5±70.5	29.9±3.0	90.0±10.4	96.8±12.0	20.5±8.8
G. cobitis	4	147.5±37.4	3.3±1.2	330.3±97.3	16.5±7.8	48.6±8.8	86.4±12.1	-2.5±7.3
G. niger	5	121.4±12.4	8.4±4.3	368.2±32.6	16.4±2.5	44.7±5.9	109.4±11.2	-19.1±5.8
Z. ophiocephalus	8	175.7±30.8	6.3±2.6	253.9±66.2	9.2±1.8	36.9±3.1	215.8±11.0	-13.6±3.3
N. fluviatilis	7	130.6±10.8	4.4±5.5	170.0±39.5	12.4±2.5	73.7±6.9	78.0±5.8	8.8±4.7
N. melanostomus	7	145.5±12.5	13.4±11.7	128.2±35.6	10.0±1.6	83.5±17.9	83.3±18.8	3.8±6.9
P. kessleri	9	157.6±13.2	2.5±0.9	457.9±68.3	44.9±7.1	99.9±6.6	104.9±11.6	-9.1±10.6
P. glenii	6	106.1±5.4	9.2±3.9	95.4±10.4	8.7±1.2	92.4±7.8	97.9±10.0	/

N, number of investigated individuals per species; TL, total length of the body (in millimeters). Abbreviations of the six acoustic variables: SR = sound rate, DUR = sound duration, NP = number of pulses, PRR = pulse repetition rate, PF = peak frequency, FM = frequency modulation.

Table S3. Percentage and cumulative percentage of variance explained by the first three axis of principal component analysis (PCA), with the loadings for these axes (i.e. factor coordinates) extracted from six acoustic variables. PC factor coordinates represent the correlations between the respective individual mean value of sound variable and each PC factor.

Variable	PC1	PC2	PC3
Percentage	35.60	27.64	17.85
Cumulative percentage	35.60	63.25	81.10
SR (s/min)	0.31	-0.37	-0.75
DUR (ms)	0.73	0.56	-0.14
NP	0.96	0.24	0.05
PRR (Hz)	0.72	-0.42	0.30
PF (Hz)	-0.22	0.69	-0.50
FM (Hz)	0.10	-0.71	-0.38

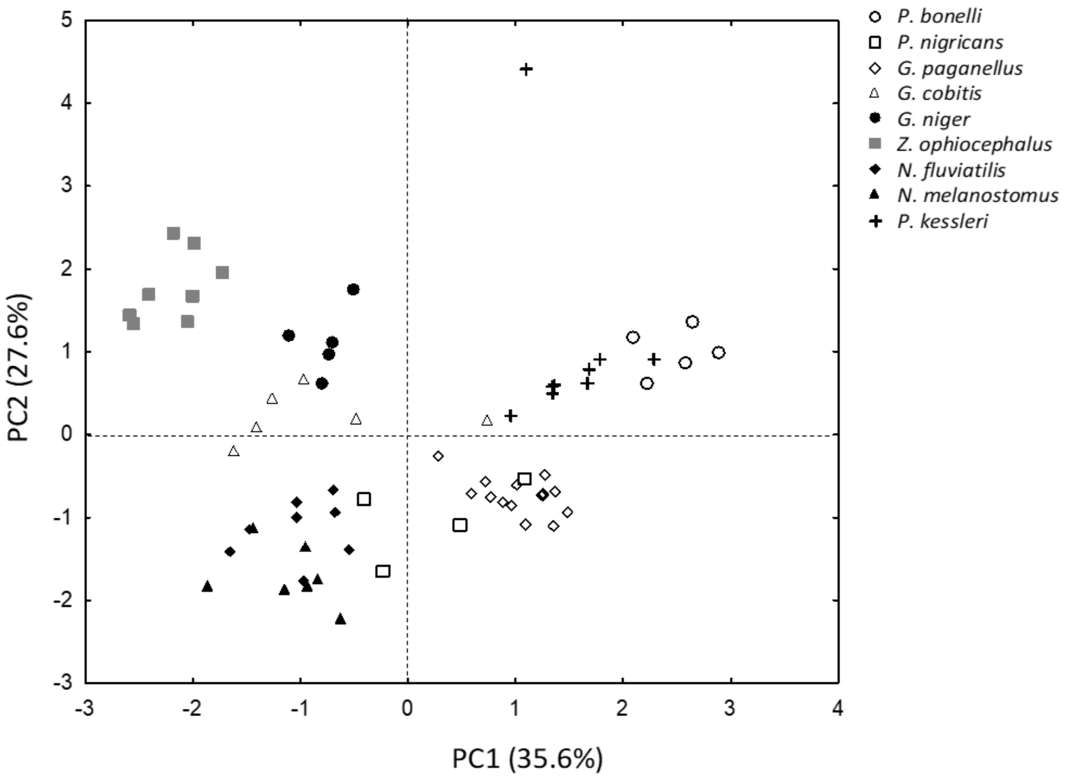

Fig S1. Scatterplot from principle component analysis (PCA) performed with individuals means of the six acoustic variables. PC1 is loaded by temporal variables number of pulses and sound duration, while PC by spectral peak frequency and frequency modulation.

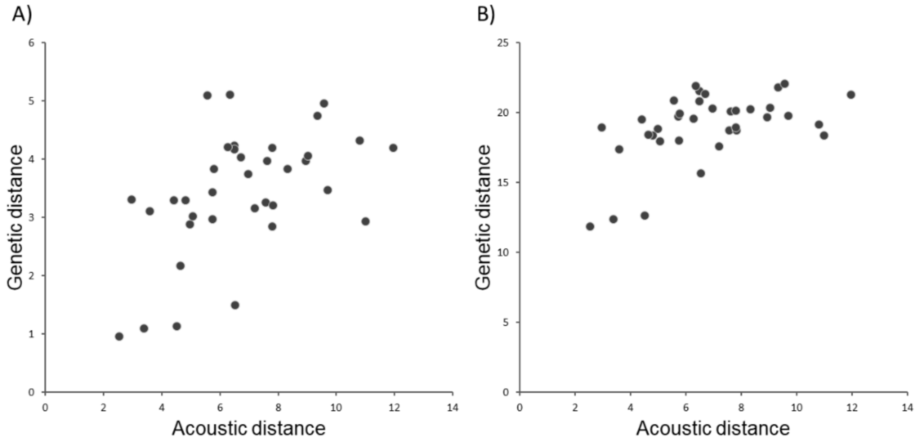

Fig. S2. Correlation between genetic distance and acoustic divergence in nine vocal *Gobius* lineage gobiids. In A), correlation was achieved (Mantel test r = 0.48, $P_{\mathrm{t.t.}}$ = 0.005) by using genetic distance obtained from p-distance method for nuclear markers (*rag1* and *rho*), while in B) correlation (Mantel test r = 0.45, $P_{\mathrm{t.t.}}$ = 0.01) was inferred from mitochondrial *cytb* and *cox1* sequences while the divergence was obtained using p-distance method. The scatterplot represents the relationship between species genetic differentiation and their acoustic distance.

3. DISKUSIJA

3.1. *Glavoči linije Gobius – modelni organizmi u bioakustičkim istraživanjima*

Svi su ciljevi obuhvaćeni ovom disertacijom izneseni u publikacijama **I**, **II**, **III** i **IV** koje su pokušale ispitati znanstvene hipoteze postavljene u okviru ove disertacije. Kao što je već navedeno, glavoči su jedna od najbrojnijih skupina vokalnih riba, s 24 dosad ispitane vrste u bioakustičkim eksperimentima, što čini oko 1,2% njihove ukupne brojnosti (Zeyl, 2016; Fricke i sur., 2020). Također, uz nekoliko drugih porodica (Pomacentridae, Batrachoidiidae, Gadidiae, Serrasalmidae), glavoči danas čine jednu od bioakustički najistraživanijih skupina riba koje se koriste u mnogim multidisciplinarnim bioakustičkim studijama kao što su akustička filogenija, fiziologija glasanja, vokalna etologija, utjecaj buke na akustičku komunikaciju i dr. (Amorim i Neves, 2007; Malavasi i sur., 2008; Parmentier i sur., 2013; Amorim i sur., 2018). Bez obzira na to, većina se znanstvene pozornosti i dalje pridaje tradicionalnim morfološko-ekološkim ispitivanjima, modernim molekularnim analizama radi rješavanja taksonomskih i sistematskih nepoznanica te dugoročnim projektima monitoringa (praćenja) glavoča određenog područja. Zbog svojih životnih navika i ekoloških karakteristika kao što su izražena teritorijalnost, bentički način života, speleofilni tip reprodukcije i dr., glavoči linije *Gobius* pokazali su se kao idealni laboratorijski (modelni) organizmi za eksperimente pasivne akustike ili *in situ* istraživanja. Za razliku od glavoča linije *Pomatoschistus* europskog područja (rodovi *Knipowitschia*, *Pomatoschistus*, *Orsinigobius*, *Ninnigobius*, *Economidichthys*), glavoči linije *Gobius* tjelesno su veći od navedenih skupina (do 27 cm ukupne dužine; Jardas, 1996), što ih čini iznimno pogodnim ribama za manipuliranje ili rukovanje tijekom laboratorijskih eksperimenata. Naime, pokazalo se kako se u eksperimentima pasivne akustike (publikacije **I**, **II** i **IV**) glavoče linije *Gobius* može, u laboratorijskim uvjetima, izazvati ili motivirati na produkciju zvukova jednostavnim, ali efektivnim etološkim „testom uljeza“ koji je postao standardni eksperimentalni postupak u bioakustičkim istraživanjima. Ukratko, u „testu uljeza“ određena jedinka domaćin (većinom mužjak) izolira se u posebnom akvariju na nekoliko sati/dana tijekom kojih razvija teritorijalno ponašanje te pri ubacivanju druge jedinke (istog ili različitog spola), počinje stvarati agresivne ili reproduktivne zvukove (publikacije **I**, **II**, **III** i **IV** i Sebastianutto i sur., 2008; Malavasi i sur., 2008, 2009). Dosad, samo kod jedne vrste glavoča, *Economidichthys pygmaeus,* zvukovi nisu zabilježeni u eksperimentima pasivne akustike tijekom reproduktivne sezone, tj. tijekom udvaranja ili parenja (Gkenas i sur., 2010). Rezultati navedenog etološko-akustičkog istraživanja navode kako je taj potencijalni sekundarni gubitak produkcije zvukova kod *E. pygmaeus*, inače jedne od najmanjih vrsta kralješnjaka na svijetu, najvjerojatnije povezan s gubitkom kopanja ili gradnje gnijezda, što je kod većine vrsta uvijek popraćeno stvaranjem akustičkih signala (Gkenas i sur., 2010; Lugli, 2015). Ribe su u eksperimentima bioakustike vrlo popularni i u posljednjih dvadesetak godina često korišteni modelni organizmi, ponajprije zbog relativno jednostavne građe središnjih (unutarnje uho) i perifernih struktura za primanje i produkciju zvukova, relativno pojednostavljene strukture svojih akustičkih signala, manjeg vokalnog repertoara u odnosu na druge kralješnjake (uz nekoliko iznimki među ribama) te zbog snažne stereotipnosti i instinktivne prirode svojih zvukova (Malavasi i sur., 2008; Amorim i sur., 2011; Ladich, 2014). Uzimajući u obzir rezultate istraživanja koji pokazuju kako ribe (uključujući i glavoče) ne mogu naučiti zvukove ili ih oponašati onako kako to rade sisavci ili ptice, već su oni kod riba urođeni (Johnston i Buchanan, 2007; Longrie i sur., 2008), još jednom dolazimo do odgovora na pitanje zašto su upravo glavoči linije *Gobius* jedna od najčešće korištenih skupina riba u eksperimentima bioakustike. Uz to, velika brojnost validnih i opisanih vrsta iz porodice Gobiidae (oko 1.250 vrsta), kao i onih koje tek čekaju svoju taksonomsku sistematsku afilijaciju (oko 500 vrsta) (Fricke i sur., 2020), ostavlja mnogo prostora za nadolazeća bioakustička istraživanja srodnih vrsta glavoča linije *Gobius* šireg mediteranskog područja.

Krenuvši od prvog cilja, publikacije **I** i **II** blisko su povezane s proširivanjem općeg znanja o vokalnom repertoaru pontokaspijskih glavoča linije *Gobius* (*Neogobius fluviatilis*, *N. melanostomus* i *Ponticola kessleri*). Do trenutka objave navedenih publikacija, vokalni repertoar s opisanim i kvantificiranim akustičkim svojstvima, kao i etološki kontekst produkcije zvukova, bio je istražen kod samo dvije vrste pontokaspijskih glavoča, mramorastog glavoča *Proterorhinus marmoratus* (Pallas, 1814) i glavočića okrugljaka *N. melanostomus* (Ladich i Kratochvil, 1989; Rollo i sur., 2007). Mramorasti glavoč je slatkovodna i morska vrsta glavoča koja se donedavno smatrala široko rasprostranjenom u euroazijskim vodotocima, uključujući i rijeku Dunav (Miller, 2004), ali su molekularne analize pokazale kako se unutar *Proterorhinus* kompleksa nalazi nekoliko kriptičnih vrsta, s naglaskom na tome kako je slatkovodna forma najvjerojatnije odvojena vrsta, *P. semilunaris* (Heckel, 1837) (Neilson i Stepien, 2009b). Budući da su Ladich i Kratochvil (1989) opisali tonalne zvukove slatkovodne forme mramorastog glavoča (lokacija: rijeka Dunav kod Beča), najvjerojatnije se radi o vrsti *P. semilunaris*, kod koje je bioakustičko istraživanje pokazalo kako se tijekom agresivnih interakcija glasaju oba spola, dok se za vrijeme parenja glasaju samo mužjaci, stvarajući kratke (240 do 260 ms) tonalne zvukove frekvencije oko 70 – 130 Hz. Opisani zvukovi *P. semilunaris* po svojoj strukturi i izgledu spektrograma djelomično nalikuju signalima dviju pontokaspijskih vrsta, *N. fluviatilis* i *N. melanostomus*, s kvantitativnim razlikama u vrijednostima akustičkih parametara (publikacija **I** i **II**). Također, vokalni repertoar *P. kessleri*, sa svojim akustičkim parametrima (publikacija **II**), omogućuje razlikovanje vrsta i skupina potporodice Benthophilinae s obzirom na akustičke signale. Neilson i Stepien (2009a) podijelili su, na temelju molekularnih analiza, grupu Benthophilinae na tri skupine (Neogobiini, Ponticolini, Bentophilini), a publikacije **I** i **II** grupiraju zabilježene kratke tonalne zvukove *N. fluviatilis*, *N. melanostomus*, koji se intenzivno brzo ponavljaju tijekom kraćeg razdoblja, u jednu grupu, dok dugi, frekvencijski modulirani tonalni zvukovi *P. kessleri* čine drugu skupinu zvukova (publikacija **II**). Umjereno dugi tonalni zvukovi *P. semilunaris* čine treću grupu, koja se nalazi između dvije već navedene skupine, što omogućuje razlikovanje glavoča skupine Neogobiini (*N. fluviatilis*, *N. melanostomus*; kratki i intenzivno ponovljeni tonalni zvukovi) od glavoča skupine Ponticolini (*P. kessleri* i *P. semilunaris*; umjereno dugi i dugi frekvencijsko modulirani tonalni zvukovi) s obzirom na strukturu akustičkog signala i njegove parametre (publikacija **II**). Nadalje, rezultati publikacije **I** i **II** pokazali su kako i dvije srodne vrste skupine Neogobiini (*N. fluviatilis* i *N. melanostomus*) dijele sličnu strukturu i izgled akustičkih signala (spektrogram i spektar snage), što ih jasno odvaja od ostalih vokalnih glavoča linije *Gobius*, iako i između navedene dvije vrste postoje jasne akustičke razlike odgovorne za njihovo međusobno odvajanje. Istovremeno, publikacije **I** i **II** potvrđuju kako grupiranje svih pontokaspijskih vrsta u jedinstven rod *Neogobius* (Berg, 1949) nije taksonomski ispravno te kako je rod *Neogobius* polifiletskog podrijetla, što su pokazali i rezultati molekularnih analiza (Neilson i Stepien, 2009a; Thacker i Roje, 2011; Medvedev, 2013).

Cilj publikacije **I**, uz opis akustičkih signala vrste *N. fluviatilis*, bio je istražiti kakvi su filogenetski odnosi između atlantsko-mediteranskih glavoča (rodovi *Padogobius*, *Gobius* i *Zosterisessor*) i navedene pontokaspijske vrste s obzirom na kvantitativne akustičke parametre. Prijašnja morfološka i molekularna istraživanja (Simonović, 1998; Thacker i Roje, 2011; Thacker, 2015) naglašavaju kako su pontokaspijski i atlantsko-mediteranski glavoči sestrinske skupine unutar linije *Gobius*, a rezultati publikacije **I** potvrdili su očekivani odnos budući da se *N. fluviatilis* grupirao na dijagramu s atlantsko-mediteranskim vrstama koje stvaraju isti tip zvuka i dijele slične akustičke parametre (*P. nigricans*, *P. bonelli* i *G. paganellus*). Ti rezultati odgovaraju na znanstveno pitanje *„Kakvi su filogenetski odnosi pontokaspijskih i atlantsko-mediteranskih glavoča iz linije Gobius rekonstruirani na temelju kvalitativnih i kvantitativnih akustičkih parametara?“*, naglašavajući kako između pontokaspijske vrste i ostalih vokalnih vrsta linije *Gobius* postoji određena filogenetska povezanost s obzirom na akustička svojstva. Također, rezultati bioakustičke analize publikacije **I** navode kako su dva temporalna akustička parametra (trajanje – DUR i stopa ponavljanja pulseva – PRR, ali i maksimalna frekvencija zvuka – PF) najvjerojatnije zaslužna za zapaženo filogenetsko odvajanje istraživanih vokalnih glavoča. Takvi su rezultati poslužili kao osnova za daljnja bioakustička istraživanja radi proširivanja vokalnog repertoara neistraženih pontokaspijskih vrsta i ispitivanja filogenetskih odnosa pontokaspijskih s atlantsko-mediteranskim vrstama linije *Gobius*, s naglaskom na rod *Padogobius* koji se prijašnjim bioakustičkim istraživanjima (Lugli, 1995, 1996; Malavasi i sur., 2008), kao i publikacijom **I**, pokazao polifiletskim. Tu je hipotezu trebalo testirati molekularnim analizama, tj. upotrebom genetičkih biljega radi ispitivanja filogenetskih odnosa vokalnih vrsta linije *Gobius*, što će poslije u Diskusiji biti objašnjeno u sklopu publikacije **IV**. Još je jedan zanimljiv rezultat publikacije **I**, koji izravno odgovara na pitanja *„Glasaju li se samo mužjaci tijekom intraspecijskih interakcija?“*, a to je zapaženo i dokumentirano glasanje ženki *N. fluviatilis* tijekom agresivnih intraseksualnih (ženka – ženka) interakcija. Takva je pojava, tj. glasanje ženki tijekom agresivnih interakcija s drugim ženkama, zabilježena kod još jedne već spomenute pontokaspijske vrste, mramorastog glavoča *P. semilunaris* (Ladich i Kratochvil, 1989). Bioakustičko istraživanje publikacije **I** pokazalo je kako su tonalni zvukovi *N. fluviatilis* izrazito stereotipni i nepromijenjeni neovisno o etološkoj kategoriji produkcije zvukova (agresivna ili reproduktivna) te nije pronađena razlika po akustičkim parametrima i ostalim kvalitativnim svojstvima (izgled spektrograma i spektra snage) među spolovima. Navedeni rezultati publikacije **I** poklapaju se sa sličnim provedenim bioakustičkim istraživanjima (Myrberg i sur., 1965; Ladich, 1990, 2007; Lagardere i sur., 2005) koja navode kako se kod riba tijekom agresivnih interakcija oba spola glasaju, za razliku od reproduktivnih interakcija gdje je većinom mužjak taj koji se glasa, a njihovi se zvukovi rijetko razlikuju s obzirom na akustičke parametre budući da se zvukovi stvaraju tijekom obrane teritorija ili hranilišta (Ladich, 2015).

Ukratko, provedena bioakustička istraživanja u sklopu ove disertacije (publikacije **I** i **II**), zajedno s prijašnjim publikacijama (Ladich i Kratochvil, 1989; Lugli, 1995, 1996; Malavasi i sur., 2008, Malavasi i sur., 2009; Parmentier i sur., 2013), potvrđuju kako su glavoči linije *Gobius*, a pogotovo pontokaspijske vrste, izrazito dobri modelni organizmi za multidisciplinarna laboratorijska ispitivanja u bioakustičke svrhe. Oni nude velik znanstveni potencijal za buduće studije koje bi mogle odgovoriti na ključna pitanja iz područja bioakustike riba (*„Zašto se ženke glavoča glasaju?“*, *„Koliko je zapravo vokalnih vrsta glavoča linije Gobius?“*, *„Kakav je utjecaj okolne buke na homeostazu glavoča?“*).

3.2. *Uvid u mehanizam za produkciju zvukova kod glavoča*

Treći je cilj ove disertacije bio istražiti potencijalni mehanizam za produkciju zvukova kod rotana (*P. glenii*), pripadnika bazalne porodice Odontobutidae unutar podreda Gobioidei (filogenija prema Thacker, 2009). Taj je cilj blisko povezan s rezultatima publikacije **III**. Naime, kod većine riba glavnu ulogu u produkciji zvukova imaju posebni (sonički) mišići koji svojim uzastopnim kontrakcijama dovode do vibracije plivaćeg mjehura, koji se zbog toga smatra jednim od najvažnijih dijelova vokalnog sustava riba (Ladich i Fine, 2006; Fine i Parmentier, 2015). Zanimljivo je, doduše, kako kod glavoča možemo naći vokalne vrste koje imaju (*Padogobius bonelli, Pomatoschistus minutus, Gobius cruentatus*) ili nemaju plivaći mjehur (*Padogobius nigricans, Neogobius melanostomus, N. fluviatilis*), samim time odbacujući njegovu istaknutu ulogu u produkciji zvukova (Zeyl i sur., 2016). Nekoliko je potencijalnih mehanizama za produkciju zvukova predloženo kod glavoča, od soničkih mišića plivaćeg mjehura (Lugli i sur., 1995), hidrodinamičnog mehanizma temeljenog na rapidnim izbacivanjima vode iz otvora škržnog poklopca (Stadler, 2002) te kranio-pektoralnih mišića (Lugli i sur., 1996). Istražujući vokalni repertoar talijanskog glavoča *Padogobius nigricans,* Lugli (1996) navodi kako bi tonalni zvukovi te vrste mogli biti stvoreni serijom mišićnih kontrakcija posebnih mišića povezanih s oplećjem. Bioakustička analiza potvrdila je kako su tonalni zvukovi frekvencijsko-modulirani, na spektrogramu s izgledom sinusoidnog vala bez dodatnih harmonika iznad jednog glavnog (oko 100 Hz), što uz činjenicu kako *P. nigricans* nema plivaći mjehur potvrđuje kako zvukovi nisu stvoreni nekom drugom rezonantnom strukturom kao što je plivaći mjehur (Lugli i sur., 1996). Nadalje, rezultati navedenog istraživanja potvrđuju pretpostavku kako temperatura okoliša, tj. vodenog medija, ima znatan utjecaj na soničke mišiće koji najvjerojatnije sudjeluju u procesu stvaranja zvukova kod talijanskog *P. nigricans* (Lugli i sur., 1996). Kao što je navedeno u Uvodu, jedina su detaljna istraživanja mehanizma za produkciju zvukova glavoča proveli Parmentier i sur. (2013, 2017), u sklopu kojih su ispitani glavoči dviju različitih porodica, *G. paganellus* (Gobiidae) i *Pomatoschistus pictus* (Gobionellidae). Obje vrste, iako iz dviju različitih porodica, dijele identičan koštano-mišićni plan građe lubanje i oplećja te imaju sposobnost stvarati dva različitija tipa zvuka istim mehanizmom (*G. paganellus* – pulsatilni i tonalni zvuk; *P. pictus* – pulsatilni/bubnjajući i tupi zvuk). Autori pretpostavljaju kako se kod obje vrste sonički mehanizam najvjerojatnije temelji na kontrakcijama kranio-pektoralnih mišića *levator pectoralis* (*pars medialis* i *lateralis*) koji povezuju lubanju s oplećjem (Parmentier i sur., 2013, 2017). Ti mišići imaju sve karakteristike brzokontrahirajućih mišića: vrpčaste miofibrile, razvijene tubule sakoplazmatskog retikuluma i razvijena područja s brojnim mitohondrijima u srži i periferiji (Parmentier i sur., 2013, 2017). Zanimljivo je napomenuti kako glavoči nemaju nikakve specifične mehaničke ili dodatne anatomske strukture, već upućuju na tipičan plan građe ostalih riba reda Perciformes (Lugli i sur., 1997; Malavasi i sur., 2008; Stadler, 2002), što pokazuje kako je mehanizam za produkciju zvukova mogao evoluirati od već postojećih struktura kroz proces egzaptacije (= iskorištavanje određene postojeće strukture za jednu funkciju kada je ona zapravo evoluirala za drugu, npr. perje ptica; Gould i Vrba, 1982). Uzimajući u obzir navedeni proces, autori pretpostavljaju kako je mehanizam za produkciju zvukova nastao od lokomotornih pokreta vezanih uz oplećje kod glavoča porodica Gobiidae i Gobionellidae (Parmentier i sur., 2013, 2017).

Jedino je istraživanje vokalnog repertoara i potencijalnog mehanizma za produkciju zvukova glavoča u širem smislu (Gobioidei) proveo Takemura (1983) koji je istražio produkciju zvukova i vokalne anatomske strukture uključene u proces akustičke komunikacije kod *Odontobutis obscura*, pripadnika porodice spavača (Odontobutidae). Rezultati istraživanja pokazali su kako mužjaci *O. obscura* stvaraju pulastilne zvukove u laboratorijskim uvjetima tijekom sezone reprodukcije (svibanj i lipanj), pri čemu se zvukovi sastoje od oko 8 pulseva trajanja 9 ms i maksimalne frekvencije ispod 1 kHz, s akustičkim parametrima (amplituda, raspon frekvencije i maksimalna frekvencija) znatno uvjetovanim veličinom ribe (veća riba – manja frekvencija i viša amplituda; Takemura, 1983). Anatomsko istraživanja *O. obscura* pokazalo je kako je stijenka plivaćeg mjehura izrazito tanka, bez izraženih mišića koji bi se vezali direktno na njega. Plivaći mjehur kod *O. obscura* nalazi se na dorzalnoj strani trbušne šupljine i ima izgled slova Y, s dva lateralna anteriorna proširenja oblika vilice koja završavaju ispod četvrtog kralješka. Dva posebna mišića, koji se anteriorno vežu na gornje ždrijelne zube, završavaju na 2. − 4. kralješku i pokrivaju dva lateralna anteriorna proširenja plivaćeg mjehura (Takemura, 1983). Dodirivanjem ždrijelnog dijela manipulirane jedinke prstima, tijekom produkcije zvukova u laboratorijskim uvjetima, osjetile bi se slabe vibracije. Također, elektrostimulacija navedenih mišića tijekom eksperimentalne manipulacije dovela bi do produkcije zvukova ribe, s naglaskom na tome kako bi zvukovi postali izrazito tihi bušenjem ili uništavanjem plivaćeg mjehura, ali ne bi u potpunosti nestali. Iz svega navedenog, autor zaključuje kako se mehanizam za produkciju zvukova kod *O.*

obscura temelji najvjerojatnije na ždrijelnom mehanizmu, tj. nastanku zvukova struganjem gornjih i donjih ždrijelnih zubi, a njihova se amplifikacija događa zahvaljujući plivaćem mjehuru (Takemura, 1983).

Publikacija **III** istražila je etološki kontekst akustičke komunikacije i anatomske strukture povezane s potencijalnim mehanizmom za produkciju zvukova kod rotana *Perccottus glenii.* Znanstvena je hipoteza publikacije **III** glasila „*Mehanizam za produkciju zvukova bazira se kod rotana Perccottus glenii (Odontobutidae) na kranio-pektoralnim mišićima, a pulsatilni zvukovi predstavljaju ancestralni/bazalni tip akustičkog signala kod glavoča u širem smislu“* s obzirom na hipotezu koju su predložili Malavasi i sur. (2008). Rezultati publikacije **III** blisko su povezani s prvim ciljem ove disertacije, tj. pokazali su kako mužjaci rotana, u laboratorijskim uvjetima, stvaraju dva tipa akustički različitih zvukova (tonalni i tupi) tijekom reproduktivne sezone, za vrijeme udvaranja (ženka izvan nastambe) ili tijekom interseksualnih interakcija u nastambi bez parenja. Zanimljivo je kako su tupi zvukovi (eng. *thumps*; < 200 ms i < 500 Hz, nemodulirani u frekvenciji) dokumentirani prvi put publikacijom **III** kod nekog pripadnika porodice Odontobutidae tijekom udvaranja ženki i njezina boravka u nastambi, dok su tonalni zvukovi (< 100 ms, 120 Hz, modulirani u frekvenciji) producirani samo tijekom udvaranja. Takav rezultat pokazuje kako oba zvuka potencijalno imaju različitu ulogu tijekom reproduktivne sezone, najvjerojatnije zbog svojih akustičkih parametara koji omogućuju frekvencijsko-moduliranim tonalnim zvukovima pokrivanje šireg spektra frekvencija u sluhu primatelja i širu transmisiju kroz okoliš, dok tupi zvukovi služe tijekom bliskih interakcija kada je ženka u nastambi. Slično su objašnjenje predložili Zeyl i sur. (2016), koji navode kako pulsatilni i tonalni zvukovi pokrivaju različite dijelove okolišnog spektra frekvencija budući da je kod sisavaca koji komuniciraju zvukovima na daljinu (npr. šišmiši i kitovi) modulacija frekvencije tonalnih zvukova česta akustička karakteristika (Wiley i Richards, 1982; Lugli i Fine, 2007). Treći je cilj publikacije **III** ispunjen etološkom analizom ponašanja vokalnih jedinki *P. glenii,* koja je potvrdila kako mužjaci produciraju tonalne zvukove pri bliskom kontaktu sa ženkama na ulazu u nastambu, a tupe zvukove tijekom interakcija izvan i unutar nastambe, s naglaskom na tome kako je većina (62%) etoloških kategorija popraćena tonalnim zvukovima, dok je kod tupih taj postotak znatno manji (23%). Anatomska analiza publikacije **III**, posebno blisko povezana s trećim ciljem disertacije, potvrdila je kako mužjaci *P. glenii* imaju koštano-mišićne dijelove lubanje i oplećja slične po svom rasporedu i strukturi onima kod ostalih glavoča, s manjim razlikama specifičnim za porodicu Odontobutidae (Winterbottom, 1974; Adriaens i sur., 1993; Li i sur., 2018). Naime, prijašnja su anatomska ispitivanja soničkog mehanizma glavoča (Parmentier i sur., 2013, 2017) istražila mišiće oplećja povezane s lubanjom (*musculus levator pectoralis*), čijim se kontrakcijama pokreće oplećje u ventro-anteriornom smjeru pri stvaranju zvukova tijekom eksperimentalnih manipulacija. Zajedno s opaženom odsutnošću pokreta bukalne i usne šupljine tijekom produkcije oba tipa zvuka kod *P. glenii*, rezultati publikacije **III** naglašavaju kako se mehanizam za stvaranje zvukova kod rotana najvjerojatnije temelji na kontrakcijama kranio-pektoralnih mišića *levator pectoralis*, koji se kod rotana dijele na tri dijela (*pars lateralis superficialis*, *pars lateralis profundus* i *pars medialis*), a na oplećju, tj. grlenjači, *levator pectoralis pars medialis* veže se dorzalno u odnosu na *pars lateralis*. Oba tipa zvukova (tonalni i tupi) kod *P. glenii* najvjerojatnije nastaju djelovanjem istog mehanizma koji se temelji na kontrakcijama kranio-pektoralnih *levator pectoralis* mišića, ali kako se točno zvukovi šire u okolišu te kako se amplificiraju, i dalje ostaje nerazjašnjeno. Mnoga istraživanja naglašavaju kako su sonički mišići poikilotermnih životinja (uključujući i ribe) pod znatnim utjecajem temperature vodenog medija (Feher i sur., 1998; Amorim i sur., 2015; Zeyl i sur., 2016), naglašavajući kako se akustički parametri znatno mijenjaju s povišenjem temperature vode zbog promjena u ritmu mišićnih kontrakcija. U slučaju *P. glenii*, oba su tipa zvukova, sa svojim akustičkim parametrima, bila pod znatnim utjecajem temperature vode (viša temperatura; viša frekvencija, viša stopa ponavljanja pulseva, veći broj pulseva, itd.), što potvrđuje kako upravo mišićne kontrakcije dovode do stvaranja zvukova i modulacije pojedinih parametara (publikacija **III**). Zanimljiv su koštani dio oplećja mnogih glavoča, uz navedene koštano-mišićne strukture lubanje, četiri velike radijalne kosti (*ossa radiales*) jače ili slabije povezane hrskavicom s kosti grljenjačom, koje su veće u odnosu na iste strukture drugih zrakoperki (Adriaens i sur., 1993). Naime, smatra se kako radijalne kosti, čineći jedinstvenu plohu nalik opni bubnja, imaju indirektnu funkciju u stvaranju zvukova kod *G. paganellus* i *P. pictus* budući da svojim vibracijama potencijalno amplificiraju zvukove stvorene kontrakcijama mišića *levator pectoralis* (Parmentier i sur., 2013, 2017). Koristeći analizu X-zraka u kombinaciji s mikrotomografijom radi istraživanja koštano-mišićnih svojstava oplećja spavača (Odontobutidae), Li i sur. (2018) navode kako se oplećje *P. glenii* sastoji od tipičnih dijelova za porodicu Odontobutidae, tj. imaju veliku, okoštanu lopaticu (*os scapula*) koja onemogućuje direktan kontakt proksimalnih radijalnih kostiju s grlenjačom (*os cleithrum*). Ako se budućim detaljnim multidisciplinarnim analizama (superbrze videokamere, elektromiografija, mikroskopija, itd.) potvrdi kako *P. glenii* dijeli morfološko-anatomske osobine i kinetičke kretnje lubanje i oplećja (klimanje ili lateralne undulacije) pri produkciji zvukova s istraženim vokalnim vrstama *G. paganellus* i *P. pictus*, onda će se moći jasnije objasniti je li mehanizam za produkciju zvukova kod glavoča u širem smislu (Gobioidei) evolucijski konzerviran i smatra li se pleziomorfnim svojstvom za navedenu skupinu riba. Zasad, publikacija **III** potvrđuje kako glavoči (Gobiidae i Gobionellidae) dijele sa spavačima (Odontobutidae) sličan raspored i organizaciju kranio-pektoralnih mišića *levator pectoralis*, predloženih kao glavni dio mehanizma za produkciju zvukova glavoča u širem smislu (Parmentier i sur., 2013, 2017).

3.3. *Akustička divergencija glavoča linije Gobius – povezanost s genetskom diferencijacijom?*

Drugi i četvrti cilj ove disertacije blisko su povezani s ispitivanjem filogenetskih interspecijskih odnosa vokalnih glavoča linije *Gobius*, dok publikacije **I** i **IV** izravno odgovaraju na znanstvene hipoteze *„Filogenetska interspecijska analiza pontokaspijskih i atlantsko-mediteranskih glavoča iz linije Gobius, temeljena na kvalitativnim i kvantitativnim akustičkim parametrima, upućuje na određen stupanj međusobne srodnosti između vokalnih vrsta. Također, pretpostavlja se kako se vokalni repertoar vokalnih glavoča linije Gobius sastoji od malog broja različitih tipova zvukova“* te *„Interspecijski filogenetski odnosi devet vokalnih vrsta glavoča linije Gobius (rodovi Neogobius, Ponticola, Zosterisessor, Gobius, Padogobius), konstruirani na temelju šest kvantitativnih akustičkih parametara, preklapaju se s rezultatima molekularnih analiza, tj. zvukovi prenose filogenetski signal odgovoran za razlikovanje srodnih vrsta“*. Generalno gledajući, filogenetski odnosi glavoča predstavljaju još uvijek neriješeni dio taksonomske zagonetke, vjerojatno zbog činjenice kako mnogi autori u svojim molekularnim istraživanjima koriste različite genetičke (jezgrene i mitohondrijske) biljege, raznovrsne metode filogenetske rekonstrukcije te brojne, ponekad nesrodne vrste, što dodatno otežava ionako kompliciranu situaciju sa sistematikom pojedinih vrsta ili linija. Cilj je publikacije **I**, a pogotovo publikacije **IV**, bio objediniti sve dotad postojeće podatke o bioakustici vokalnih glavoča linije *Gobius* te u početnom dijelu ove disertacije (publikacija **I**) istražiti postoji li bilo kakva akustička srodnost među ispitanim vrstama. Pozitivan je rezultat poslije poslužio kao osnova za dublja komparativna istraživanja (publikacija **IV**).

U publikaciji **I**, čiji je cilj bio istražiti akustičku srodnost pontokaspijske vrste *N. fluviatilis* s dotad dokumentiranim vokalnim atlantsko-mediteranskim glavočima (Lugli i sur., 1995, 1996, 1997; Malavasi i sur., 2008), pokazalo se kako bi zvukovi glavoča mogli poslužiti kao kvalitetan fenotipski karakter u ispitivanjima filogenetskih odnosa vokalnih vrsta linije *Gobius*. Publikacija **I** je, uz to što joj je cilj bio istražiti vokalni repertoar *N. fluviatilis*, multivarijatnim statističkim pristupom ispitala i akustičku srodnost navedene vrste sa šest vrsta rodova *Gobius, Zosterisessor* i *Padogobius*. Rezultati akustičke analize potvrdili su kako su zvukovi istraživanih vrsta vokalnih glavoča izrazito specifični, a dodatna statistička analiza grupirala je vokalne jedinke (tj. njihove zvukove izražene s pet akustičkih parametara) u pripadajuće vrste s velikom razinom točnosti. Istovremeno, analiza je pokazala kako od nekoliko kvantitativnih akustičkih parametara samo temporalna (trajanje i stopa ponavljanja pulseva) i spektralna (maksimalna frekvencija) svojstva nisu korelirana s bilo kojim drugim parametrom, što je omogućilo kasnije korištenje u komparativnim analizama. Rezultati publikacije **I** pokazali su kako su se pojedine vrste smjestile na dijagramu u klastere s obzirom na sličnost i strukturu produciranog zvuka, a zanimljivo je kako su se vrste roda *Padogobius* (*P. bonelli* i *P. nigricans*) koje stvaraju tonalne zvukove grupirale s *N. fluviatilis* i *G. paganellus*, vokalnim glavočima koji produciraju također tonalne zvukove. Pogotovo je zanimljiv rezultat publikacije **I** kako su se dvije nesrodne vrste, *P. nigricans* i *N. fluviatilis*, grupirale zajedno s obzirom na akustičke parametre na dijagramu, što je pokazalo kako bi rod *Padogobius* mogao biti polifiletskog podrijetla s obzirom na to da je druga vrsta (*P. bonelli*) bila smještena na suprotnoj strani dijagrama u odnosu na *P. nigricans* i *N. fluviatilis*. Takav je rezultat, tj. polifiletsko podrijetlo roda *Padogobius*, predložen prijašnjim istraživanjima temeljenima na genetičkim biljezima (Penzo i sur., 1998; Huyse i sur., 2004). Stoga je publikacija **I** poslužila kao osnova za kasnija multidisciplinarna istraživanja temeljena na akustičko-molekularnim podacima koja su detaljnije istražila filogenetske odnose vokalnih glavoča linije *Gobius* i njihovu akustičku divergenciju (publikacija **IV**).

Zvukovi mnogih životinja, pogotovo ptica, žaba, sisavaca i kukaca, važno su komunikacijsko sredstvo za prijenos i izmjenu informacija, a koriste se tijekom agresivnih (borba za hranu, briga za potomstvo, signaliziranje o prisutnosti predatoru) i reproduktivnih (privlačenje partnera, udvaranje i parenje) interspecijskih ili intraspecijskih interakcija, zbog čega su kod nekih vokalnih skupina akustički signali povezani s njihovom evolucijom pa imaju izraženu taksonomsku vrijednost u razlikovanju vrsta, pa čak i populacija (Slabbekoorn, 2004; Velasquez i sur., 2013; Wilkins i sur., 2013). Kod pojedinih skupina, kao što su cikade i cvrčci, određene brzoevoluirajuće, ali fenotipski vrlo slične vrste mogu se odrediti samo na temelju međusobnih razlika u akustičkim signalima (Mendelson i Shaw, 2005; Marshall, 2008). Također, rezultati modernih komparativnih filogenetskih studija naglašavaju kako su akustički signali izrazito specifično fenotipsko svojstvo za vrstu ili rod te kako se morfološke/genetske razlike među vrstama mogu izraziti i razlikama u akustičkim signalima

(Mendelson i Shaw, 2005; Marshall, 2008), što potvrđuje kako akustička divergencija među vrstama otkriva i upućuje na obrazac interspecijske diversifikacije unutar rodova (Seddon, 2008). Ti rezultati potvrđuju kako akustički signali imaju izraženu filogenetsku ulogu u divergenciji pojedinih vokalnih skupina, i u ranim (prezigotna izolacija, prije same reprodukcije) i u kasnim (post-zigotna izolacija) fazama specijacije (Wilkins i sur., 2013). Nadalje, pojedine publikacije naglašavaju kako su akustički signali izrazito bitno fenotipsko svojstvo kod simpatrijskih i srodnih vrsta (kao što su neke vrste glavoča rodova *Pomatoschistus*, *Knipowitschia* i *Neogobius*) koje ih stvaraju tijekom reproduktivnih interakcija budući da služe kao sredstvo filogenetske diskriminacije te na taj način izravno sudjeluju u procesu razdvajanja populacija i nastanka novih vrsta (Ritchie, 2007; Pedroso i sur., 2013; Wilkins i sur., 2013; Blom i sur., 2016; Zeyl i sur., 2016).

Cilj je publikacije **IV** istražiti filogenetsku srodnost vokalnih glavoča linije *Gobius*, rekonstruiranu na temelju DNK sekvenci četiriju genetičkih biljega (dva mitohondrijska: Citokrom *b* - cyt *b* i Citokrom oksidaza podjedinica I - COI i dva jezgrena: Rodopsin - Rho i Rekombinacijski aktivacijski gen 1 - RAG1) te dobivene rezultate usporediti s akustičkim podacima (tj. matricom interspecijske akustičke udaljenosti) kako bismo potvrdili postoji li poklapanje akustičke divergencije s genetičkom. Zanimljivo je kako se filogenetski odnosi mnogih životinja rekonstruirani na temelju akustičkih podataka često poklapaju s odnosima utvrđenima koristeći morfološke ili molekularne podatke, kao što je to slučaj kod kukaca, žaba, ptica i sisavaca (Oates i Trocco, 1983; Hoikkala i sur., 1994; Cocroft i Ryan, 1995; Desutter-Grandcolas, 1997; McCracken i Sheldon, 1997; Conner, 1999; Peters i Tonkin-Leyhausen, 1999; Robillard i Desutter-Grandcolas, 2004). Do danas niti jedna znanstvena publikacija nije kvantitativno istražila i empirijski usporedila, u smislu komparativne filogenetske analize, zvukove riba zrakoperki s molekularnim podacima, što je bio jedan od glavnih ciljeva publikacije **IV**. Uz to, publikacija **IV** ponudila je alternativnu hipotezu u odnosu na postojeću koju su predložili Malavasi i sur. (2008) povezanu s evolucijom akustičke divergencije glavoča linije *Gobius* (rodovi *Gobius*, *Zosterisessor*, *Padogobius*, *Neogobius* i *Ponticola*) s naglaskom na evolucijske sile (determinističke ili stohastičke) koje su potencijalno mogle dovesti do interspecijskog akustičkog razdvajanja istraživanih vrsta kao i opažene korelacije s molekularnim podacima. Rezultati publikacije **IV** potvrdili su kako se akustički signali istraživanih devet vokalnih glavoča linije *Gobius* međusobno razlikuju na temelju svih šest kvantitativnih akustičkih parametara, filogenetski su specifični za svaku vrstu te kao takvi omogućuju njihovo razlikovanje. Taj su rezultat potvrdile i multivarijatne statističke analize koje su s velikim postotkom klasifikacije (oko 92%) grupirale zvukove pojedinih jedinki u odgovarajuće vrste te istaknule temporalne parametre zvuka (pogotovo trajanje – DUR i stopu ponavljanja pulseva – PRR, uz maksimalnu frekvenciju – PF) kao odgovorna svojstva za opaženo grupiranje/razdvajanje vrsta. Važno je napomenuti kako su publikacijom **IV** objedinjeni gotovo svi podaci povezani s vokalnim glavočima linije *Gobius,* dok su dvije dodatne vokalne vrste (*Proterorhinus marmoratus/semilunaris* i *Gobius cruentatus*) izostavljene zbog tehničkih razloga. Iz toga slijedi da je gotovo većina vokalnih glavoča linije *Gobius* bila uključena u istraživanje u sklopu publikacije **IV**, a inicijalna je bioakustička analiza pokazala kako bi pojedine vrste (*P. nigricans* s vrstama roda *Neogobius*) mogle biti srodnije nego što se to prije smatralo. Filogenetski odnosi prezentirani u obliku filograma (tj. filogenetskog stabla) i rekonstruirani na temelju četiriju genetičkih biljega koristeći metode maksimalne vjerodostojnosti i Bayesovski pristup (ML i BI), potvrdili su kako su pontokaspijske vrste roda *Neogobius* (*N. fluviatilis* i *N. melanostomus*) blisko povezane i srodne s talijanskim glavočem *P. nigricans*, s kojima još dijele i ekološko-morfološke karakteristike (nedostatak plivaćeg mjehura, raspored glavenih papila i kanala, stanište, itd.; Miller, 2003, 2004), dok je druga vrsta iz roda *Padogobius* (*P. bonelli*) bila smještena kao sestrinska vrsta svim ostalim istraživanim skupinama budući da se odvojila prva na filogenetskom (kombiniranom) stablu, odmah nakon vanjske grupe (*P. glenii*). Lugli i sur. (1996) naveli su, u sklopu bioakustičkog istraživanja zvukova talijanskog glavoča *P. nigricans,* kako bi dvije vrste roda *Padogobius* mogle biti filogenetski udaljene te kako se razlikuju po akustičkim signalima, što je poslije i potvrđeno molekularnim istraživanjima (Geiger i sur., 2014). Publikacija **IV** potvrdila je, uzimajući u obzir komparativne akustičke i molekularne rezultate, kako je rod *Padogobius* najvjerojatnije polifiletskog, dok su pontokaspijske vrste u cjelini gledano monofiletskog podrijetla. Za ostale vrste *Gobius* roda (*G. paganellus, G. cobitis* i *G. niger*), kao i za *Z. ophiocephalus*, filogenetski odnosi iz publikacije **IV** ostaju nerazriješeni na dubljoj taksonomskoj razini, što bi buduća molekularna istraživanja radi detaljnijeg rješavanja njihovih odnosa trebala riješiti uzimajući u obzir veći broj srodnih vrsta. Ipak, rezultati publikacije **IV** naglašavaju kako bi vrsta *Z. ophiocephalus* mogla biti srodna ostalim vrstama *Gobius* roda, pogotovo *G. niger*.

Mnoge multidisciplinarne studije navode kako akustički signali kod određenih skupina životinja prenose filogenetski signal zaslužan za interspecijsko razlikovanje vrsta, što potvrđuje opažena pozitivna kvantitativna korelacija između akustičke i genetičke divergencije (Päckert i sur., 2004; Percy i sur., 2006; Irwin i sur., 2008; Toews i Irwin, 2008;

Thinh i sur., 2011; Velásquez i sur., 2013; Lee i sur., 2016). Ukratko, moderne komparativne studije navode kako se između filogenetski srodnijih vrsta može opaziti statistička ovisnost u vrijednostima njihovih filogenetskih osobina upravo zbog njihove bliže genetičke srodnosti (Felsenstein, 1985; Revell i sur., 2008), tj. tendencije srodnih vrsta da više nalikuju jedna drugoj nego vrstama nasumično izabranim s filogenetskog stabla, što je zapravo definicija filogenetskog signala (Blomberg i Garland, 2002; Münkemüller i sur, 2012). U sklopu publikacije **IV** komparativna akustičko-molekularna analiza potvrdila je kako postoji znatna korelacija između interspecijske genetske diversifikacije (uključujući matrice udaljenosti konstruirane za svaki pojedinačni gen ili sve gene zajedno) i akustičke divergencije, još jednom potvrđujući taksonomsku specifičnost akustičkih signala svake vokalne vrste glavoča linije *Gobius* iz ove disertacije. Taj rezultat pokazuje, prvi put dosad, kako se evolucija zvukova najvjerojatnije mijenjala (i dalje mijenja) u istom smjeru i jednakom stopom kao i genotip, bez obzira na tip i vrstu korištenih genetičkih biljega te kako stohastički procesi imaju dominantnu ulogu u oblikovanju opažene akustičke divergencije glavoča linije *Gobius*. Stoga rezultati publikacije **IV** izravno potvrđuju hipotezu *„Interspecijski filogenetski odnosi devet vokalnih vrsta glavoča linije Gobius (rodovi Neogobius, Ponticola, Zosterisessor, Gobius, Padogobius), konstruirani na temelju šest kvantitativnih akustičkih parametara, preklapaju se s rezultatima molekularnih analiza, tj. zvukovi prenose filogenetski signal odgovoran za razlikovanje srodnih vrsta“*. Također, iz opažene akustičko-molekularne korelacije slijedi kako zvukovi glavoča linije *Gobius* najvjerojatnije imaju filogenetski signal, kao što je to slučaj kod mnogih drugih vokalnih životinja (Freckleton i sur., 2002; Blomberg i sur., 2003; Escalona i sur., 2019) iako bi detaljna istraživanja trebala kvantitativno ispitati u kojoj je mjeri on prisutan u pojedinačnim temporalnim ili spektralnim parametrima zvuka.

Ova je disertacija istražila interspecijsku korelaciju akustičke divergencije s genetičkom diferencijacijom glavoča linije *Gobius*, što je zasad prvi pokušaj kvantificiranja filogenetske ovisnosti zvukova o genskoj divergenciji kod bilo koje skupine riba zrakoperki. Na temelju rezultata publikacija **I**, **II**, **III** i **IV** konstruirana je hipoteza o evoluciji zvukova istraživanih vokalnih glavoča linije *Gobius*, koja znatno proširuje hipotezu koju su predložili Malavasi i sur. (2008). Ti su autori predložili kako su najvjerojatnije upravo pulsatilni zvukovi predstavljali ancestralnu (zajedničku) karakteristiku svih glavoča, vjerojatno zbog činjenice kako je većina vrsta imala sposobnost stvoriti samo taj tip zvuka i kako je kod sestrinske skupine svih ostalih glavoča u širem smislu, Odontobutidae (sa predstavnikom *O. obscura*), zabilježen samo pulsatilni tip akustičkog signala (Takemura, 1983; Malavasi i sur., 2008). Dvije vrste iz sestrinske porodice svih ostalih glavoča, *P. glenii* i *O. obscura* (Odontobutidae), mogu producirati dva različita tipa zvukova (tonalni i tupi – pulsatilni). Iz toga slijedi kako su osnovni elementi za razvoj frekvencijsko-moduliranih i kompleksnih zvukova zabilježenih kod linije *Gobius* bili upravo kratki tonalni zvukovi (koji su prisutni kod pontokaspijskih vrsta *Neogobius* roda i talijanskog *P. nigricans*) te pulsatilni zvukovi (rodovi *Gobius* i *Zosterisessor*).

4. ZAKLJUČAK

- Filogenetska interspecijska analiza pontokaspijskih i atlantsko-mediteranskih glavoča iz linije *Gobius* potvrdila je kako pojedine vokalne vrste iz ove disertacije dijele određen stupanj međusobne akustičke srodnosti (primjer vrste roda *Neogobius* s *Padogobius nigricans* i *Gobius niger* sa *Zosterisessor ophiocephalus*), dok se akustički repertoar glavoča iz linije *Gobius* sastoji od tri različita tipa zvukova.

- Tijekom intraspecijskih interakcija ne glasaju se samo mužjaci, već su i ženke vokalni spol (primjer *N. fluviatilis*).

- Sposobnost stvaranja zvukova zahvaljujući mehanizmu temeljenom na kontrakcijama kranio-pektoralnih mišića (mišići oplećja i lubanje) karakteristično je svojstvo za sve glavoče u širem smislu (Gobioidei). Također, za porodicu Odontobutidae (primjer *P. glenii*), zabilježena su dva tipa zvukova koji predstavljaju ancestralni vokalni repertoar glavoča u širem smislu (Gobioidei).

- Interspecijska akustička divergencija znatno je korelirana s genetičkom diferencijacijom glavoča linije *Gobius* te slijedi isti evolucijski obrazac bez obzira na tip genetičkog biljega (nDNA ili mtDNA), što pokazuje kako zvukovi prenose filogenetski signal zaslužan za razlikovanje srodnih vrsta linije *Gobius*.

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6. ŽIVOTOPIS

Sven Horvatić rođen je 8. svibnja 1991. godine u Zagrebu. Nakon završene osnovne škole u Rugvici, 2006. godine upisuje se u Opću gimnaziju u Dugom Selu koju završava 2010. godine. Iste godine upisuje se na Prirodoslovno-matematički fakultet (PMF) u Zagrebu, na preddiplomski smjer Znanosti o okolišu. Titulu sveučilišnog prvostupnika struke znanosti o okolišu stječe 2013. godine te se iste godine upisuje na diplomski smjer Eksperimentalne biologije (modul Zoologija) na PMF-u u Zagrebu. Diplomski rad „Produkcija zvukova pontokaspijskog riječnog glavočića (*Neogobius fluviatilis* Pallas, 1814) i njegova srodnost s mediteranskim vrstama s obzirom na akustična svojstva“ izradio je 2015. godine te ga uspješno obranio pod mentorstvom prof. dr. sc. Davora Zanelle. Tijekom diplomskog studija sudjelovao je u programu ERASMUS studentske mobilnosti, u sklopu kojeg je proveo stručnu praksu u ljetnom semestru 2014. godine na Sveučilištu Ca' Foscari di Venezia. Za vrijeme stručne prakse radio je pod mentorstvom prof. dr. sc. Stefana Malavasija, tijekom koje je većina znanstvene pozornosti bila posvećena provedbi laboratorijskih bioakustičkih eksperimenata i akustičkoj obradi zvukova europskih glavoča porodice Gobiidae. Poslije završetka diplomskog studija 2015. godine, upisuje prvu godinu poslijediplomskog studija Biologije na PMF-u u Zagrebu pod mentorstvom prof. dr. sc. Davora Zanelle. Godinu dana poslije zaposlio se na Biološkom odsjeku PMF-a u Zagrebu kao znanstveni novak - asistent na stručnom projektu „Provođenje programa praćenja stanja u slatkovodnom ribarstvu u 2016. godini (grupa Es – Ribolovno područje Jadran)“. Od 2017. godine radi kao stručni suradnik na projektu „Biološka ispitivanja nadzemnih voda na HE Varaždin, HE Čakovec i HE Dubrava“ na Zoologijskom zavodu Biološkog odsjeka PMF-a. U sklopu poslijediplomskog studija sudjelovao je na nekoliko znanstvenih usavršavanja iz područja bioakustike izvan Hrvatske: u Italiji (Ca' Foscari Sveučilište u Veneciji, mentor: prof. Stefano Malavasi) i Belgiji (Sveučilište u Liègeu, mentor: prof. Eric Parmentier). Autor je 13 izvornih znanstvenih radova (10 u CC bazi po *WOS*-u), dok je na njih šest prvi autor. Također, usmeno je prezentirao dva izlaganja na međunarodnim kongresima (Švicarska i Njemačka), dok je na jednom koautor (Zagreb). Prvi je autor na tri posterska izlaganja prezentirana na međunarodnim kongresima (Švicarska, Bosna i Hercegovina i Hrvatska). Od 2016. godine vodi praktikumsku nastavu iz kolegija Vertebrata, Kralješnjaci, Zoologija 3, Terenska nastava i Ihtiologija i ribarstvo slatkih voda, za studente preddiplomskih, diplomskih i integriranih preddiplomskih i diplomskih studija na PMF-u.

ZNANSTVENE PUBLIKACIJE:

Tutman, P., Zanella, D., **Horvatić, S.**, Hamzić, A., Adrović, A., Dulčić, J., Glamuzina, B. Freshwater gobies (Gobiidae) of Bosnia and Herzegovina: a review of the current status and distribution. Journal of Vertebrate Biology. 69 (2020), 4; 1-15.

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Horvatić, S., Špelić, A., Amorim, MCP,. Fonseca, PJ., Zanella, D. Endemic goby calling: acoustics and vocal behavior of *Orsinigobius croaticus*. Goby meeting 2020. (2020)

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Horvatić, S., Marčić, Z., Mrakovčić, M., Mustafić, P., Buj, I., Ćaleta, M., Zanella, D. Ichthyofauna structure of the Save River within the riprap zone as a specific habitat type, with emphasis on the presence of the round goby *Neogobius melanostomus* (Pallas, 1814). FINS II conference. (2016)

ZNANSTVENI PROJEKTI

Utjecaj klimatskih promjena na bioraznolikost koralja - istraživanje slučaja masovnih ugibanja u Jadranskom moru“ (IP-2019-04-3389**)**. Nositelj: Izv. prof. dr. sc. Petar Kružić, Prirodoslovno-matematički fakultet, Biološki odsjek, Zagreb. Hrvatska zaklada za znanost (HRZZ).

STRUČNI PROJEKTI

Biological study of surface water of the hydropower systems Varaždin, Čakovec and Dubrava in 2016, 2017, 2018 and 2019. Perica Mustafić i sur., Prirodoslovno-matematički fakultet, Biološki odsjek, Zagreb.

Monitoring project of Croatian freshwater ichthyofauna in 2016, 2017, 2018 and 2019 (zone E – Adriatic). Perica Mustafić i sur., Prirodoslovno-matematički fakultet, Biološki odsjek, Zagreb.

Research and optimisation of ichthyocenosis of Butoniga Reservoir for the purpose of reducing the trophic level in 2017, 2018 and 2019. Perica Mustafić i sur., Prirodoslovno-matematički fakultet, Biološki odsjek, Zagreb.

Usluga istraživanja riba i školjkaša u rijeci Sutli u projektu Veze prirode (Fishes and Unionidae of Sutla River). Zoran Marčić i sur., Prirodoslovno-matematički fakultet, Biološki odsjek, Zagreb.

Stručna podloga za Plan upravljanja (s akcijskim planom) za vrste roda *Salmo* (982617). Ivana Buj i sur., Hrvatsko ihtiološko društvo, Zagreb.

Usluga izrade stručne podloge – svijetlica i kapelska svijetlica u sklopu projekta „Izrada prijedloga Planova upravljanja strogo zaštićenim vrstama (s akcijskim planovima)“. (178923). Zoran Marčić i sur., Hrvatsko ihtiološko društvo, Zagreb.

RADIONICE, ZNANSTVENO USAVRŠAVANJE I SURADNJA:

Training workshop on fish. The International Commission for the Protection of the Danube River (ICPDR). (2018)

Znanstveno usavršavanje izvan Hrvatske, prekogranična akademska mobilnosti prema visokoškolskim i znanstvenim ustanovama, Sveučilište u Liègeu, Belgija. (2019)

Znanstveno usavršavanje izvan Hrvatske, Sveučilište Ca' Foscari u Veneciji, Italija (2019, 2018, 2017)

Upoznavanje sa sintaksom jezika R i njegova primjena u osnovnoj statističkoj i grafičkoj analizi podataka (S720). Sveučilište u Zagrebu , Sveučilišni računski centar. (2018)

NASTAVNA AKTIVNOSTI I INSTITUCIJSKA ZADUŽENJA:

Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu. Biološki odsjek. Kolegij: Kralješnjaci (40873) - asistent (2016 – danas)

Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu. Biološki odsjek. Kolegij: Vertebrata (40923) - asistent (2016 – danas)

Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu. Biološki odsjek. Kolegij: Zoologija 3 (40896) - asistent (2016 – danas)

Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu. Biološki odsjek. Kolegij: Terenska nastava (40876) - asistent (2016 – danas)

Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu. Biološki odsjek. Kolegij: Terenska nastava iz biološke, geografske i geološke zaštite okoliša (73836) - asistent (2016 – danas)

Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu. Biološki odsjek. Kolegij: Ihtiologija i ribarstvo slatkih voda (60228) - asistent (2019 – danas)

- predstavnik asistenata i poslijedoktoranata na Vijeću Biološkog odsjeka Prirodoslovno-matematičkog fakulteta (2018 – danas)

- zamjenik predstavnika asistenata i poslijedoktoranata na Vijeću Kolegija Biološkog odsjeka (2018 – danas)

- član Studentskog zbora Prirodoslovno-matematičkog fakulteta (2019 – danas)

- član organizacijskog odbora manifestacije “Noć Biologije (Dan i noć PMF-a 2019)”. (2019)

- član Hrvatskog ihtiološkog društva (HID) (2016 – danas)