



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 phylogenetic 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*, 2015, **00**, 000–000.

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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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 phylogenetic 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 (Colley & 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 Take-mura (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	<i>Gobius</i>	<i>Gobius niger</i> , <i>G. paganellus</i> , <i>G. cobitis</i> , <i>G. cruentatus</i> , <i>Zosterisessor</i> <i>ophiocephalus</i> , <i>Padogobius</i> <i>bonelli</i> , <i>P. nigricans</i>	<i>Proterorhinus</i> <i>marmoratus</i> , <i>Neogobius</i> <i>melanostomus</i>	Tonal, pulsatile and complex (sounds composed of both pulsatile and tonal segments)
Gobionelline-like	<i>Pomatoschistus</i>	<i>Pomatoschistus marmoratus</i> , <i>P. pictus</i> , <i>P. minutus</i> , <i>P. microps</i> , <i>P. canestrinii</i> , <i>Knipowitschia panizae</i> <i>K. punctatissima</i>		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 *NEOGOBIOUS 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

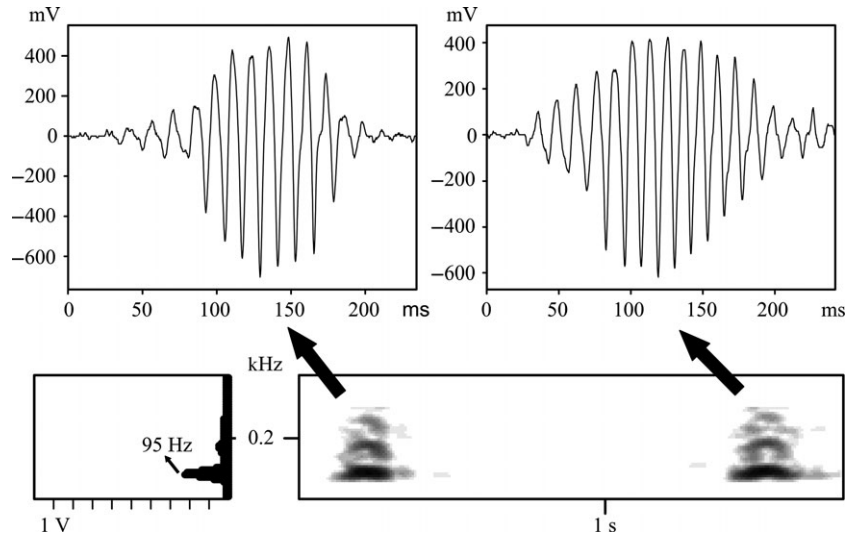


Figure 1. Structural details of two consecutive, representative sounds of *Neogobius fluviatilis*: below, spectrogram (right) and power spectrum (left); above, the wave form of both sounds.

Table 2. Individual means (\pm 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 \pm 8.3	116.2 \pm 11.8
Reproductive	128.5 \pm 11.2	–
Temperature ($^{\circ}$ C)		
Aggressive	20.2 \pm 0.4	19.8 \pm 0.4
Reproductive	20 \pm 0	–
DUR (ms)		
Aggressive	179 \pm 29.5	163.62 \pm 20.5
Reproductive	161 \pm 38.5	–
NP		
Aggressive	12.7 \pm 1.3	12.2 \pm 1.3
Reproductive	12 \pm 2.9	–
PRR (Hz)		
Aggressive	72.6 \pm 8.8	74.4 \pm 2.4
Reproductive	74.8 \pm 1.0	–
PF (Hz)		
Aggressive	77.2 \pm 6.6	78.4 \pm 2.4
Reproductive	78.8 \pm 3.6	–
FM (Hz)		
Aggressive	9.4 \pm 2.1	11.8 \pm 3.5
Reproductive	8.3 \pm 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 fre-

quency 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 *GOBIOUS* 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

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.

* $P < 0.05$.

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.

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

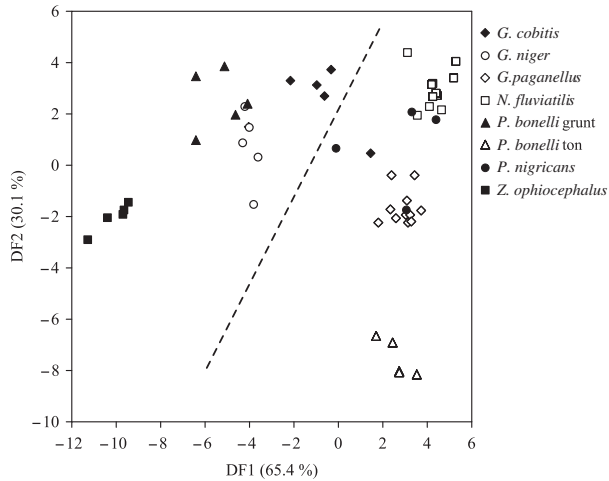


Figure 2. Bivariate comparison between discriminant functions DF1 and DF2 where individuals are delineated by species (different symbols represent different species). The dashed line separates species producing sounds with a high pulse repetition rate (tonal sounds, right side) from species producing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

Acoustic property	Standardized coefficients		Factor structure coefficients	
	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 phylogenetic 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

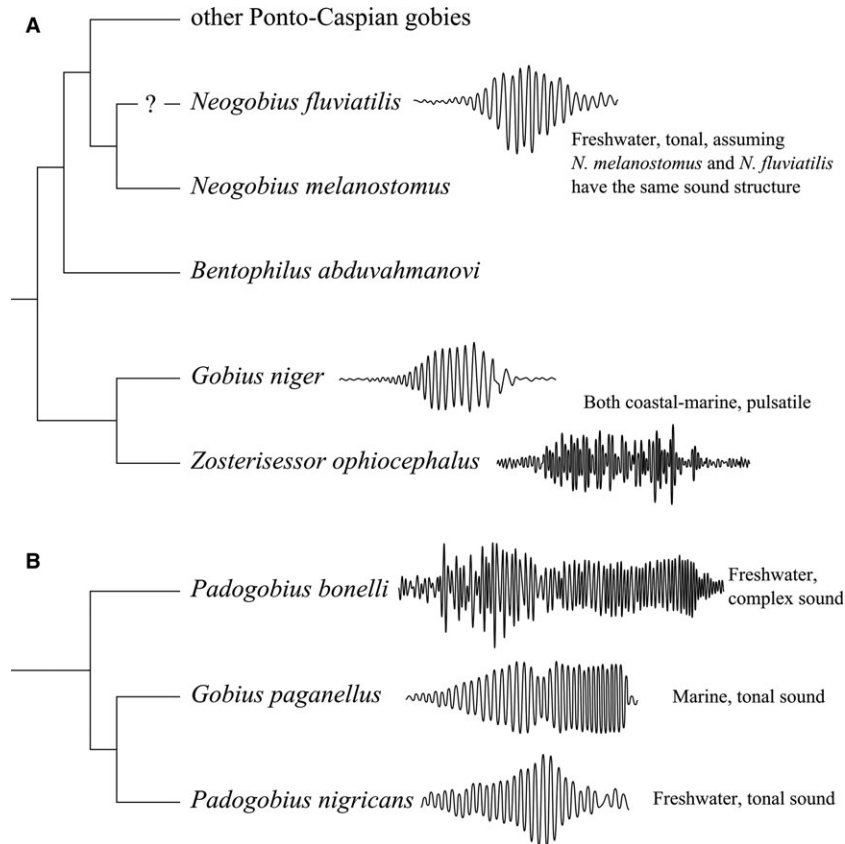


Figure 3. Two clusters extracted and adapted from Thacker & Roje (2011) (A) and Huyse *et al.* (2004) (B) showing the relationships between certain soniferous gobies, and the wave forms of their typical sounds.

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 (Huysse *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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