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"Molecular evidence for the freshwater lifestyle conquer in two Italian endemic representatives of the Gobiidae family: *Padogobius nigricans* and *Padogobius bonelli*"

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Molecular evidence for the freshwater lifestyle conquer in two Italian endemic representatives of the Gobiidae family: *Padogobius nigricans* and *Padogobius bonelli.*

Abstract

Evolutionary transitions between marine and freshwater habitats are rare events that can have profound impacts on aquatic biodiversity. Marine–freshwater transitions are a particularly interesting aspect of the evolutionary biology of many fish groups. Within Mediterranean fish biodiversity, one of the most enigmatic group in terms of adaptive radiation on evolutionary time scale is represented by the Gobiidae family (Teleostei). Mediterranean representatives of Gobiidae are divided in three distinct evolutionary lineages: *Pomatoschistus* lineage, with marine and freshwater species; *Aphia* lineage, composed of exclusively marine species inhabiting the Mediterranean and North-East Atlantic; *Gobius* lineage, including a radiation in the inland Ponto-Caspian region (Benthophilines), as well as species inhabiting the Mediterranean, North-eastern Atlantic, and Atlantic coast of Africa. Within *Gobius* lineage, one intriguing open question is related to the origin of the genus *Padogobius*. *Padogobius* is represented by only two species: *P. nigricans* Canestrini, 1867, endemic to Central and Northern Italy, and *P. bonelli* (Bonaparte, 1846), known from the North-Eastern Italy and

adjacent areas. Both species are pure freshwater inhabitants. Nevertheless, whether the adaptation to the freshwater habitats evolved only once or independently is still debated.

In this dissertation I inferred a more comprehensive analysis of species-level relationships of the major representatives of Mediterranean gobies, linking evolutionary marine/freshwater ecological transitions to the paleohistory of the Mediterranean basin. I implemented a time calibrated species tree inference with ancestral state reconstruction encompassing the search for a sister group of *Padogobius* to test the monophyly of the freshwater habit. All the reconstruction proposed in this study converge in the definition of the polyphyletic origin of the genus *Padogobius.* The results provide strong support for a multiple independent origin of the freshwater habit in the two endemic Italian species *P. bonelli* and *P. nigricans*. Additionally, the results from species tree inference propose new insight in the relationships among members of Mediterranean *Gobius* lineage, previously not considered. Molecular dating of the radiation of *Gobius* species and *Padogobius* representatives are consistent with the oceanographic and tectonic history of the basin, enhancing of the role of the Messinian Salinity Crisis in shaping Mediterranean biodiversity.

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Overview

Why lineages of organisms persisted over million years mostly unchanged while others have spread over a wide variety of ecological and phenotypic adaptations? The ecological diversification of species observed in nature is a marvel narration that has amazed evolutionary biologists since they first began drawing the tree of life. Some groups of organisms (e.g African Ciclids, Sticklebacks, Darwin's Finches) appeared to display a greater plasticity for ecological diversification, seeming to be better 'adapted for adaptation'. Colonizations of new environments can be considered the starting point for adaptation and speciation processes, promoting the incoming of new selective pressures that can stimulate adaptive differentiation among populations (Kornfield and Smith 2000; Streelman and Danley 2003; Rundle and Schluter 2004). However, why organisms should drift into a new environment, especially with strong differences in its biotic or abiotic conditions, remains an open question. From a general point of view, ecological release or ecological opportunity can be pointed out as main goal of the process of colonization of a novel biome: the new environment offers the chance of reduced predation or competition for resources, or the availability of new resources absent or rare in the ancestral environment (Bloom & Lovejoy, 2012; Ortì et al., 2012; Bloom et al., 2013; Losos and Mahler 2010). Nevertheless, transitions between different adaptive zones can be considered a rare event over an evolutionary time scale, suggesting the presence of considerable abiotic and biotic filters.

Within aquatic ecosystems, the interface between marine and fresh waters represents an imposing physiological challenge for aquatic species and constitutes a barrier that a number of entire phyla (Echinodermata, Brachiopoda) and clades within other groups have failed to overcome (Lee & Bell 1999; Vermeij and Dudley 2000; Vermeij and Wesselingh 2002; Bloom

and Lovejoy 2011). The suite of osmoregulatory mechanisms that facilitate permanent freshwater or marine residency are therefore thought to represent a significant evolutionary adaptation for biota (Lee & Bell, 1999). Marine–freshwater transitions are a particularly interesting aspect of the evolutionary biology of many fish groups (Lovejoy & Collette, 2001; Yokoyama & Goto, 2005; Wilson et al., 2008; Bloom & Lovejoy, 2012, 2014; Bloom et al., 2013; Huyse et al., 2004; Almada et al., 2009; Davis et al., 2012; Betancur, 2010; Ortì et al., 2012; Konijnendijk et al., 2015). Some fish lineages have apparently achieved the feat on multiple occasions (Lovejoy et al., 2006; Betancur, 2010; Yamanoue et al., 2011), whereas radiations in several otherwise ecologically diverse fish lineages (Cypriniformes, Characiformes, Labridae, Acanthuridae) have been limited to either marine or freshwater environments (Lee & Bell, 1999). In the light of ecological plasticity, one of the most enigmatic group is represented by the Gobiidae family.

The Gobiidae comprise more than 1700 valid species (Gill and Mooi, 2012) and represent the most diverse families of vertebrates on Earth. Most species are small and cryptobenthic and depict the dominant components of the ichthyofauna of subtropical and tropical marine and estuarine habitats as well as freshwater stream communities on tropical islands and temperate regions. To date, several studies have demonstrated that different gobies clades underwent on explosive adaptive radiation driven by ecological divergence and microhabitat partitioning, as in the case of shrimp gobies, mudskippers and cleaner gobies (Rüber et al., 2003; Huyse et al., 2004; Yamada et al., 2009; Neilson & Speien, 2009; Tougard et al., 2014; Thacker, 2015). Furthermore, after gobies diverged into these ecologically-distinct clades, speciation has been constant or perhaps even accentuated as we approach the present (Thacker, 2009). Despite of a great numbers of molecular investigations on this group of fishes, links between ecologically-divergent species within lineages are still largely unresolved (Thacker & Roje, 2011; Rüber &

Agorreta, 2011), preventing the chance to increase the resolution on the evolutionary processes moulding gobioid's tree of life. In this perspective, gobies represent an understudied but promising model group for evolutionary studies inquiring a multitude of biotic and abiotic mechanisms of diversification across a number of evolutionary time scales.

In this dissertation I present a phylogenetic investigation of the Gobiidae that sheds light onto the patterns and processes behind ecological transitions in gobies. Specifically, the object of my thesis is to test the hypotheses on the origins and outcomes of marine/freshwater transitions within Mediterranean Gobiidae (Teleostei), focusing on two freshwater endemic species of Italian rivers: *Padogobius bonelli* (Bonaparte, 1846) and *Padogobius nigricans* Canestrini, 1867.

Introduction

Mediterranean Sea acted as a performing stage for vicariant speciation: it has been forced by strong geological perturbations, promoting one of the richest marine environment in terms of biodiversity and endemisms (Bianchi & Morri, 2000; Boudouresque, 2004; Bianchi, 2007; Coll et al., 2010). During the Miocene, the proto-Mediterranean (Paratethys) was a large and complex oceanic seaway between the Atlantic and the Indian Ocean. After the Arabian and Anatolian plates collision (15–14 million years ago) the Eastern Mediterranean connection closed (Rögl, 1999; Seidenkrantz et al., 2000), promoting an independent evolution of Mediterranean and Indo-West Pacific fauna. Most of the paratethyan ancestral fauna fell into a massive extinction during the Messinian Salinity Crisis (5.971–5.33 Ma; Roveri et al., 2014, and references therein), which led to the origin of several hyper- and hyposaline lakes (Bianchi & Morri, 2000; Rouchy & Caruso, 2006). This remarkable environmental shift lasted for about one million years, ending with the re-flooding of Atlantic waters in to the basin. Several author suggested that the origin of a number of the endemic freshwater fishes of the Mediterranean and Balkan area dates back to the Miocene–Pliocene period, when the Messinian salinity crisis led to the formation of different lake-sized sea (Penzo et al., 1998; Almada et al., 2009; Miller 1990). According to this hypothesis, the progressive evaporation of Mediterranean waters and the consequent disappearance of marine habitats might have forced some species to adapt to a freshwater environment. Following the reflooding of the Mediterranean Basin by the Atlantic Ocean through the Gibraltar Strait, these species retained their freshwater habitats and thus remained isolated from the euryhaline ancestors. Contemporary Mediterranean marine fish biodiversity show significant evidence of the role of the Messian Salinity Crisis in shaping

local biodiversity, composed by a puzzle of different biogeographical clusters: Atlantic subtropical (Quaternary interglacial remnants), Atlantic boreal (Quaternary glacial remnants), and endemic species (Miocene or Pliocene origin) (Bianchi & Morri, 2000; Boudouresque, 2004; Bianchi, 2007; Coll et al., 2010).

Within Mediterranean fish biodiversity, the most specious fish group is represented by the Gobiidae, one of the little known marine fish species flock. Mediterranean representatives of Gobiidae are divided in three distinct evolutionary lineages (see Supplementary Material Fig.1a,b): *Pomatoschistus* lineage, with marine and freshwater species; *Aphia* lineage, composed of exclusively marine species inhabiting the Mediterranean and North-East Atlantic; *Gobius* lineage, including a radiation in the inland Ponto-Caspian region (Benthophilines), as well as species inhabiting the Mediterranean, North-eastern Atlantic, and Atlantic coast of Africa (Nielson & Stepien, 2009; Thacker & Roje, 2011; Agorreta et al., 2013; Thacker, 2015). The history of Mediterranean *Gobius* lineage clade is deeply connected to the geological dynamism of the basin. A time calibrated phylogeny suggests divergence from the Indian Ocean ancestors around 25 Ma, followed by a diversification into an array of species during the migration through the Tethys seaway into the Atlantic Ocean (Thacker, 2015). They spread into a great number of different ecological adaptations, which also include freshwater lifestyle. Within modern Mediterranean freshwater gobies, the most representative, in terms of species, are the Benthophilines, a monophyletic radiation of hardy freshwater species native of the Aral, Azov, Black and Caspian Seas and their drainages (Agorreta et al., 2013; Thacker & Roje 2011). Benthophilines species flock is in general concordance with major geological events in the Ponto-Caspian basin, consisting in fluctuation of salinity levels from the Messinian Salinity Crisis over the last 5 My, promoting lineage separations on multiple temporal scales (Neilson &

Stepien, 2009; Thacker, 2015). Within this group, one intriguing open question is related to the origin of the genus *Padogobius*.

Padogobius is represented by only two species: *P. nigricans*, endemic to Central and Northern Italy, and *P. bonelli*, known from Northern Italy and adjacent areas (Slovenia and Croatia) (see Supplementary Material Fig. 2a,b). Both species are pure freshwater inhabitants. Nevertheless, whether the adaptation to the freshwater habitats evolved only once or independently is still debated (Ahnelt & Patzner, 1996; Herler et al., 1999; Patzner, 1999; La Mesa et al., 2006; Thacker & Roje, 2011; Agorreta et al., 2013). To date, there is only one phylogenetic reconstruction of the relationship between the two Italian endemic freshwater gobies. In this study (Penzo et al..1998), the authors aimed at resolving the phylogenetic relationship among some representatives of Western Mediterranean marine gobies and *Padogobius* species, tracing the evolution of freshwater habit in relation to the geological history of the Mediterranean region. Based on mitochondrial markers, the authors proposed an independent origin of the freshwater condition within the genus *Padogobius* (as their allopatric distribution seems to indicate), with an early Pliocene origin. In conclusion, although genetically very close, *Padogobius* species did not appeared as sister species with *G. paganellus*, a moderately euryhaline marine species, sister to the freshwater *P. nigricans* (Fig.3). Nevertheless, the low resolution of this phylogenetic reconstruction could not rule out the alternative hypotheses that freshwater lifestyle took place only once during the Messinian Salinity Crisis, (in the ancestor of *P. bonelli*, *P. nigricans*, and *G. paganellus*), with a subsequent adaptation to marine lifestyle in *G. paganellus*. Likewise, any other Benthophilines representatives were included in the analyses, leaving unresolved the relation between Ponto-Caspian species flock and Mediterranean *Gobius* species.

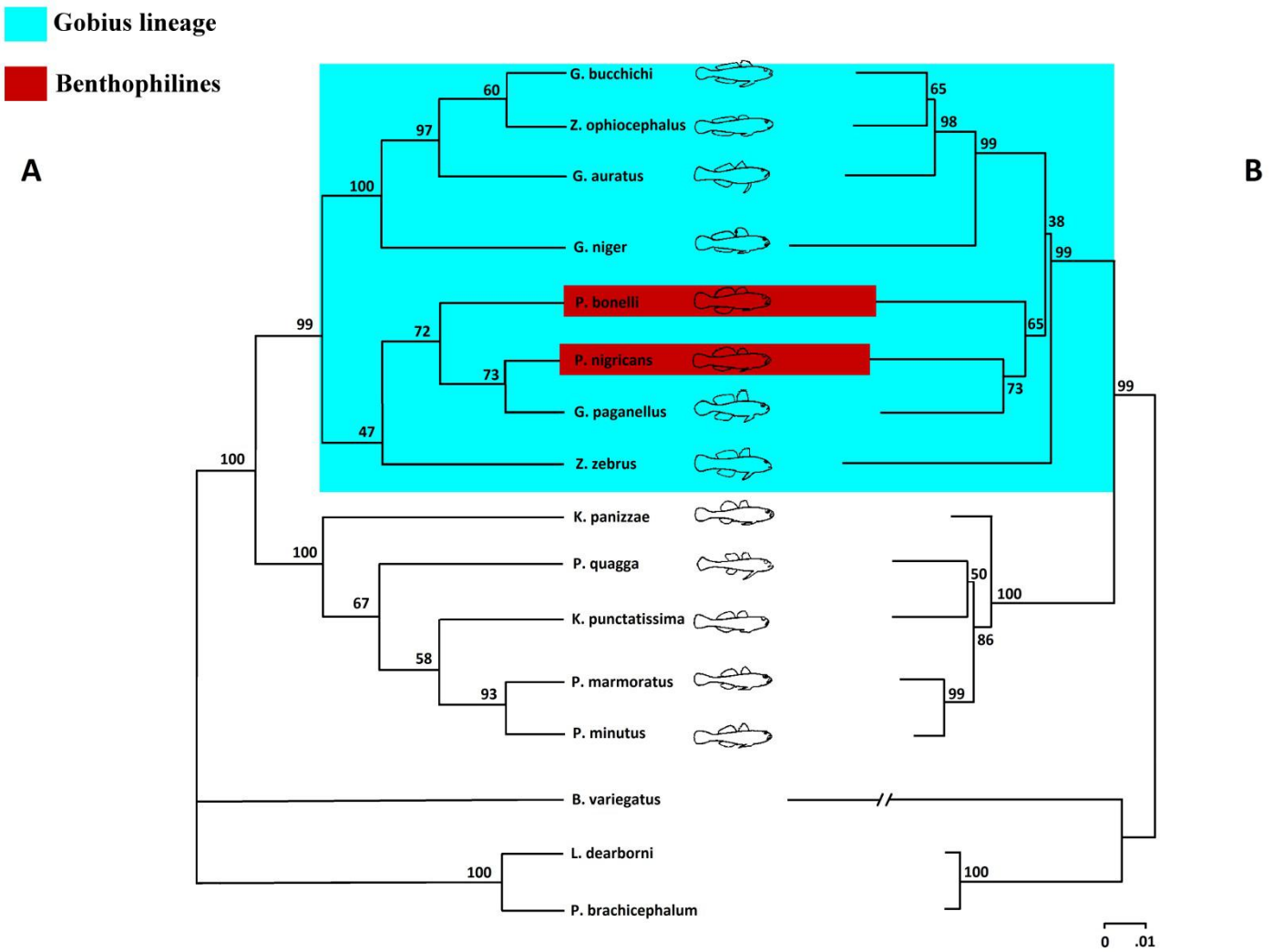

Fig.3—,Neighbour-joining tree (A-right side) representing the phylogenetic relationships of the 13 gobiid species included in Penzo et al., 1998: *Bovichthys variegatus* (Notothenioidea), *Lycodichthys pearborni*, and P*achychara brachycephalum* (Zooarcoidea) were used as outgroups. Pairwise distances between taxa were calculated according to the two-parameter Kimura (1980) model. The scale bar indicates 0.01 sequence distance. Numbers at branches refer to bootstrap values after 1,000 replicates. B, Majority-rule consensus tree (left side) after 1,000 bootstrap replicates using maximum parsimony; bootstrap values are reported for each node. Modified from Penzo et al., 1998.

Compared to studies on other diverse groups of fishes (i.e. cyprinids, cichlids, labrids), phylogenetic studies on gobies have been slow to encompass the use of nuclear loci in deep-scale phylogenetic studies (Rüber & Agorreta, 2011; Tornabene et al., 2013). Mitochondrial DNA contains important genes, whose protein products are vital for the mitochondrial oxidative phosphorylation process (Ballard & Whitlock 2004). There are evidence, in fact, that mtDNA undergoes substantial amounts of adaptive evolution and that the rate of adaptive evolution is correlated to the diversity of the species being considered. These results have important implications for molecular ecology. MtDNA is widely used as a neutral genetic marker; however, it is important to take count that a number of not synonymous substitutions could have been fixed by positive selection (James et al., 2016). MtDNA diversity will, at least in part, reflect the amount of time since the last selective sweep, rather than demographic processes affecting the population. Thus, investigation on phylogenetic relationships between species connected with different environment should include in the final dataset also proper nuclear markers to avoid unbalanced phylogenetic signals.

Among the most prominent molecular phylogenetic studies building relationships within Mediterranean gobies, only few have employed nuclear loci (in combination with mtDNA), providing a small fraction of suitable markers. Moreover, despite huge advances both in evolutionary theory and in sequencing technology, estimating the “Tree of Life” even for a small subset of species can be challenging. Better mathematical models and more data improve our ability to infer a single gene phylogeny, but a gene history may be different from the species phylogeny. The potential for a discrepancy between the gene tree and the species tree has been known for decades and is especially problematic for closely related species (Heled & Drummond, 2010). Research into multigene phylogenetics demonstrates that the common approach of concatenating sequences from multiple genes generates the wrong kind of average

(Degnan and Rosenberg 2006) and can lead to poor estimation of the species tree (Kubatko & Degnan, 2007). For these reason, a robust phylogenetic investigation on ecological transition ask for more extended tools, both in terms of dataset and inference methods.

Aim of the present study is to set up a more comprehensive analysis of species-level relationships between *Padogobius* and the major representatives of Mediterranean gobies. In addition to the common approach of concatenating sequences from multiple genes, I've implemented a species tree approach to infer the phylogeny. In order to link ecological transitions events to paleoclimate history of Mediterranean basin, I calibrate species tree analysis and ancestral state reconstruction using fossil data, encompassing the search for a sister group of *Padogobius* to test the monophyly of the freshwater habit.

Material and methods

Taxon sampling, DNA extraction, amplification and sequencing

A total of 28 samples including specimens of genus *Gobius* Linnaeus, 1758, *Zosterisessor* Whitley, 1935, *Knipowitschia* Iljin, 1927, *Zebrus* de Buen, 1930, *Chromogobius* de Buen, 1930 and *Padogobius* Berg, 1932 were collected and fixed in 95% ethanol for further DNA analyses. Extraction of DNA from sampled specimens were performed using tissues. DNA has been extracted using proteinase-K digestion followed by a standard phenol–chloroform protocol (Sambrook et al. 1989). Purification and sequencing of PCR products were carried out by Macrogen Inc. Acquired dataset, composed of the partial mitochondrial (mtDNA) D-loop

control region, 12s rRNA, 16s rRNA and partial nuclear (nDNA) recombination activating gene 1 (RAG1) and S7 ribosomal protein intron markers, has been implemented with available sequences downloaded from GenBank, including *gobius*-lineage outgroup as proposed in Agorreta et al. (2013). Collection data and accession numbers are listed in Table 1. For mitochondrial and nuclear markers, all primer sequences and PCR protocols are shown in the Supplementary Material (Table 2).

Data analysis

Sequencing results were controlled by eye using Chromas 2.31 (Technelysium Ltd.) and aligned using Mafft v7 (Katoh & Standley 2013), checking for any obvious misalignment. Protein-coding sequences were translated into amino acids for confirmation of alignments. In order to improve the phylogenetic signal of each marker, I submitted the full dataset on the G-blocks Web Server workbench (Castresana, 2000) with low stringency options (Talavera and Castresana, 2007) to remove gaps and alignment ambiguities. For each data set (16s, 12s, d-loop, RAG1 and RSP7), the best-fit model of evolution has been estimated according to the Bayesian Information Criterion (BIC) as implemented in jModeltest 2.1.7 (Darriba et al. 2012).

Phylogenetic analysis using genes tree.

To infer phylogenetic relationships using genes tree, I employed two different methods: Maximum Likelihood (ML) and Bayesian Inference (BI). Partitioned ML tree searches were performed using RAxML GUI v.1.5 BETA (Silvestro and Michalak, 2012), a graphical front-end for RaxML v.8 (Stamatakis, 2014), set with 10 random addition replicates and 1000 non

Classification	Species	16s	D-loop	12s	RAG1	RSP7	Locality	References
Odontobutidae	*Percottus glenii*	NC020350	NC020350	NC020350	KF415837	n.a.	----------	Xue et al., 2013; Agorreta et al., 2013
Eleotridae	Eleotris acanthopoma	NC004415	NC004415	NC004415	KF235448	n.a.	----------	Miya et al., 2003; Tornabene et al., 2013
Eleotridae	*Mogurnda adspersa*	NC024058	NC024058	NC024058	GU288532	n.a.	----------	Perini et al., 2016; Adamson et al., 2010
Butidae	*Bostrychus sinensis*	NC017880	NC017880	NC017880	n.a.	JF358013	----------	Wei & Xu, 2013; GenBank
Acanthogobius lineage	*GIllichthys seta*	NC012908	NC012908	NC012908	KF486319	n.a.	----------	Bucciarelli et al., 2009; Ellingson et al., 2014
Acanthogobius lineage	*Gymnogobius petschiliensis*	NC008743	NC008743	NC008743	KF486315	n.a.	----------	Kim et al., 2004; Ellingson et al., 2014
Pomatoschistus lineage	*Knipowitschia punctatissima*	AF067273	This study	This study	This study	This study	Viarolo River, Italy, 44°52'30.17"N/ 10°14'27.59"E. 1 m depth	This study; Penzo et al., 1998
Gobius l. (Benthophilines)	*Padogobius nigricans*	KM406308	This study	This study	This study	This study	Amaseno river, Italy, 41°30'10.06"N/ 13°15'43.61"E. 1m depth	This study; GenBank
Gobius l. (Benthophilines)	*Padogobius nigricans*	AF067270	This study	This study	This study	This study	Amaseno river, Italy, 41°30'10.06"N/ 13°15'43.61"E. 1m depth	This study; Penzo et al., 1998
Gobius l. (Benthophilines)	*Padogobius nigricans*	This study	This study	This study	This study	This study	Amaseno river, Italy, 41°30'10.06"N/ 13°15'43.61"E. 1m depth	This study
Gobius l. (Benthophilines)	*Padogobius bonelli*	KM406309	This study	This study	This study	This study	Taro River, Italy, 44°42'3.13"N/ 9°51'59.42"E. 1 m depth	This study; GenBank
Gobius l. (Benthophilines)	*Padogobius bonelli*	EF218652	This study	This study	This study	This study	Taro River, Italy, 44°42'3.13"N/ 9°51'59.42"E. 1 m depth	This study; Giovannotti et al., 2007
Gobius l. (Benthophilines)	*Ponticola kessleri*	KM583832	KM583832	KM583832	FJ526934	FJ526934	----------	Kalchhauser et al., 2016; Neilson & Stepien, 2009
Gobius l. (Benthophilines)	*Ponticola kessleri*	NC025638	NC025638	NC025638	n.a	n.a	----------	Kalchhauser et al., 2016;
Gobius lineage	*Zebrus zebrus*	AF067279	This study	This study	This study	This study	Nice, France, 43°41'38.32"N/ 7°16'41.89"E. Tide pool	This study; Penzo et al., 1998
Gobius lineage	*Zebrus zebrus*	This study	This study	This study	This study	This study	Izola, Slovenia, 45°32'51.31"N/ 13°41'18.58"E. 3m depth	This study
Gobius lineage	*Zosterisessor ophiocephalus*	EF218649	This study	This study	This study	This study	Venice lagoon, Italy, 45°13'19.45"N/ 12°16'13.30"E. 7m depth	This study; Giovannotti et al., 2007
Gobius lineage	*Zosterisessor ophiocephalus*	KF415492	This study	This study	This study	This study	Venice lagoon, Italy, 45°13'19.45"N/ 12°16'13.30"E. 7m depth	This study; Agorreta et al., 2013

Table 1. Species, sequence's GenBank accession numbers and sampling locality for the 113 sequences used in this study. Classification (simple = outgroups family; *italics*= lineage within Gobiidae family) according to Agorreta et al., 2013. Habitat of each species are presented with orange shading for freshwater and light blue for marine.

Classification	Species	16s	D-loop	12s	RAG1	RSP7	Locality	References
Gobius lineage	*Chromogobius quadrivittatus*	This study	n.a.	This study	This study	This study	Sabaudia lake, Italy, 41°14'54.57"N/13° 2'22.19"E. 1m depth	This study
Gobius lineage	*Gobius ateriformis*	n.a.	This study	This study	This study	This study	Sal Is., Capo Verde	This study
Gobius lineage	*Gobius geniporus*	FJ460200	This study	This study	This study	This study	Giglio Is., Italy, 42°22'27''N /10°52'47''E. 22m depth	This study; GenBank
Gobius lineage	*Gobius bucchichi*	AF067268	This study	This study	This study	This study	Tusa, Sicily, Italy, 38° 0'42.27"N/ 14°15'4.02"E. 4m depth	This study; Penzo et al., 1998
Gobius lineage	*Gobius bucchichi*	This study	This study	This study	This study	This study	Tarifa Is., Spain 36° 0'20.55"N/ 5°36'30.23"W. 4m depth	This study
Gobius lineage	*Gobius cobitis*	EF218644	This study	This study	This study	This study	Sabaudia lake, Italy, 41°14'54.57"N/13° 2'22.19"E. 1m depth	This study; Giovannotti et al., 2007
Gobius lineage	*Gobius cobitis*	FJ460198	This study	This study	This study	This study	Tarifa Is., Spain 36° 0'20.55"N/ 5°36'30.23"W. 4m depth	This study; GenBank
Gobius lineage	*Gobius cruentatus*	EF218641	This study	This study	This study	This study	Giglio Is., Italy, 42°22'27''N /10°52'47''E. 30m depth	This study; Giovannotti et al., 2007
Gobius lineage	*Gobius cruentatus*	This study	This study	This study	This study	This study	Tarifa Is., Spain 36° 0'20.55"N/ 5°36'30.23"W. 10m depth	This study
Gobius lineage	*Gobius gasteveni*	n.a.	This study	This study	This study	This study	Tarifa Is., Spain 36° 0'20.55"N/ 5°36'30.23"W. 7m depth	This study
Gobius lineage	*Gobius niger*	FJ460203	This study	This study	This study	This study	Venice lagoon, Italy, 45°13'19.45"N/ 12°16'13.30"E. 7m depth	This study; GenBank
Gobius lineage	*Gobius niger*	KJ128783	This study	This study	This study	This study	Sabaudia lake, Italy, 41°14'54.57"N/13° 2'22.19"E. 1m depth	This study; GenBank
Gobius lineage	*Gobius vittatus*	GQ485305	This study	This study	This study	This study	Giglio Is., Italy, 42°22'27''N /10°52'47''E. 30 m depth	This study; GenBank
Gobius lineage	*Gobius auratus*	AF067267	This study	This study	This study	This study	Giglio Is., Italy, 42°22'27''N /10°52'47''E. 30m depth	This study; Penzo et al., 1998
Gobius lineage	*Gobius paganellus*	FJ460204	This study	This study	This study	This study	Astypalaia, Greece, 36°32'28.49"N/26°20'31.44"E. 5m depth	This study; GenBank
Gobius lineage	*Gobius paganellus*	EF218651	This study	This study	This study	This study	Roscoff, France, 48°43'43.31"N/ 3°59'23.69"W. Tide pool	This study; Giovannotti et al., 2007
Gobius lineage	*Gobius paganellus*	AF067271	This study	This study	This study	This study	Sabaudia lake, Italy, 41°14'54.57"N/13° 2'22.19"E. 1m depth	This study; Penzo et al., 1998

Table 1. Species, sequence's GenBank accession numbers and sampling locality for the 113 sequences used in this study. Classification (simple = outgroups family; *italics*= lineage within Gobiidae family) according to Agorreta et al., 2013. Habitat of each species are presented with orange shading for freshwater and light blue for marine.

parametric bootstrap replicates, applying the general time-reversible model with CAT model of rate heterogeneity (GAMMACAT). Due to lower computational and memory costs, CAT, which is a rate heterogeneity and fast approximation of the gamma model, appears to yield better log likelihood scores even when calculated under a real gamma model (Stamatakis, 2006). Under the GTR + CAT model, the default number of categories (c = 25) and random starting trees were employed.

BI analyses were performed in BEAST v.1.8.4 (Drummond et al.,2012). Among partitions, I set unlinked nucleotide substitution and clock models, linking tree model among partitions. As prior for molecular clock I set Relaxed Uncorrelated Lognormal Clock for all genes, with Yule process of speciation selected as tree prior. All the others priors were left with default settings. BEAST was run three times with 100 million generations, sampling every 10000 generations. The different runs were combined with LogCombiner (Drummond et al.,2012) and checked for convergence using Tracer v 1.6 (Rambaut and Drummond, 2007) (burn-in = 10%), ensuring that all effective sample sizes parameters (ESS) were higher than 200. The final tree has been generated by TreeAnnotator v.1.8.4 (Drummond et al.,2012) with a burnin of 1000.

Phylogenetic analysis using species tree, divergence time estimation and ancestral state reconstruction.

To simultaneously estimate and calibrate the multilocus species tree reconstruction, I assigned divergence time calibration using fossil data. To incorporate appropriate fossil calibration points, I included species from throughout the families Eleotridae, Butidae and Odontobutidae. These families (plus Rhyacichthyidae, Milyeringidae and Thalasseleotrididae, neither of which were available for this study) collectively comprise the Gobioidei and represent appropriate out-groups for the analysis

(Thacker, 2009; Gill & Mooi, 2012; Thacker et al., 2015). I calibrated the reconstruction basing on a well-preserved whole-body fossil of an upper Oligocene Butidae species (Gierl et al., 2013), the most recent common ancestor of *Bostrychus sinensis*; the TMCRA was set to a minimum of 23 Ma (prior settings: lognormal distribution; mean, 1.5; standard deviation, 0.8; offset, 23).

I further employed ancestral state reconstruction. In order to trace ecological character evolution over *Gobius* representatives, I classified species using literature sources (see Supplementary Material Tab.4). Habitat type was coded as marine/freshwater, with estuarine species categorized as marine, since rarely found in entirely freshwater habitats, and likely do not reproduce in freshwater habitats. Ecological transitions need a physical continuity between biomes. Transitions between oceans and continental freshwater are far more difficult that transitions between continental freshwater and continental saline lakes (Adamowicz et al. 2010). As principal abiotic variables discerning coastal habitat, I therefore included depth and substrate in the character matrix. Species were categorized as inshore (0-50m) and offshore (50-200m), with notation on the preferred substrate according to the Marine Species Identification Portal (http://species-identification.org; accessed on 05/06/2015) and Fish Base (http://www.fishbase.org; accessed on 05/06/2015). Behavioural attribute, such the ability of producing sounds were also included in the matrix. This peculiar attitude has been documented in at least 21 gobioid species belonging to ten different genera (Bass & McKibben, 2003; Polgar et al., 2011; Horvatic et al., 2015). 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). Within Mediterranean gobies, several species are considered soniferous (Horvatic et al., 2015) (see Supplementary Material Fig.4). Whereas different in the structural details, I coded sound emission as a binary character (0=present; 1=absent). To determine the timing of biome transition, the calibrated

species tree inference was implemented with ancestral character reconstruction; the analysis was performed using the multi-species coalescent-based method (species tree) implemented in the *BEAST extension (Heled and Drummond 2010) of the BEAST 1.8.4 package (Drummond et al. 2012) setting linked tree models for the mitochondrial genes and unlinked for nuclear genes. Substitution models and relaxed clock models were unlinked across all the partitions. I specified a Yule Birth-Death process of speciation as tree prior and a random starting tree. Due to the computational cost and to prevent an overload of variables, only habitat type (marine or freshwater) was included in the character matrix. All *BEAST analyses were run three times, 100 million iterations per run, sampling every 10000 steps. Results of the runs were analysed, combined and summarized with Tracer v1.6, LogCombiner and TreeAnnotator. Consensus tree representing the posterior distribution were visualised and edited in FigTree v1.4.

I employed *BEAST species trees output to perform additional ancestral characters reconstruction on Mesquite v.2.6 (Maddison and Maddison 2011) using Maximum Likelihood as inference method. Maximum likelihood determines the likelihood of a character state at each internal node using the Mk model (Pagel 1999). It gives equal probability (or rate) for changes between any two character state, providing a measure of uncertainty for character states, while taking into account branch length information (Schluter et al. 1997). Ml character reconstructions were further summarized on the *BEAST consensus tree.

Results

Successful amplification and sequencing was completed for all gene regions used in this study, with a total of 113 new sequences out of the 167 employed. However, sequencing failures occurred for some markers for a few individuals (see Table 1). The initial sequence alignment, including gaps, consist of 3442 bp in length. GBlocks was used to further refine the alignment, removing ambiguously aligned regions, resulting in final alignments of 2868 bp.

Phylogenetic relationships within Mediterranean Gobius lineage

Phylogenies inferred from Maximum Likelihood and Bayesian analyses of the combined dataset produced largely congruent topologies. Overall, I found higher Bayesian Posterior Probabilities support than the Bootstrap Support when comparing BI and ML trees. The phylogenetic placement of all the main evolutionary lineage included in this study are in concordance with the ones proposed by Agorreta et al. (2013), with hight statistical support at each node. Whereas the placement of *Padogobius bonelli* differs between BI (Fig.5) and ML (Fig.6) topologies, there is convergence in the rejection of its placement as sister species of *P nigricans*, with *P. bonelli* rooted in a basal position of the *Gobius* lineage clade compared to the other Benthophilines. Both Ml and BI topologies reject the monophyly of the genus *Gobius*, with *Z. ophiocephalus,* sister species of *G. bucchichi* and *P. nigricans* and *P. kessleri* sister group of *G. cobitis*, *G.paganellus* and *G. ateriformis*.

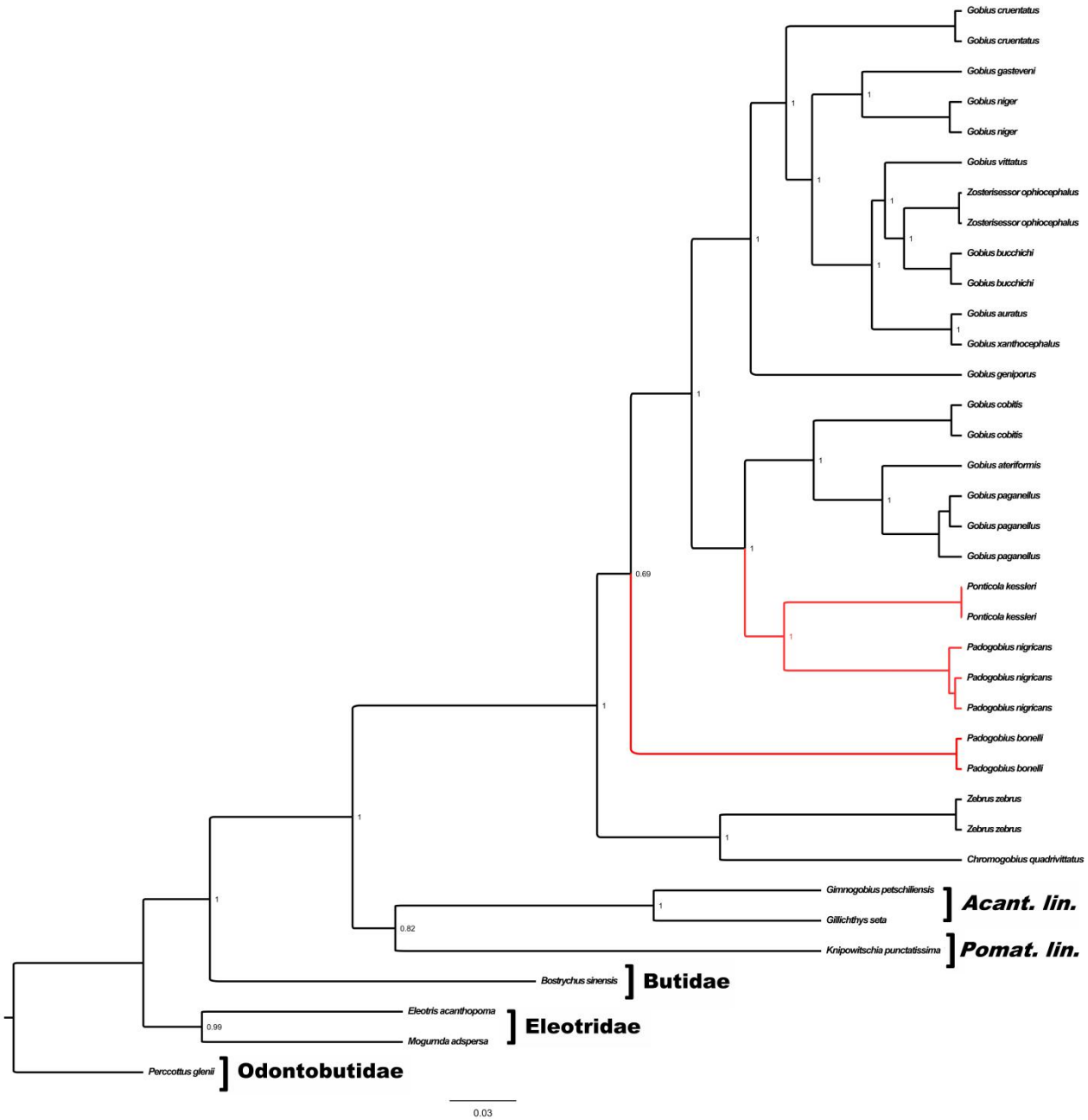

Fig.5: phylogenetic relationships based on Bayesian analyses of concatenated dataset. Bayesian posterior probabilities (three independent runs of 100 million generations combined, with a burnin of 10%) are reported for each nodes. Red branches represent *Padogobius bonelli* and Benthophilines.

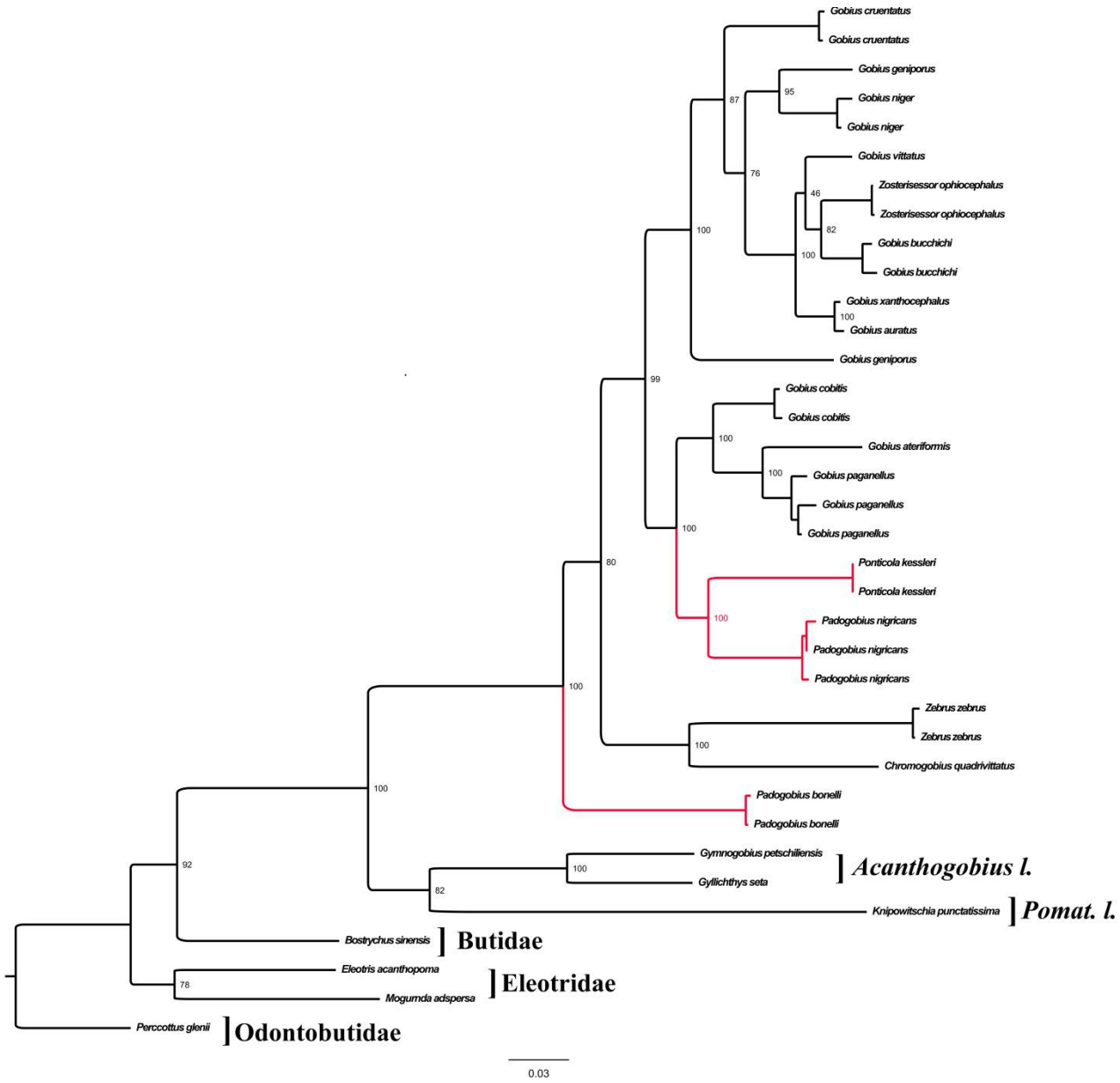

Fig.6: phylogenetic relationships within *Gobius* lineage based on Maximum Likelihood analyses of the concatenated dataset. Likelihood bootstrap values (10 random addition replicates with 1000 non-parametric bootstrap replicates) are reported for each nodes. Red branches represent *Padogobius bonelli* and Benthophilines.

Species tree reconstruction (Fig.7) confirm the relation between the different evolutionary lineage and family; however, significant differences can be found in the *Gobius* lineage phylogenetic framework. Excepting for the inclusion of *Z. ophiocephalus* (see discussion) *Gobius* is here represented as a monophyletic clade, with Benthophilines (*P. nigricans* and *P. kessleri*) as sister clade. The polyphyletic origin for the genus *Padogobius* is reaffirmed in the species tree reconstruction, with hight level of statistic support (BPP=1). As in BI and ML inferences, *P. bonelli* is rooted in a basal position compared to the other Benthophilines.

Molecular dating and ancestral state reconstruction

Molecular dating results are shown in Table 3 (see Supplementary Material), along with the 95% highest probability density (HPD) intervals. The chronogram (Fig.8) obtained by the time-calibrated multispecies coalescent phylogeny proposes the divergence between Gobiidae and Butidae at around 35 million years ago. Within Gobiidae family, the divergence between the different evolutionary lineages is placed within Miocene, with *Pomatoschistus* lineage diverging around 23 million years ago followed by the *Acanthogobius* lineage separation from *Gobius* lineage around 20 million years ago. Within *Gobius* lineage, the majority of splits are placed in a short time span of about 3 million years after the Messinian Salinity Crisis. The root of Benthophilines clade is placed in correlation with the beginning of the environmental drift of the Mediterranean in to the “Lago-Mare” condition, with the split of *P. kessleri* from *P. nigricans* 4,6 million years ago. The molecular dating on *Padogobius bonelli* proposes its ancestral placement (9,14 Ma) compared to the other Benthophilines, rejecting its evolutionary origin during the Messinian Salinity Crisis.

Bayesian and Maximum Likelihood ancestral state reconstruction on marine/freshwater transitions proposes Mediterranean *Gobius* lineage as an ancestrally marine group (see Supplementary Material Fig.8,9 and Tab.5, node 12,14 and 15). The hypothesis for which the conquer of freshwater lifestyle within *Padogobius* representatives has been independent and built up from a marine ancestor is therefore corroborated. Benthophilines are presented as a Messinian monophyletic radiation of freshwater species, with a common freshwater ancestor (see Fig.8; Supplementary Material Fig.9 and Tab.5, node 13) occurred 4,61 Ma. Within *Gobius* clade, four subclusters can be highlighted according to the substrate preference (see Supplementary Material Fig.10,11 and Tab.5): NODE 1, with inshore marine species (*G. paganellus, G. cobitis, G. ateriformis, G. geniporus*), with ecological preferences for rocky substrates; NODE 5, with *G. gasteveni* and *G. niger*, mainly connected with sandy bottoms with a wide range of depth (0-200m); NODE 7, with *G. auratus*, *G. vittatus* and *G. xanthocephalus,* which share same habitat conditions (stony substrate of coralligenous bottoms, usually in sympatry) and NODE 10, with *G. bucchichi* and *Z. ophiocephalus* who inhabit shallow water sandy bottoms. Ancestral state reconstruction supports the preference for sandy substrate and a wider range of depth (0-200m) as an independent recent radiations with significative likelihood scores. Character history reconstruction on sound emission outcomes largely equivocal, as only terminal nodes resulted statistically supported (see Supplementary Material Fig. 12 and Tab.5).

Fig.7: phylogenetic relationships within *Gobius* lineage based using the multispecies coalescent analyses of the concatenated dataset. Bayesian posterior probabilities (three independent runs of 100 million generations combined, with a burnin of 10%) are reported for each nodes. Red branches represent *Padogobius bonelli* and Benthophilines.

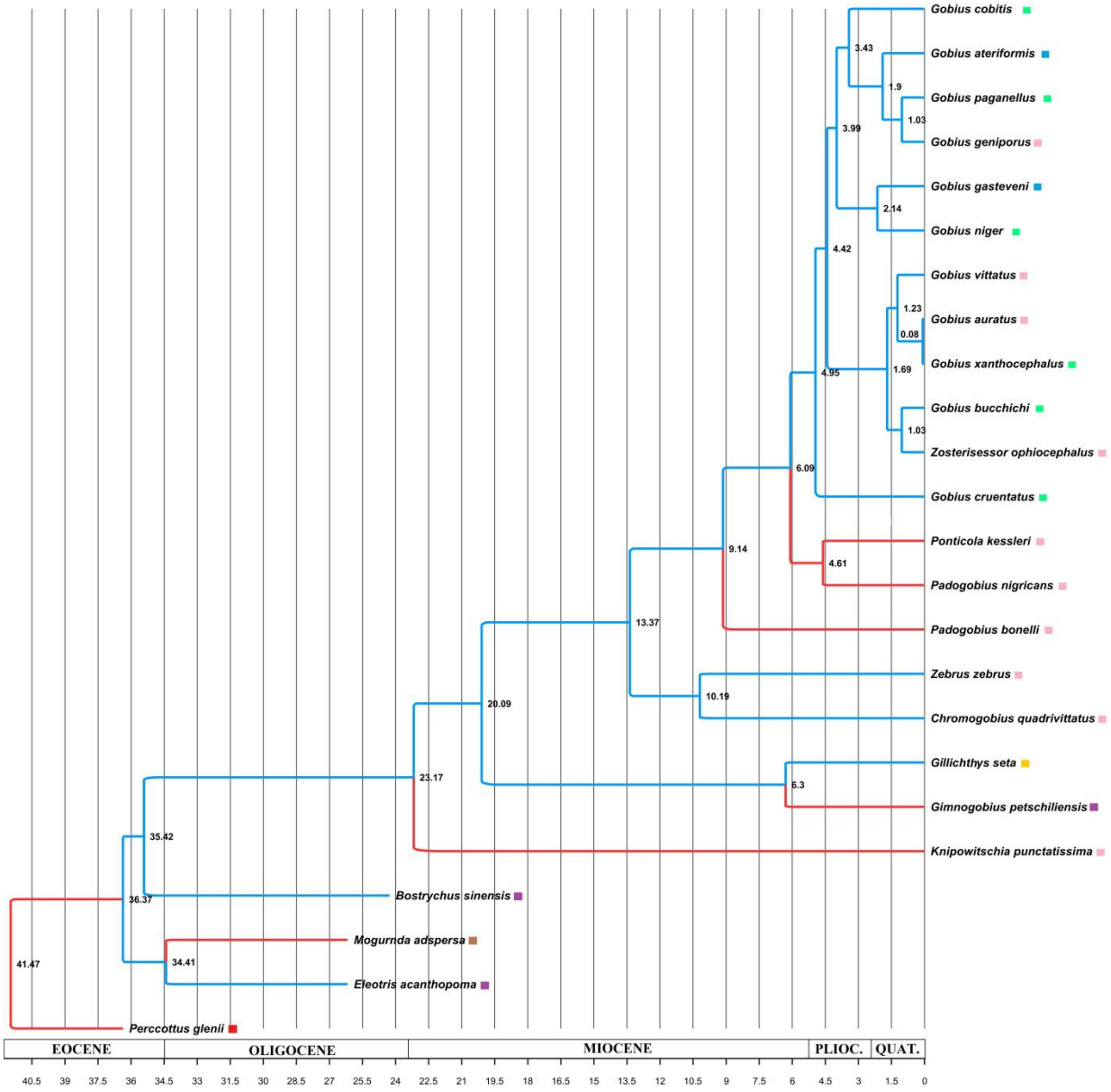

Fig.8: BEAST* ancestral state reconstruction inferred on a time-calibrated multispecies coalescent phylogeny. Branch colour identify freshwater (red) and marine (blue) habitat state reconstruction. States at nodes indicate age in million years. Coloured squares at branch tips indicate the current geographical distributions of taxa: green-Eastern Atlantic/Mediterranean; pink-Mediterranean; blue-Eastern Atlantic; yellow-Eastern Pacific; violet-Western Pacific; brown-Oceania; red-Asia

Discussion

For a long time, speciation events over the Tree of Life have been enforced in molecular investigations in the context of gene trees or concatenation approaches. Discordance among genes is common (Knowles and Carstens 2007; Cranston et al. 2009), and although multiple potential sources for gene-tree heterogeneity exist (Degnan and Rosenberg 2006, 2009), a universal source of discord arises from the random coalescence of gene lineages (Kingman, 1982). Compared to concatenation methods, multilocus coalescent approach allows for better accurate estimation of the divergence history (Heled and Drummond 2010). Moreover, because the timing of gene divergences necessarily predates the actual speciation event, conclusions based on gene trees will always overestimate divergence times in contrast to multilocus coalescent approaches (Carstens & Knowles, 2007). Estimates of the timing of divergence are central to testing the underlying causes of ecological transitions; the implementation of relaxed molecular clocks and fossil calibration have improved these estimates. In the past, few molecular study has been very informative with respect to phylogenetic relationships among representatives of Mediterranean gobies. In this study I employed different methods to infer phylogenies within Mediterranean *Gobius* lineage in order to screen how discrepancies between methods could affect final results. A more extended taxon sampling, the addition of nuclear molecular markers and the implementation of calibrate multi-locus coalescent approaches and ancestral state reconstruction enabled the detection of new relationships between and within genera, providing insights into previously open questions.

The higher-level relationships of lineages recovered in both species (Fig.7) and genes trees (Fig.5-6) are consistent with aforementioned investigations (Agorreta et al., 2013; Thacker & Roje, 2011; Neilson &

Stepien, 2009). Nevertheless, within *Gobius* lineage, species tree reconstruction proposes different topologies compared to the Maximum Likelihood and Bayesian Inference analysis, especially in rooting *Padogobius* species. These results remark how inference of species relationships can be sensitive to the method used.

Molecular dating of the radiation of *Gobius* species and its freshwater sister clade is consistent with the oceanographic and tectonic history of the basin. According to widely-accepted geologic reconstructions, for less than seven hundred thousand years during the Messinian Salinity Crisis (5.971–5.33 Ma; Roveri et al., 2014), hyperhaline and, later, hypohaline hydrological conditions characterized the Mediterranean, which was armoured from free hydrological exchanges with oceans (e.g., Blanc, 2000). The isolation of the Mediterranean from the Atlantic took place after the closure of the Betic and Rif straits (Müller & Hsü, 1987). During the last period, the basin was dominated by wide non-marine deposits of the so called “Lago - Mare”, featured by brackish faunas of paratethyan biogeographic affinity. (Taviani, 2002; Neubauer et al., 2015). The hypohaline hydrological conditions came suddenly to end when the Atlantic breached into the Mediterranean through the Gibraltar portal at the beginning of the Pliocene (Cita & McKenzie, 1986; Iaccarino et al., 1999), and oceanic marine fauna re-entered the basin. From a biological perspective, such long-lasting, basin-wide extreme saline and following hypohaline conditions produce the same result: the complete annihilation of all stenoecious benthic marine life. This scenario gives insights to the *Gobius* and Benthophilines species radiation.

Gobius, whose ancestral state reconstruction proposes a marine origin, are probably a monophyletic radiation of marine fishes originated after the ingression of Atlantic waters in the end of the Messinian Salinity Crisis. Atlantic *Gobius* ancestors could have re-colonized the newly formed Mediterranean Sea and adaptation to new and free ecological niches might have led to a rapid radiation resulting in the present day *Gobius* species. The age of the basal root of the genus (95% HPD = 4,3-6,02 Ma) proposed

in this study closely fit with the geological and hydrological events described above. These results are also kindred to the ones proposed for the recolonization of the basin by *Pomatoschistus* lineage representatives 3,9 million years ago (Huyse et al., 2004).

From a taxonomical perspective, the inclusion in *Gobius* clade of *Z. ophiocephalus* would seem to commend a paraphyletic condition of the genus. As a matter of fact, the evidence that *Z. ophiocephalus* (which is the only representative of its genus) turns out to be genetically very close to *Gobius* species in several other studies (McKay and Miller 1991; Miller et al. 1994; Penzo et al., 1998; Medvedev et al., 2013; Agorreta et al., 2013), suggests the opportunity to revise its systematic status and to eventually include it in the genus *Gobius.*

The Ponto-Caspian gobies (Benthophilines) were here represented by two genera, *Ponticola* and *Padogobius.* All the reconstruction proposed in this study converged in the definition of the polyphyletic origin of the genus *Padogobius,* as it was in Penzo et al. (1998). Nevertheless, species tree topology differs from ML and BI reconstructions in placing *Padogobius* + *Ponticola* clade outside the *Gobius* clade, as sister group. Previous study of Ponto-Caspian gobies relationships, including the genus *Ponticola*, (but not *Padogobius*) has demonstrated that the tribe is monophyletic (Thacker & Roje, 2011) and sister group of *Gobius*. Thus, given the close affinity between *Ponticola kessleri* and *Padogobius nigricans*, the latter can be place within Benthophilines. Analysis support that the split between *Gobius* and Benthophilines occurred in a distinct event compared to *Padogobius bonelli*. Comparison between 95% highest posterior density (HPD) intervals for the supported nodes (Tab.3) reveal no overlap, enforcing the independent origin of the freshwater habit.

In the species tree reconstruction, there are correlation between subclusters within *Gobius* lineage and ecological constrains. These evidences are strongly supported by ancestral state reconstruction. The split between Benthophilines and *Gobius* occurred 6,09 Ma from a common marine ancestor, probably

inhabiting shallow waters before the incoming of the Messinian Salinity Crisis. This age is in accordance with the first evidence of extreme conditions at sea surface observed at around 6.3 Ma (Sierro et al., 1999), when planktonic foraminifera began to disappear. From that moment the basin experienced a constant increasing in salinity and suboxic or completely anoxic waters hampered the survival of benthic organisms (Manzi et al., 2007; Dela Pierre et al., 2012). Such conditions might have forced marine organisms to adapt to a freshwater rifugia, as in the case of Benthophilines ancestor. *Padogobius nigricans* and *Ponticola kessleri* share different geographic distribution, with consistent geological boundaries in between. Towards the final stages of the Messinian Salinity Crisis much of the Mediterranean basin contained water of reduced salinity (the Lago- Mare phase, Briggs, 1995). Thus, this landscape may have provided the rout through which the freshwater ancestor of Benthophilines reached both Ponto-Caspian and Western Italian freshwater systems. After the refilling of the basin by Atlantic waters, the different geographical populations have underwent to different evolutionary history. The role of the "Lago - Mare" phase as a dispersal vector for freshwater species during the Messinian has been also confirmed by several other studies on fishes (Almada et al., 2009; Huyse et al., 2004).

The present distribution of *P. nigricans* (Fig.2b) may be explained by the ancient river connections occurred after the colonization of the region (Bianco & Miller, 1990; Miller, 1990): the paleohistory of the Arno basin (Tirrenic side of Central Italy) revealed several connections and disconnections between the Arno river and the Tiber over the last 4 million years (Bartolini & Pranzini, 1981). Since those two rivers represent the major freshwater system of Central Italy, with no connections with any other freshwater network, the modern distribution of *P. nigricans* is the result of geological and hydrological boundaries of this region.

The time calibrated reconstruction proposes an earlier root for *Padogobius bonelli* freshwater conquer (95% HPD = 7,74-10,66 Ma), in correlation with the Tortonian. During this time span, a weak cooling

trend is recorded worldwide, leading to an increase in seasonality in the circum-Alpine area (Bruch et al., 2007). The retreat of the Paratethys also reduced influence in the Pannonian domain and probably also in the eastern portion of the Po-Plain basin, with a consequent decrease in temperatures and increase in seasonality (Fauquette et al., 2006; Bruch et al., 2007). Corroboration of this climatological scenario comes from herpetological fossil assemblages, suggesting a significant increase of rainfall (Böhme et al., 2008). The uplift of the Alps and the Carpathians, provided increasing amount of clastic sediments within North Adriatic basin; the rivers dumping the sediment load into the estuarine areas may have promote the origin of wide flat alluvial plains, as it has been proved for the Paratethyan realms (Juhasz, 1991). Paleontological analysis of the fossil faunas collected in the Venetian-Friulian deposit are characterised by mixing of brackish and truly marine species. The stable isotope values obtained from ostracod valves from the Venetian-Friulian Basin indicate that valves were moulted in an non marine water body or, more likely, in a marginal marine environment where mixing with seawater took place (Ligios et al., 2012). Whereas on a smaller geographical scale, this scenario closely resembles the condition in which the Benthophilines approach the freshwater lifestyle. The coexistence of marine and brackish-freshwater interfaces correlated with a dynamism in coastal shape due to massive sediment dumping might have promote the formation of wide areas of transitional waters which may have driven the ecological transition.

Ancestral state reconstruction of ecological constrains, such as substrate and depth range demonstrate that most of the species involved in this study share ancestral attribute, such as preference for shallow water rocky bottom. The adaptation to sandy or wider depth range condition can be interpreted as a recent conquer, shared by monophyletic groups.

Conclusion

In this dissertation I inferred a more comprehensive analysis of species-level relationships of the major representatives of Mediterranean gobies, linking evolutionary marine/freshwater ecological transitions to the paleohistory of the Mediterranean basin. I implemented a time calibrated species tree inference with ancestral state reconstruction encompassing the search for a sister group of *Padogobius* to test the monophyly of the freshwater habit. All the reconstructions proposed in this study converge in the definition of the polyphyletic origin of the genus *Padogobius.* The Results provide strong support for a multiple independent origin of the freshwater habit in the two endemic Italian species *P. bonelli* and *P. nigricans* . Moreover, the results from species tree inference propose new insight in the relationships among members of Mediterranean *Gobius* lineage previously not considered. Molecular dating of the radiation of *Gobius* species and Benthophilines, its freshwater sister clade, is consistent with the oceanographic and tectonic history of the basin. Specifically:

j) *P. nigricans* is placed within Benthophilines, sister species of *P. kessleri*. The split between Benthophilines and *Gobius* occurred 6,09 Ma from a common marine ancestor, probably inhabiting shallow waters before the incoming of the Messinian Salinity Crisis, which might have forced the adaptation to freshwater rifugia.

jj)P. bonelli is rooted in a basal position compared to the Benthophilines, in correlation with the Tortonian (9,14Ma). Whereas the lack of fish fossil data prevents the chance to fix a much more detailed reconstruction, adaptation to the freshwater lifestyle might have been forced by similar constrains as to the ones occurred in the end of the Messinian Salinity Crisis.

jjj) *Gobius* is presented as a monophyletic radiation of marine fishes originated after the ingression of Atlantic waters in the end of the Messinian Salinity Crisis from an Atlantic ancestor. The adaptation to

new and free ecological niches lead to a rapid radiation resulting in the present diversity of *Gobius* species.

Giving the aforementioned results, Mediterranean gobies can be advocate as a model group in evolutionary studies on adaptive radiation. They underwent to wide ecological transitions imposed by the paleohistory of the basin, resulting in a high rate of endemism and niche partitioning. Further research will deepen the process of ecological radiation within the Mediterranean basin using Gobius species as candidate model organism.

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Supplementary material

List of tables

Gene	Primer pair	Pcr programme	Prod. size	Reference
Mitochondrial **16S rDNA**	**F**- CGCCTGTTTATCAAAAACAT **R-**CCGGTCTGAACTCAGATCACGT	94 C°/ 2'; [94C°/45sec; 51C°/60 sec ; 72C°/90 sec] x 35 cycles; 72C°/10'	508 bp	Palumbi *et al*, 1991
Mitochondrial **12S rDNA**	**F**- TGCATAACTGATNTCATGAGC **R-**TTCTAGAACAGGCTCCTCTAGGGGGG	94 C°/ 5'; [94C°/30sec; 57C°/45 sec ; 72C°/60 sec] x 40 cycles; 72C°/10'	541 bp	Present study
Mitochondrial **d-D-loop**	**F**- CCGGCCGCCGGAGGTTA **R-**YYACGAGTTTAWGGGGGGTAG	94 C°/ 5'; [94C°/30sec; 57C°/45 sec ; 72C°/60 sec] x 40 cycles; 72C°/10'	1066 bp	Present study
Nuclear **RAG1**	**F**-GGGACAGGYTAYGAYGARAAGATGGT **R-**ATYTCATCYTGRAAGATTTTGTARAACTC	94 C°/ 5'; [94C°/45sec; 51.5C°/45 sec ; 72C°/120 sec] x 40 cycles; 72C°/10'	725 bp	Present study
Nuclear **RSP7**	**F**-TGCATAACTGATNTCATGAGC **R-**GACTCATTCCCCTCGCTGGC	94 C°/ 5'; [94C°/60sec; 60C°/45 sec ; 72C°/120 sec] x 40 cycles; 72C°/10'	581 bp	Present study

Tab2: Primers and PCR protocols used for the amplification of the molecular markers used in this study.

Split/Clade	Age (Ma)	Height 95%HPD
Gobius / *Zoosterisessor*	1.03	0.32 / 1.71
Gobius	4.95	4.3 / 6.02
Gobius / Benthophilines	6.09	5.14 / 7.1
Ponticola / *Padogobius*	4.61	2.78 / 5.99
Padogobius bonelli	9.14	7.74 / 10.66
Zebrus / *Chromogobius*	10.19	7.52 / 12.64
Gobius lineage / *Acanthogobius* l.	20.09	17.34 / 22.43
Pomatoschistus lineage	23.17	22.77 / 23.61
Gobiidae / Butidae	35.42	33.19 / 37.51

Tab.3: Age, in million years (Ma), and node 95% highest posterior density (HPD) intervals for the major supported nodes in the time-calibrated multispecies coalescent phylogeny (Fig. 7).

SPECIES	Habitat type	Depth range	Prefer. substrate	Sound emissions
Bostrychus sinensis(*)	*marine*	*inshore*	*sandy*	/
Chr. quadrivittatus	*marine*	*inshore*	*rocky*	/
Eleotris acanthopoma(*)	*marine*	*inshore*	*sandy*	/
Gillichthys seta	*marine*	*inshore*	*sandy*	/
Gobius ateriformis	*marine*	*inshore*	*rocky*	/
Gobius auratus	*marine*	*offshore*	*rocky*	/
Gobius bucchichi	*marine*	*inshore*	*sandy*	/
Gobius cobitis	*marine*	*inshore*	*rocky*	*complex*
Gobius cruentatus	*marine*	*offshore*	*rocky*	*complex*
Gobius gasteveni	*marine*	*offshore*	*sandy*	/
Gobius geniporus	*marine*	*inshore*	*rocky*	/
Gobius niger	*marine*	*offshore*	*sandy*	*pulsatile*
Gobius paganellus	*marine*	*inshore*	*rocky*	*tonal*
Gobius vittatus	*marine*	*offshore*	*rocky*	/
Gobius xanthocephalus	*marine*	*offshore*	*rocky*	/
Gymn. petschiliensis	*freshwater*	*inshore*	*sandy*	/
Knipow. punctatissima	*freshwater*	*inshore*	*rocky*	*pulsatile*
Mogurnda adspersa	*freshwater*	*inshore*	*sandy*	/
Padogobius bonelli	*freshwater*	*inshore*	*rocky*	*complex*
Padogobius nigricans	*freshwater*	*inshore*	*rocky*	*tonal*
Percottus glenii	*freshwater*	*inshore*	*sandy*	/
Ponticola kessleri	*freshwater*	*inshore*	*rocky*	/
Zebrus zebrus	*marine*	*inshore*	*rocky*	/
Zosterisessor ophiocephalus	*marine*	*inshore*	*sandy*	*pulsatile*

Tab.4: Ecological characters of species considered in this work. Marine species were considered as follow: inshore, depth range 0-50 m; offshore, depth range 50-200 m; euryhaline species (*) categorized as marine, as they are rarely or never found in entirely freshwater habitats; preferred substrate, according to bibliographical references; sounds emission during mating ritual according to Hrvatich et al, 2015. Retrieved from: Marine Species Identification Portal (http://species-identification.org; accessed on 05/06/2015) and Fish Base (http://www.fishbase.org; accessed on 05/06/2015).

Node number	Marine /Fresh. Likelihood (Fig.8)	Inshore/Offshore Likelihood (Fig.9)	Rocky/Sandy Likelihood (Fig.10)	Sound emission (y/n) Likelihood (Fig.11)
1	1/0	0.93/0.07	0.98/0.02	0.5/0.5
2	1/0	0.96/0.04	0.98/0.02	0.54/0.46
3	1/0	0.96/0.04	0.98/0.02	0.73/0.27
4	1/0	0.82/0.18	0.96/0.04	0.5/0.5
5	1/0	0.08/0.92	0.09/0.91	0.5/0.5
6	1/0	0.84/0.16	0.85/0.14	0.4/0.6
7	1/0	0.05/0.95	0.98/0.02	0.3/0.7
8	1/0	0/1	1/0	0/1
9	1/0	0.63/0.37	0.85/0.15	0.4/0.6
10	1/0	0.97/0.03	0.07/0.93	0.5/0.5
11	1/0	0.84/0.16	0.97/0.03	0.5/0.5
12 *	0.70/0.30	0.91/0.09	0.97/0.03	0.5/0.5
13	0.19/0.81	0.93/0.07	0.97/0.03	0.5/0.5
14 *	0.63/0.37	0.92/0.08	0.96/0.04	0.5/0.5
15	0.67/.033	0.92/0.08	0.91/0.09	0.5/0.5
16	0.73/0.27	0.92/0.08	0.93/0.07	0.5/0.5
17	0.53/0.47	0.92/0.08	0.55/0.45	0.5/0.5
18	0.50/.050	0.92/0.08	0.07/0.93	0.5/0.5
19	0.50/0.50	0.91/0.09	0.5/0.5	0.5/0.5
20	0.48/0.52	0.50/0.50	0.50/0.50	0.5/0.5
21	0.48/0.52	0.91/0.09	0.50/0.50	0.5/0.5
22	0.48//0.52	0.92/0.08	0.04/0.96	0.5/0.5

Tab.5: Maximum likelihood scores of ancestral state reconstruction inferred on the multispecies coalescent phylogeny using Mesquite v.2.6 (Maddison and Maddison 2011). Probability of each state at each node calculated on the average frequencies of a state across all of the trees possessing that node.

Supplementary material

List of figures

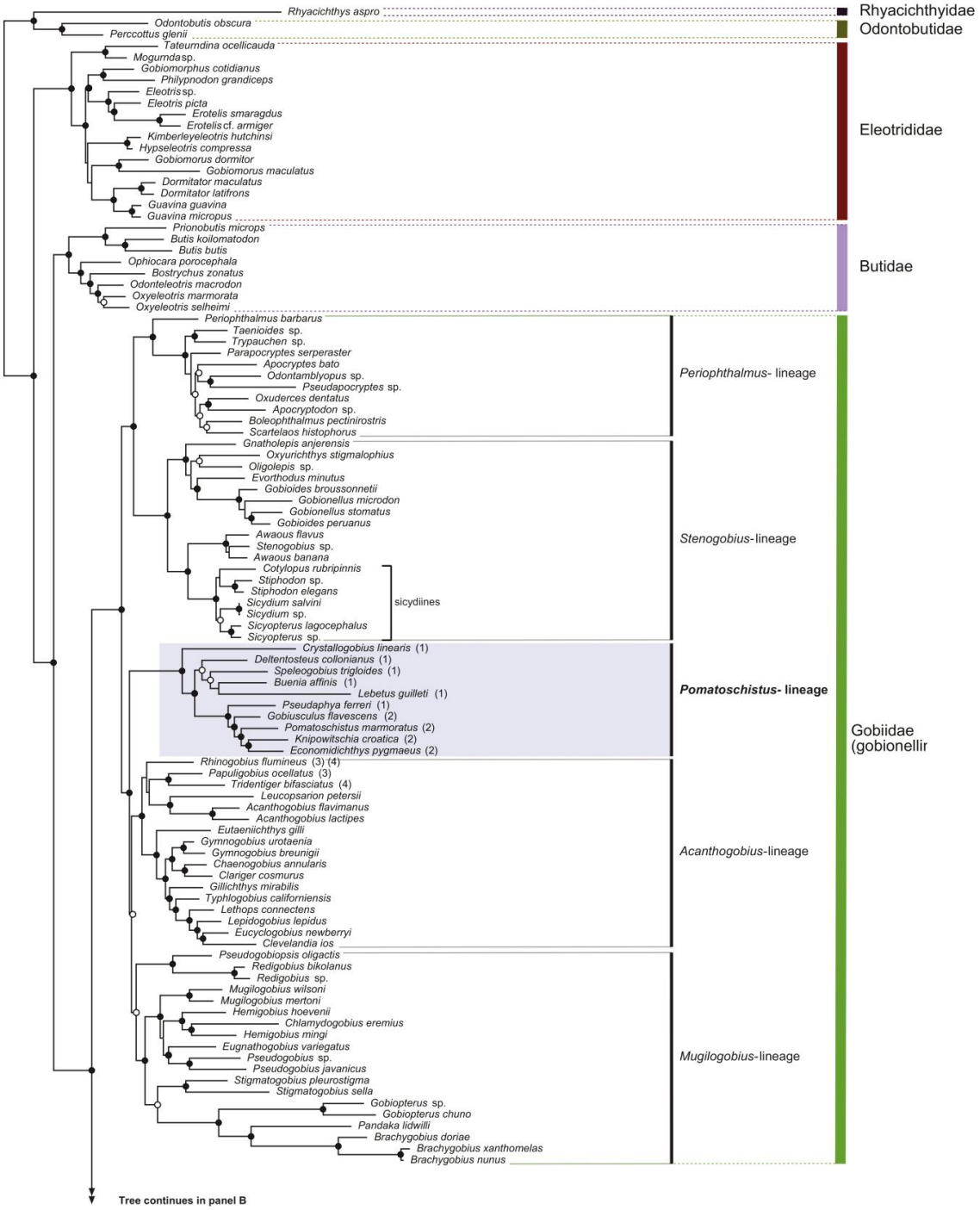

Fig.1a: From Agorreta et al., 2013. Phylogenetic relationships within *Pomatoschistus* lineage based on the main dataset comprising of two mt (rRNA, cytb,) and three nuclear (rag1, zic1, and sreb2) markers. Filled bullets on nodes denote support for both BI (PP P0.95) and ML (BP P70%). Open bullets on nodes denote support for BI(PP P 0.95)but not for ML (BP < 70%). Absence of bullet on a node denotes no support for either BI (PP < 0.95) or ML (BP < 70%). Families and supra-generic groups are indicated on the right. Scale bar indicates substitutions per site site.

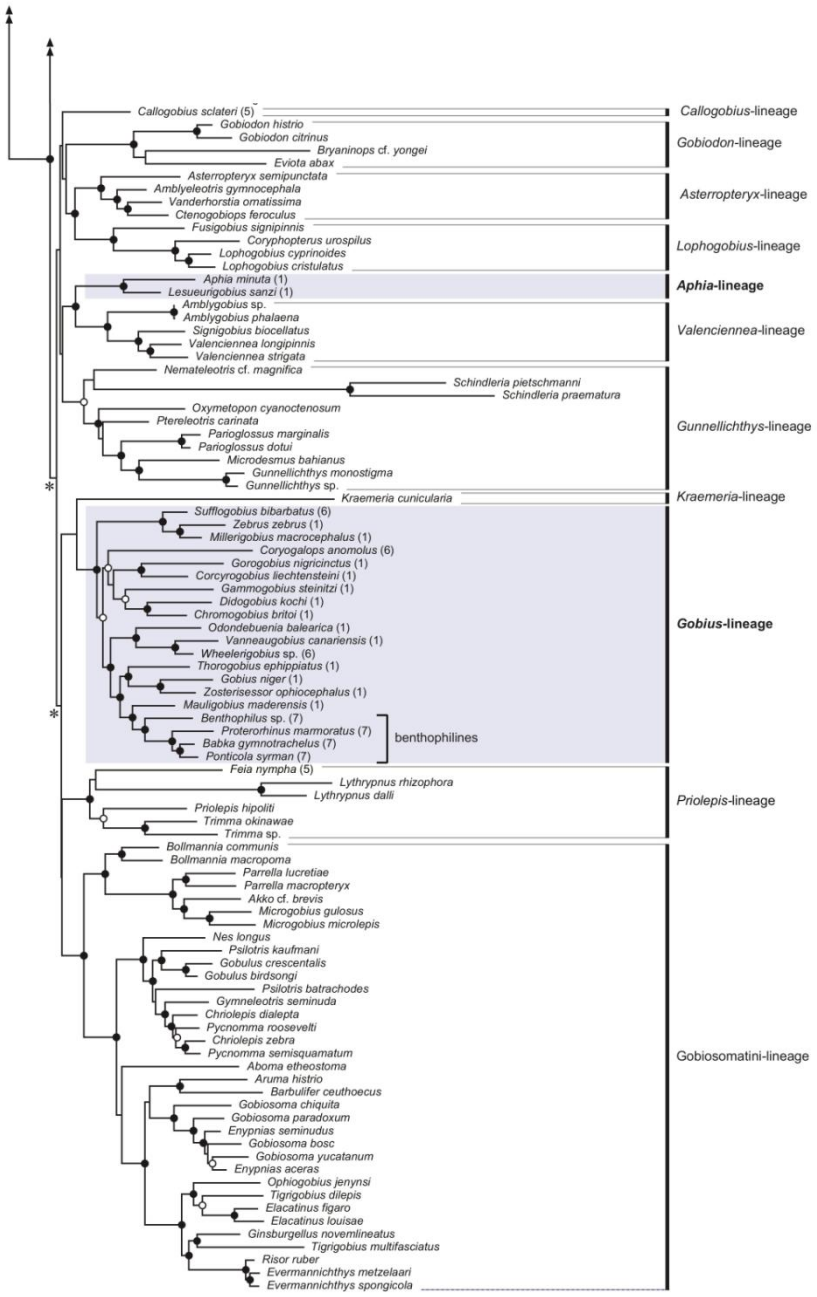

Fig1b: From Agorreta et al., 2013. Phylogenetic relationships within *Gobius* lineage based on the main dataset comprising of two mt (rRNA, cytb,) and three nuclear (rag1, zic1, and sreb2) markers. Filled bullets on nodes denote support for both BI (PP P0.95) and ML (BP P70%). Open bullets on nodes denote support for BI(PP P 0.95)but not for ML (BP < 70%). Absence of bullet on a node denotes no support for either BI (PP < 0.95) or ML (BP < 70%). Families and supra-generic groups are indicated on the right. Scale bar indicates substitutions per site.Numbers in parenthesis next to some species names denote belonging to groups recovered in previous studies: (1) Mediterranean and North-East Atlantic gobies in Thacker and Roje (2011), (6)African gobies (South-East Atlantic and Western Indian Ocean) in Thacker and Roje (2011), and (7) Ponto-Caspian gobies in Thacker and Roje (2011) and Benthophilinae in Neilson and Stepien (2009).

Fig.2a: geographical distribution of *Padogobius bonelli*

Fig.2b: geographical distribution of *Padogobius nigricans*

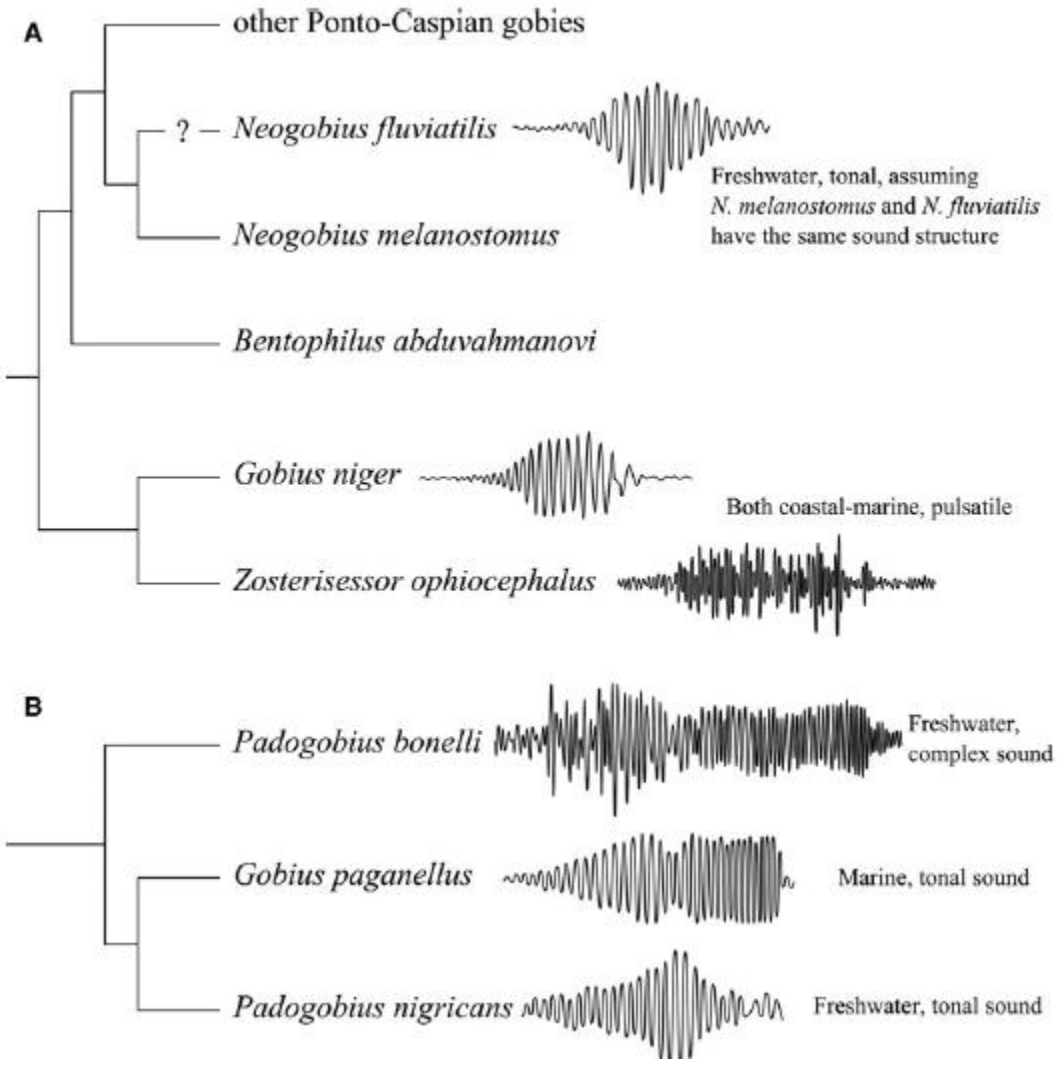

Fig.4: Two clusters extracted and adapted from Thacker & Roje (2011) (A) and Huyse et al. (2004) (B) showing the relationships between certain soniferous gobies, and the wave forms of their typical sounds.

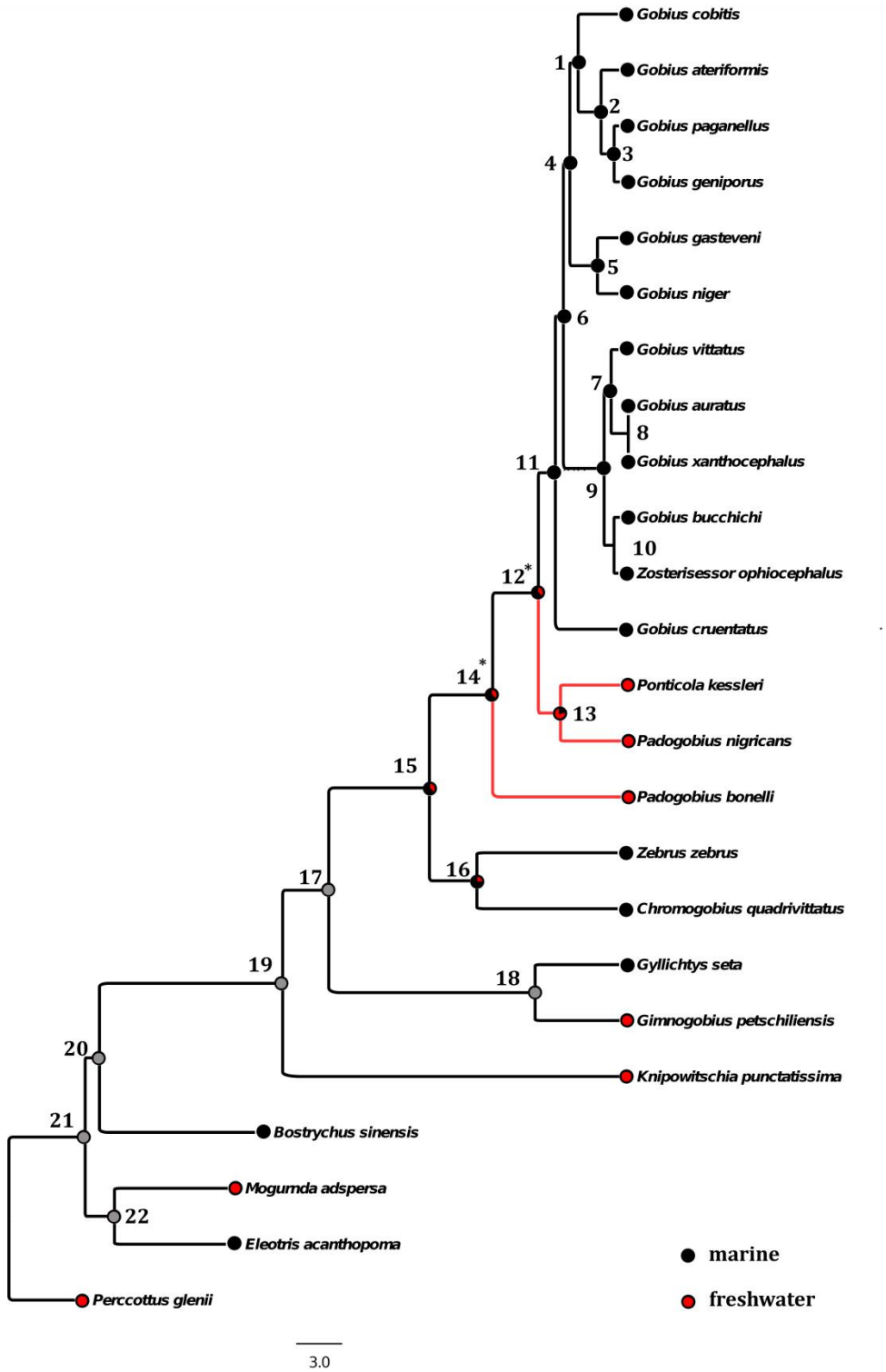

Fig.9: *BEAST consensus tree with marine (red pie) / freshwater (black pie) habitat evolution reconstructed with Mesquite v.2.6 (Maddison and Maddison 2011). Pie diagrams indicate the relative degree of support for alternative character states. Grey pie diagram representing equivocal node.

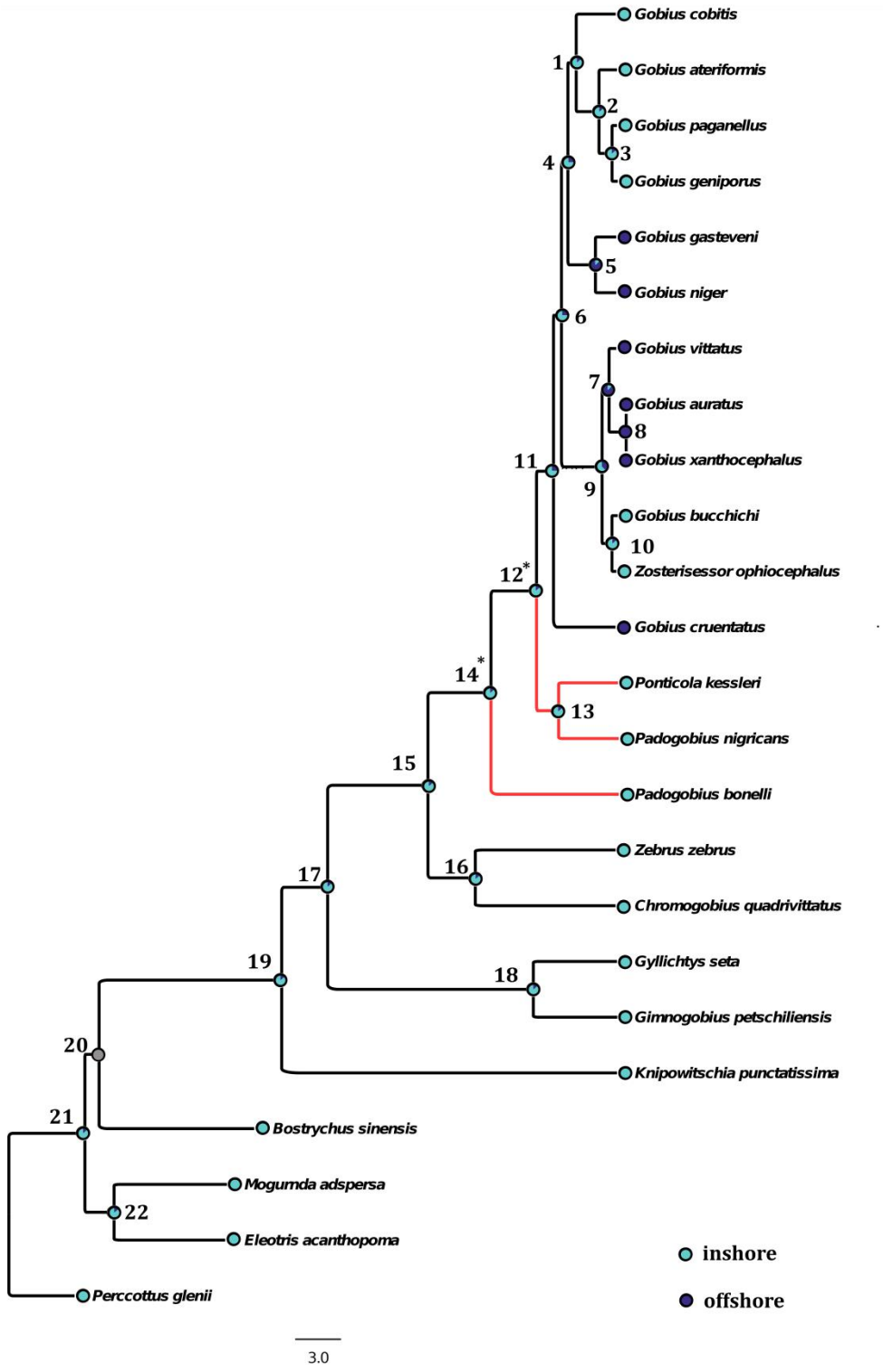

Fig.10: *BEAST consensus tree with inshore (light blue pie) / offshore (dark blue pie) habitat evolution reconstructed with Mesquite v.2.6 (Maddison and Maddison 2011). Pie diagrams indicate the relative degree of support for alternative character states. Grey pie diagram representing equivocal node.

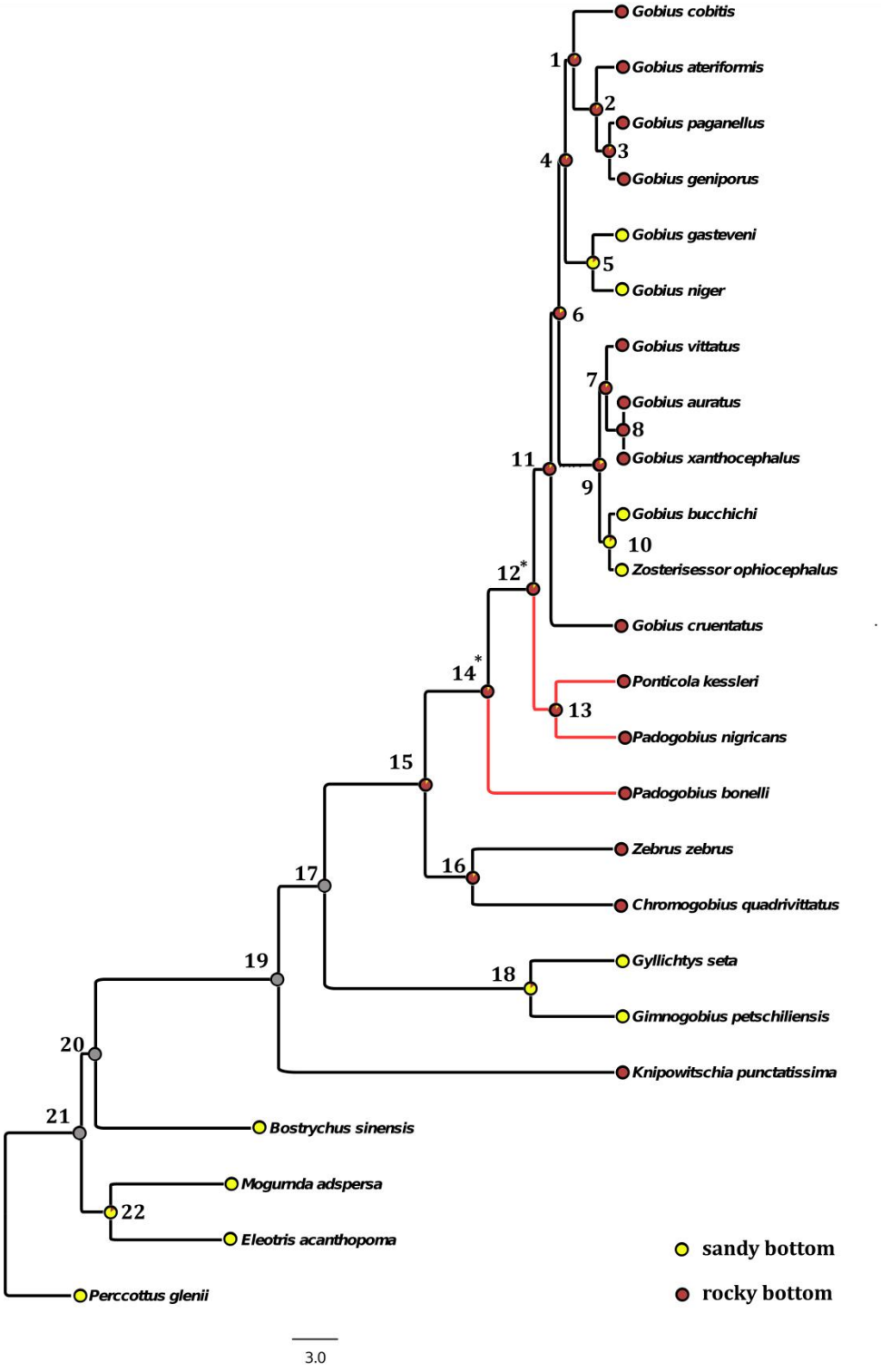

Fig.11: *BEAST consensus tree with rocky (brown pie) / sandy (yellow pie) habitat evolution reconstructed with Mesquite v.2.6 (Maddison and Maddison 2011). Pie diagrams indicate the relative degree of support for alternative character states. Grey pie diagram representing equivocal node.

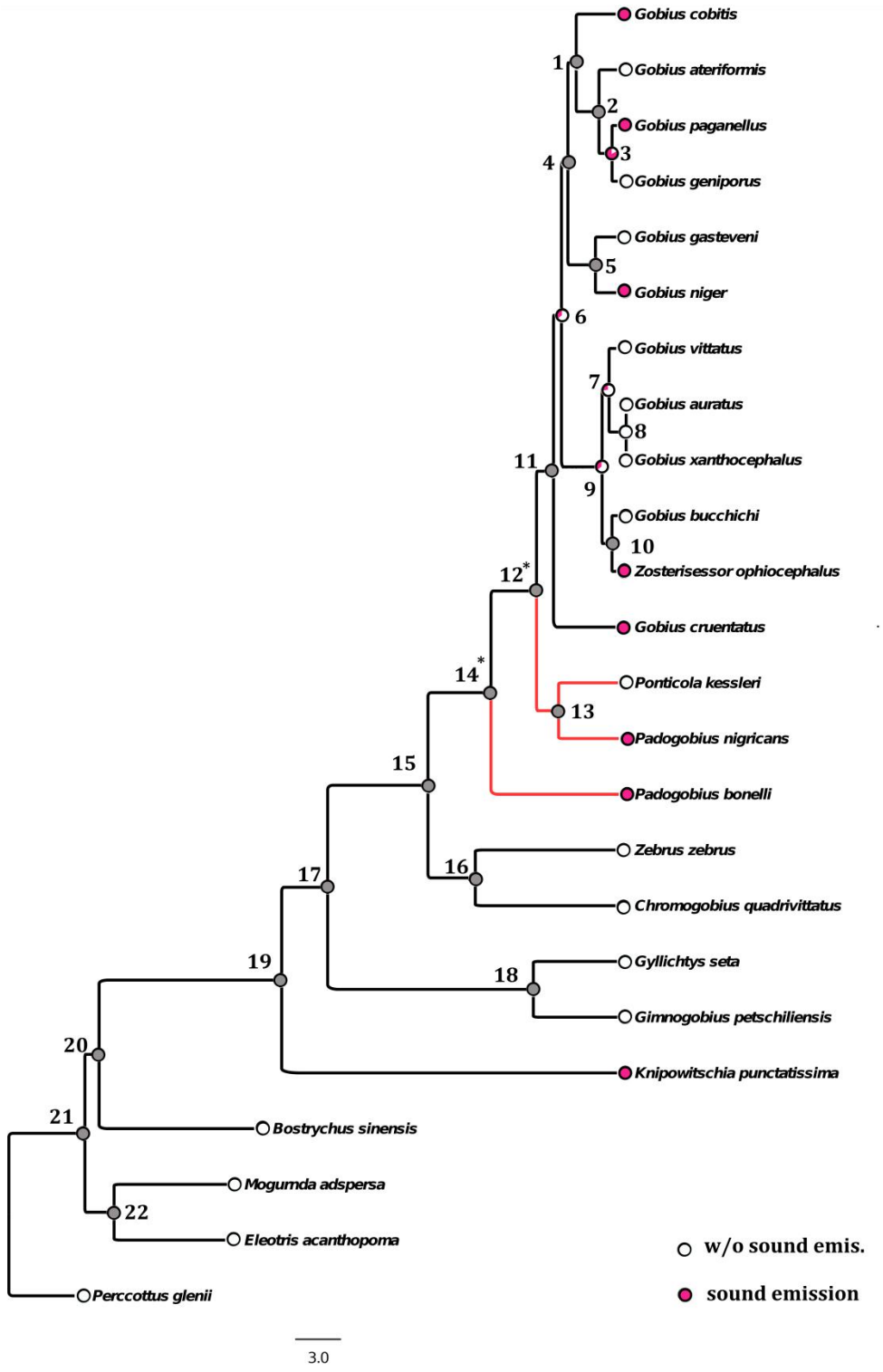

Fig.12: *BEAST consensus tree with sound emission (red pie) / w/o sound emission (white pie) character evolution reconstructed with Mesquite v.2.6 (Maddison and Maddison 2011). Pie diagrams indicate the relative degree of support for alternative character states. Grey pie diagram representing equivocal node.