

The phylogeny of a reduced ‘sand goby’ group based on behavioural and life history characters

STEFANO MALAVASI^{1*}, CHRISTOS GKENAS², IOANNIS LEONARDOS²,
PATRIZIA TORRICELLI¹ and DEBORAH A. MCLENNAN³

¹*Department of Environmental Sciences, Informatics and Statistics, Castello 2737/b Università Ca’ Foscari Venezia, 30122 Venice, Italy*

²*Department of Biological Application and Technology, University of Ioannina, PC 45110, Ioannina, Greece*

³*Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks St. Toronto, ON, Canada M5S 3B2*

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Phylogenetic analysis of 27 behavioural and life history traits for five Mediterranean sand goby species (Perciformes, Gobiidae) produced one tree with a consistency index (excluding uninformative characters) of 0.756. This tree agreed with previous molecular analyses in providing strong support for the monophyly of the sand gobies, indicating that *Pomatoschistus* and *Knipowitschia* are paraphyletic and helping to resolve the ambiguous position of *Economidichthys pygmaeus*, placing it as the basal member of the reduced data set. Although the tree was completely resolved, the branches above *E. pygmaeus* were only moderately supported in the bootstrap analysis. Overall, the behavioural data provide information that may eventually help clarify the speciation bursts within the Mediterranean sand goby clade as much as is possible.

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INTRODUCTION

The family Gobiidae is a perciform taxon represented by at least 1950 described species (Nelson, 2006) that have radiated into marine, brackish and inland waters of tropical and temperate regions (Gandolfi *et al.*, 1991). Within this large group, approximately 60 species inhabit the Mediterranean basin and surrounding regions. A recent molecular analysis (Thacker & Roje, 2011) indicated that the majority of these species form a monophyletic group in which an eastern Atlantic/Mediterranean lineage, represented by *Gobius* and *Zosterisessor*, is the sister group to gobies found in the Black, Caspian, Azov and Aral Seas/drainages (the Ponto-Caspian clade; e.g.

Mesogobius, *Proterorhinus*, and *Ponticola*). Interestingly however, one eastern Mediterranean species, the sand goby, *Pomatoschistus minutus*, clustered well outside this group, appearing as the basal member of the Gobiidae. The sand goby was the only representative in Thacker & Roje’s study (2011) of a larger group, the sand gobies (*Pomatoschistus*, *Gobiusculus*, *Knipowitschia*, *Economidichthys*: sensu Huyse, Van Houdt & Volckaert, 2004), whose relationships have plagued ichthyologists for several decades. The monophyly of the sand gobies, established based on a unique pattern of infraorbital neuromast organs (McKay & Miller, 1997), has been supported by molecular data (Penzo *et al.*, 1998; Huyse *et al.*, 2004; Neilson & Stepien, 2009), but neither their placement within Gobiodei (McKay & Miller, 1997; Neilson & Stepien, 2009; Thacker & Roje, 2011) nor the

*Corresponding author. E-mail: mala@unive.it

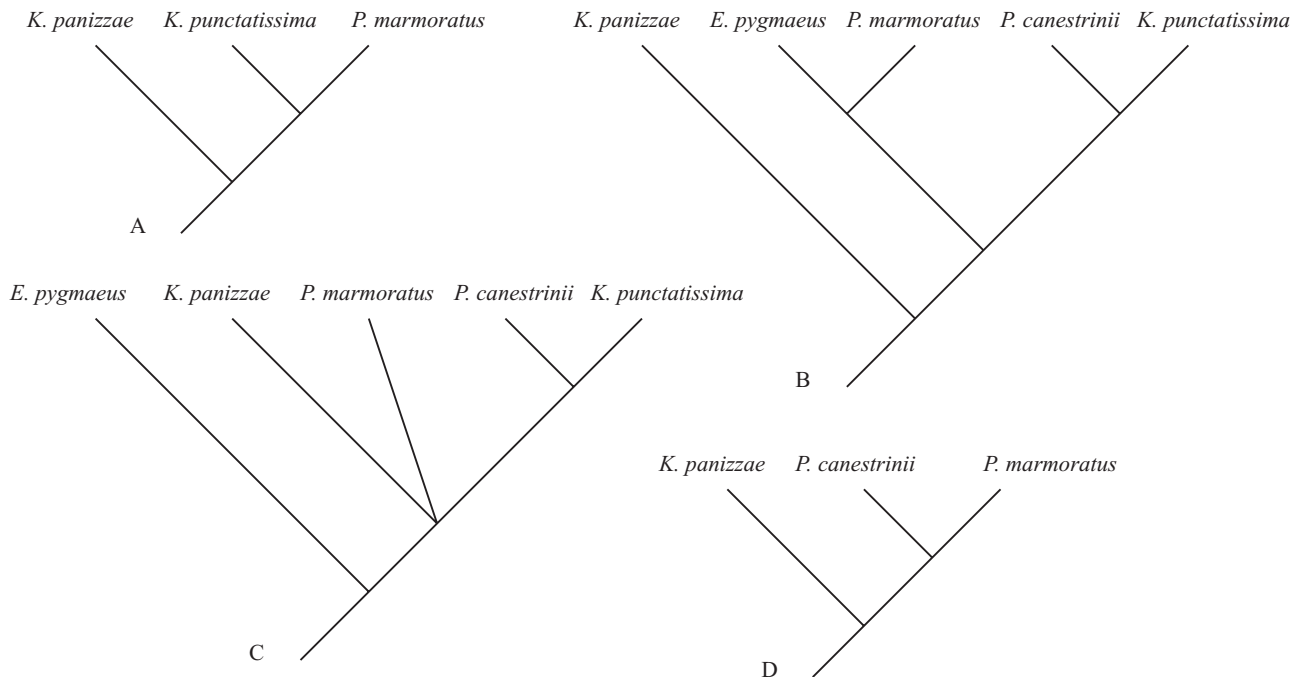


Figure 1. Previous phylogenetic hypotheses of sand goby relationships redrawn to highlight only the species used in this study. A, 867 bp from 16S/12S rRNA (Penzo *et al.*, 1998); B, 800 bp from 16S/12S mtDNA (Huyse *et al.*, 2004); C, 815 bp from 16S/12S rDNA (Vanhove *et al.*, 2011); D, presence/absence of allozymes, consensus of 12 equally parsimonious trees (McKay & Miller, 1997).

relationships amongst members of the sand goby clade (McKay & Miller, 1997; Penzo *et al.*, 1998; Huyse *et al.*, 2004; Vanhove *et al.* 2012: Fig. 1) have achieved a consensus amongst researchers.

Huyse *et al.* (2004) suggested that the sand gobies might have speciated very rapidly during the phase during and immediately after the Messinian salinity crisis (MSC) at the end of the Holocene. Such rapid bursts of speciation often make it difficult to reconstruct phylogenetic relationships. Indeed, bootstrap values on the Huyse *et al.* (2004) molecular tree were low, with several branches not significantly different from zero. Polytomies are a fact of life when dealing with rapid episodes of microvicariance or peripheral isolates speciation (see e.g. Walsh *et al.*, 1999; Avise, 2000). Polytomies can also arise from data-related issues, such as a high amount of homoplasy or an insufficient number of characters given the number of taxa being examined (e.g. Maddison, Donoghue & Maddison, 1984). One way to differentiate between explanations based on speciation rates and explanations based on lack of data is to add more data to the analysis. Studies based on allozymes and 16S/12S rDNA sequences have not reached a consensus about phylogenetic relationships within the sand gobies. If this lack of resolution is simply caused by too few

characters in the analysis, then adding an additional source of data to the system should help solve the problem. In this paper we discuss the potential for such additional information to come from behavioural characters.

Biologists have traditionally been concerned about the use of behavioural traits in reconstructing genealogical relationships. This concern is based upon the assumption that behaviour is too responsive to fluctuations in environmental and social variables, and hence too 'plastic' to be informative (for an extensive discussion see McLennan, Brooks & McPhail, 1988; Brooks & McLennan, 1991, 2002). Over the past three decades these assumptions have been tested by reconstructing phylogenies based upon behaviour then comparing those patterns with trees based upon morphological and/or molecular data sets. The result of these studies is clear, behavioural traits are useful in phylogenetic reconstruction; in other words, behaviour is not more homoplasious than other types of data (e.g. de Queiroz & Wimberger, 1993; Cap *et al.*, 2008). Unfortunately, despite substantial progress in technologies for recording behavioural characters and increased refinement in the way in which those characters are described for systematic analysis (e.g. Stuart, Hunter & Currie, 2002; Robillard *et al.*, 2006),

studies incorporating behavioural traits are still relatively rare. The reasons for this slow progress are twofold: persistent, and as discussed above, incorrect, assumptions about the homoplasious nature of behavioural traits and, more importantly, the fact that it is time consuming to record such traits.

The record to date does show that investing in behavioural data is worthwhile for systematists. For example, phylogenetic analyses based solely on behavioural/life history characters of sticklebacks (Gasterosteidae: McLennan *et al.*, 1988; McLennan, 1993; Mattern & McLennan, 2004) and Pacific coast salmon/trout (Salmonidae: Esteve & McLennan, 2007) produced trees that were highly congruent with analyses using morphological and/or molecular characters. We were therefore curious about whether behavioural/life history traits could be used as an additional source of data to help resolve relationships within the sand gobies. The social systems of most gobiid fishes are resource-based and characterized by male parental care. Males build nests under various kinds of shelters, using vibrant courtship displays to attract females to the nest. Eggs are released on the ceiling of the shelter by means of adhesive structures, fertilized by sperm trails, and then guarded by the male until hatching. Many species produce sounds during both aggressive and reproductive interactions (Myrberg & Lugli, 2006; Malavasi, Collatuzzo & Torricelli, 2008). Depending upon the species, males vocalize to females during courtship, prespawning (in the nest, while females are inspecting prospective nests), and/or during spawning (Lugli *et al.*, 1997; Myrberg & Lugli, 2006). Although the behaviour and behavioural ecology of gobies have been the object of numerous studies (Magnhagen & Kvarnemo, 1989; Lindström, 1992; Magnhagen, 1992, 1998; Kvarnemo, Forsgren & Magnhagen, 1995; Lindström & Lugli, 2000; Svensson & Kvarnemo, 2003; Forsgren *et al.*, 2004; Lindström, Mary & Pampoulie, 2006), to date there has been no attempt to use these traits as systematic characters.

In this study we used behavioural/life history characters to reconstruct the phylogenetic relationships amongst five sand goby species, with two species from the clade including larger Mediterranean gobies (belonging to the genera *Gobius*, *Padogobius*, and *Zosterisessor*) as outgroups. We asked: (1) do behavioural traits produce a well-supported phylogeny for this reduced sand goby group? and (2) if so, how does the tree compare with previous studies based on molecular and allozyme data? In essence, we are interested in discovering whether behavioural and life history traits are phylogenetically informative enough to help disentangle the relationships within this enigmatic group of fishes.

MATERIAL AND METHODS

THE STUDY GROUP

Pomatoschistus marmoratus (Risso) is widespread in the eastern Atlantic, Mediterranean, Black and Azov seas and the Suez Canal (Miller, 1986; Whitehead *et al.*, 1986), and is one of the most abundant gobiid species in estuaries, coastal lagoons and freshwater habitats connected to the sea (Malavasi *et al.*, 2005). Populations are subdivided into two main areas in eastern and western sections of the Mediterranean (Mejri *et al.*, 2011). *Pomatoschistus canestrinii* (Ninni) is endemic to the lagoons and estuaries of the northern Adriatic Sea (Miller, 1986) from Monfalcone to the Po River Delta (Gandolfi, Torricelli & Cau, 1982), and the Jadro estuary, Dalmazia (Kolombatovich, 1891). Its occurrence in the Ionian Sea (Gulf of Taranto) and in Lake Trasimeno is probably the result of recent introductions (Gandolfi *et al.*, 1982; Freyhof, 1998). It lives in the shallow waters of the lagoons and river-mouths in poorly vegetated salt marshes and creeks, selecting salinities ranging from 2 to 20 P.S.U. (Practical salinity Units) and never exceeding 30 P.S.U. (Franco *et al.*, 2005). *Knipowitschia panizae* (Verga) is a euryhaline species that has been collected from lagoons and estuaries of the northern and eastern Adriatic (the Istrian Peninsula), southern Italy and the Ionian Sea, usually at a depth below 2 m (Ahnelt & Bianco, 1990; Miller, 2004; Vanhove *et al.*, 2012). The typical habitat is characterized by stable environmental conditions undisturbed by sea or river currents, on a soft substrate of mud or clay with rich aquatic vegetation and bivalve beds (Gandolfi *et al.*, 1991). The species has also been introduced to freshwater lakes, where it apparently flourishes. *Knipowitschia punctatissima* (Canestrini) inhabits spring waters in which the temperature is constantly low and the bottom substrate is sandy or muddy with rich vegetation; its geographical distribution is restricted to north-eastern Italy. *Economidichthys pygmaeus* (Holly) is an endemic species from the freshwaters of west Greece (Epirus and Lefkas) and regarded as the most distinctive element in the freshwater fish fauna of Greece (Daoulas *et al.*, 1993; Huyse *et al.*, 2004). It is a short-lived species inhabiting both flowing and stagnant shallows with abundant vegetation and detrital substrate (Gkenas *et al.*, 2010). We chose *Padogobius nigricans* (Canestrini) and *Gobius paganellus* (Linnaeus) as outgroups for this study because previous upper level analyses based on mtDNA and nuclear gene sequences (Penzo *et al.*, 1998; Huyse *et al.*, 2004; Giovannotti *et al.*, 2007; Vanhove *et al.*, 2011) unambiguously placed these two species in a large clade distinct from the sand gobies. Species from this large clade, including *Pa. nigricans* and *G. paganellus*, have been used as outgroups in subsequent

studies investigating phylogenetic relationships within sand gobies (Huyse *et al.*, 2004; Larmuseau *et al.*, 2010; Vanhove *et al.*, 2012).

Padogobius nigricans displays a strong preference for stony substrates, but is geographically isolated from the congeneric goby *Padogobius bonelli* (Günther), formerly *Padogobius martensii*, being distributed on the Tyrrhenian side of central Italy (Gandolfi *et al.*, 1991). The rock goby *G. paganellus* is distributed along the Mediterranean and Black Sea and eastern Atlantic from Senegal to Scotland, showing a preference for inshore rocky shallow environments (Miller, 1986). It clusters as the sister species of *Pa. nigricans* within a clade containing the rock-dwelling, freshwater *Pa. bonelli*.

THE DATA SET

The behavioural characters were described for this study based on detailed analysis of videotapes from previous research on bioacoustics and courtship in the relevant goby species (Torricelli, Lugli & Pavan, 1990; Lugli *et al.*, 1997; Lugli & Torricelli, 1999; Malavasi *et al.*, 2003, 2008; Gkenas *et al.*, 2010). Three to ten individuals were observed from each species, corresponding to a total recording time ranging from 720 to more than 2000 min, with an average of 240 min of recording for each individual. In these studies a male who had established a territory around an artificial nest was presented with one or more gravid females and the display/acoustical characters simultaneously recorded by connecting a video camera with a VHS video-recording system to a hydrophone positioned next to the back opening of the nest. Descriptions of acoustic data and the behavioural context of sound production also incorporated information reviewed in Malavasi *et al.* (2008) and Gkenas *et al.* (2010). Life history traits were obtained from the literature (see Miller, 2004). Detailed character descriptions are given in the Appendix.

PHYLOGENETIC ANALYSIS

Characters were assigned a state of 0 or 1 (polarized) using the two outgroups, *Pa. nigricans* and *G. paganellus*. The data matrix (Table 1) was analysed using the software program PAUP* 4.0b4a (Swofford, 1999). Characters that are unique to only one taxon (autapomorphies) were included in the matrix even though they are not phylogenetically informative because they represent evolutionary change and thus may be useful when more taxa are added in future studies (de Queiroz & Wimberger, 1993). As autapomorphies can inflate measures of how well the data fit resultant tree topologies (the consistency index,

Table 1. Data matrix for 27 behavioural and life history characters. See the Appendix for explanations of the characters and character states

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
<i>Economichthys pygmaeus</i>	2	0	0	0	0	0	0	0	1	0	0	0	1	1	1	2	2	2	2	1	1	1	0	1	1	1	3
<i>Pomatoschistus canestrinii</i>	2	2	1	0	1	0	0	2	1	0	1	1	1	1	1	2	0	0	1	1	1	1	1	1	1	1	2
<i>Knipowitschia punctatissima</i>	1	0	0	1	0	1	0	1	1	0	1	0	1	1	1	2	1	1	1	1	1	1	1	1	0	1	1
<i>Knipowitschia panizzae</i>	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3
<i>Pomatoschistus marmoratus</i>	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
<i>Gobius paganellus</i>	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Padogobius nigricans</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CI), we report both the value for the unaltered CI and the value for the 'CI excluding uninformative characters', which calculates the goodness of fit after autapomorphies have been removed. Of the 27 characters, 19 were binary, represented by 0 or 1 in the data matrix, and eight were multistate (0, 1, 2). All characters were run unweighted and unordered, which means that no a priori constraints were placed on the direction of evolutionary change (Fitch, 1971). Both accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN) optimizations (Farris, 1970; Swofford & Maddison, 1987) were used to determine whether different patterns of character evolution affected tree topologies. ACCTRAN and DELTRAN hypotheses of character evolution only differ when there are two or more equally parsimonious ways to represent a homoplasious character transformation series on a tree. We tested the monophyly of the five species ingroup by using the basal polytomy option in PAUP; other rooting options incorporate the assumption of ingroup monophyly. The data were investigated via an exhaustive search, the most robust algorithm for building trees in PAUP, followed by bootstrap analysis, which gives a measure of character support for each node based, in this case, on 1 000 000 random repetitions.

RESULTS AND DISCUSSION

The behavioural/life history data set produced one tree (Fig. 2; CI = 0.766, CI excluding uninformative characters = 0.756, rescaled consistency index = 0.538; f-ratio = 0.616) whether the rooting option was set at a basal polytomy or by designating the ingroup as monophyletic and whether ACCTRAN or DELTRAN was used to optimize characters. This tree is congruent with previous molecular-based analyses (Penzo *et al.*, 1998; Huyse *et al.*, 2004; Vanhove *et al.*, 2012) in several respects. First, it provides additional support for the monophyly of the sand goby clade, as already suggested by morphology, allozymes (McKay & Miller, 1997) and molecules. This support is extremely strong, being based on 11 synapomorphies, although those traits must be tested against a much larger data set including species from within and outside of the putative sand goby group. Second, it postulates that *Pomatoschistus* and *Knipowitschia* are paraphyletic; in this case because *P. marmoratus* clustered within *Knipowitschia*. Given that several authors have also reached this conclusion (McKay & Miller, 1997; Penzo *et al.*, 1998; Huyse *et al.*, 2004; Vanhove *et al.*, 2012), it is clearly time for an extensive investigation of the taxonomy of these groups. Third, the behavioural-based analysis

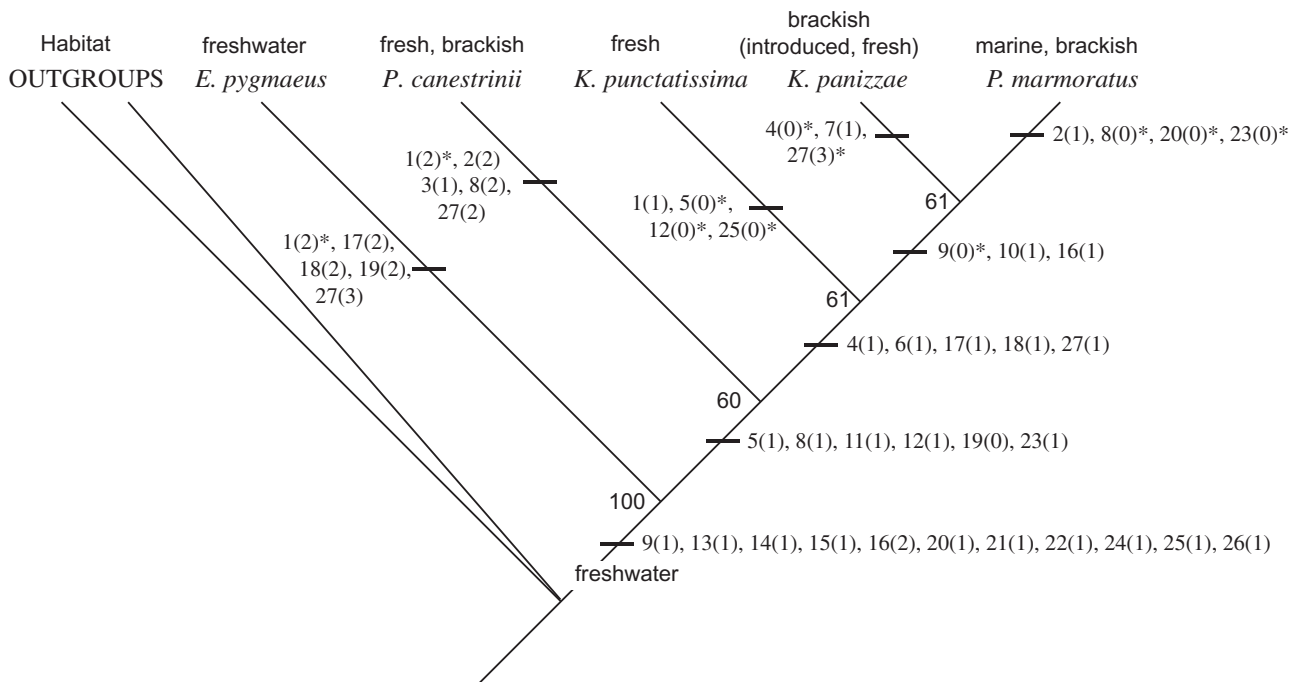


Figure 2. Single phylogenetic tree based on an exhaustive search analysis of 27 behavioural and life history traits in PAUP. *, homoplasious traits. Numbers in parentheses refer to character states. Bootstrap values (1 000 000 iterations) are shown at each node. For character descriptions see the Appendix. Habitat preferences for each species are mapped across the top of the tree, indicating that living in freshwater is the plesiomorphic state for this reduced sand goby group.

provides strong support for the placement of *E. pygmaeus* as the basal member of this five species clade. Previous molecular data were ambiguous with respect to the position of this species (Huyse *et al.*, 2004); however, more recent analyses based on the rhodopsin gene *RH1* (Larmuseau *et al.*, 2010) and 16S/12S rDNA sequences (Vanhove *et al.*, 2012) also placed *E. pygmaeus* as the basal member of a reduced sand goby clade.

Finally, the distribution of habitat types on our reduced tree (Fig. 2) supports Huyse *et al.*'s (2004) conclusion that the adaptive ability to colonize freshwater ecosystems originated only once (in other words, is not homoplasious) in the sand gobies, even though the species inhabiting freshwater are members of different genera. Although the study of palaeoecological and palaeoenvironmental changes during and immediately after the MSC is a matter of controversy (Rouchy & Caruso, 2006), the most recent scenarios depict a triple event, related to subsequent changes in water levels of the Mediterranean basin and Parathethys, with different degrees of connectivity and exchanges between the main water bodies and associated drastic changes in water salinity (Clauzon *et al.*, 2005; Rouchy & Caruso, 2006). The patterns shown in this study and that by Huyse *et al.* (2004) support a scenario in which basal sand gobies originated in isolated brackish and freshwater, with derived species radiating during subsequent phases, characterized by higher sea levels, reflooding and salinity increases. A third phase, corresponding to Pleistocene glaciations and consequent additional fluctuations in sea levels, could have determined further population subdivision and new bursts of speciation (Stefanni & Thorley, 2003; Neilson & Stepien, 2009; Larmuseau *et al.*, 2010).

To summarize then, this study has demonstrated the utility of behavioural and life history traits in the reconstruction of phylogenetic relationships in gobies. Our analysis produced one well-supported phylogenetic tree that was congruent with previous data sets in many ways (e.g. monophyly of the sand gobies, paraphyletic status of *Pomatoschistus* and *Knipowitschia*). The analysis provided a large suite of putative synapomorphies for the sand goby clade, which must be tested further by collecting behavioural data from additional gobiid species. Behavioural traits also supported the basal position of the enigmatic *Economidichthys*, at least in a reduced data set, a placement that has only recently been suggested by both *RH1* and mitochondrial DNA sequences. On their own, the behavioural data produced one tree; however, there are three equally parsimonious trees just one step longer. The 50% majority rule consensus of those three trees produced a similar topology to the most parsimonious tree shown in Figure 2, except

that *P. canestrinii* and *K. punctatissima* collapse into a polytomy. This is interesting because two of the molecular-based trees (Fig. 1B, C) suggest that *P. canestrinii* and *K. punctatissima* should be more closely related than the behavioural traits inferred.

It has been difficult to produce a robust tree for the sand gobies, possibly because, as discussed in the Introduction, they have undergone rapid bursts of speciation. Indeed a consensus of the patterns shown in Figure 1 would produce one large polytomy. The only way to distinguish between soft (data-based problems) and hard (an outcome of speciation processes) polytomies is to accumulate as large a database for the ingroup as possible. Overall, we believe that this study has demonstrated that behavioural traits should be added to the database for the sand gobies. The question should never have been about the utility of a particular type of data, but rather the utility of individual characters; some characters within every data set are more subject to homoplasy, be it via selection, long branch attraction, or genetic introgression, than others (Esteve & McLennan, 2007 and references therein). Kluge (1998) proposed that combining all types of data in one total evidence analysis was the most effective way to construct robust phylogenetic trees. We therefore hope that this study encourages other researchers to continue building the database for such a total evidence study by collecting behavioural, ecological and life history information from the more poorly studied species of sand gobies and their relatives. In conjunction with adding information from nuclear genes to the analysis, this may be the only way to resolve the speciation bursts within the enigmatic Mediterranean sand goby clade as much as is possible.

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APPENDIX

CHARACTER LIST

- Male leading:** the male comes out of the nest, approaches the female and then returns to the reproductive site. A typical courtship behaviour in gobies. The ‘return or lead’ is described as: 0 = straight swim; 1 = zigzag swim and circling; 2 = zigzag swim.
- Male’s body position when leading:** 0 = body and head straight; 1 = arched body and head down; 2 = straight body, head down.
- Male rattling:** the male produces a sound by rapid shaking of his head and his pectoral fins, performed repeatedly during the lead when the male is next to the female and at the end of the lead when he is back in the nest, especially if the female has followed. Within the ingroup, this character is autapomorphic for *Pomatoschistus canestrinii*. 0 = absent; 1 = present.
- Male quivering:** when the female approaches the nest, the male swims into the nest, rests horizontally with his head sticking out and rapidly vibrates his entire body. 0 = absent; 1 = present.

5. *Female vibration*: female vibrates her body when the male approaches her. 0 = present; 1 = absent.
6. *Male prodding*: the male head-butts the female during spawning. 0 = present; 1 = absent.
7. *Female prodding*: the female rapidly and repeatedly nudges the male's body during the lead to the nest. Within the ingroup, this character is autapomorphic for *Knipowitschia panizzae*. 0 = absent; 1 = present.
8. *Male digging*: the male sweeps sand away from the sides of the nest. 0 = absent; 1 = with pectoral fins; 2 = with pectoral fins and mouth.
9. *Male fanning during prespawning and spawning phase*: rhythmical movement of pectoral fins performed to promote water circulation inside the nest. Typically performed in gobies during parental care. 0 = absent; 1 = present.
10. *Upside-down*: the male turns upside down in the nest so that his genital papilla comes in contact with the nest ceiling, during the leading phase and well before the female enters the nest to start egg deposition. 0 = present; 1 = absent.
11. *Female hopping*: female orientates head-up at about 135° and propels herself forward with her pectoral fins in a series of slow jumps displaying the yellow belly coloration towards the nest (Malavasi, Valerio & Torricelli, 2009): 0 = absent; 1 = present.
12. *Habitat preference*: 0 = structured substrate (vegetation, rocks, pebbles); 1 = unstructured (sandy or muddy substrate).
13. *Longevity*: 0 = more than 3 years; 1 = 3 years or fewer.
14. *Age at sexual maturity*: 0 = 1–2 years; 1 = less than 1 year.
15. *Duration of the spawning period*: 0 = short (2–3 months), 1 = long (> 3 months).
16. *Nest materials*: 0 = rocks and pebbles; 1 = shells; 2 = reeds, vegetation, and various randomly selected materials.
17. *Context of sound production*: 0 = during agonistic and courtship interactions; 1 = only during courtship; 2 = not applicable (does not produce sound).
18. *Courtship sounds produced*: 0 = during the leading phase (male and female out of the nest); 1 = only when the female is in the nest; 2 = not applicable (does not produce sound).
19. *Sound type*: 0 = tonal (low pulse rate and long duration); 1 = grunt (train of pulses); 2 = not applicable (does not produce sound).
20. *Female nuptial body coloration*: yellow spot on a female's belly of varying intensity. 0 = absent; 1 = present.
21. *Female mask*: the presence of a black mask around the eyes. 0 = absent; 1 = present.
22. *Partial coloration*: a dark band or a black spot on the female's first dorsal fin (a complete dark coloration was rarely observed). 0 = absent; 1 = partial (spot or band).
23. *Male mask*: male's face becomes completely dark. This differs from a female's mask, which is more intensely black and only around the eyes. 0 = absent; 1 = present.
24. *Band on male's first dorsal fin*: 0 = light; 1 = dark.
25. *Black spot on the male's first dorsal fin*: 0 = absent; 1 = present.
26. *Dark blotches on the male's second dorsal fin*: 0 = present; light band; 1 = absent.
27. *Male nuptial body coloration*: 0 = full dark coloration; 1 = partially covered with vertical lateral dark bars; 2 = scattered dark spots along the body; 3 = absent.