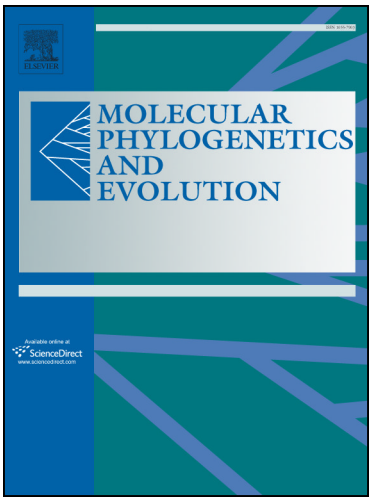

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An exhaustive phylogeny of the combtooth blenny genus *Salaria* (Pisces, Blenniidae) shows introgressive hybridization and lack of reciprocal mtDNA monophyly between the marine species *Salaria basilisca* and *Salaria pavo*

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Abstract

A comprehensive phylogeny of the genus *Salaria* based on mitochondrial and nuclear markers grouped the extant species of the genus in well-characterised marine and freshwater clades, thus rejecting the hypothesis of a polytypic origin of the freshwater *Salaria* populations and supporting the occurrence of a single invasion event of the inland waters by the genus.

Based on both mitochondrial and nuclear DNA datasets, the *Salaria* species of the freshwater clade proved to be vicariant taxa originating from a common ancestor which could possibly spread throughout the circum-Mediterranean inland waters during the late Miocene Messinian salinity crisis, then experiencing a process of allopatric differentiation after the re-flooding of the Mediterranean basin.

Within the marine clade, although the nuDNA datasets showed the existence of well-supported subclades in accordance to the morphological identification of the studied specimens, one of the two subclades obtained in the phylogenetic tree based on the mtDNA dataset included both *S. basilisca* and *S. pavo* specimens, thus failing to find the two species as reciprocally monophyletic. Such a mito-nuclear discordance is here ascribed to multiple mtDNA unidirectional introgression events from *S. basilisca* to *S. pavo*, and the molecular diversity pattern of the marine *Salaria* species is here ascribed to a Pleistocene speciation event nowadays partly concealed by the occurrence of introgressive hybridization phenomena between the two taxa.

Our results urge for prudence when implementing DNA barcoding approaches since, in the presence of mito-nuclear discordance phenomena, single-marker mtDNA-only analyses might lead to significant misidentifications.

Keywords: Mito-nuclear discordance; Speciation without monophyly; Introgressive hybridization; Asymmetrical mtDNA introgression.

1. Introduction

Modern taxonomical, phylogenetic and biogeographical researches routinely implement the analysis of molecular data, thus raising the need of a sound theoretical framework defining, inter alia, what a species (or any other taxon of a given rank) is, and how it could be reliably identified and delimited (e.g. De Queiroz, 2007; Butlin et al., 2009; Seifert, 2014); accordingly, several analytical tools for DNA-based species delimitation have been recently developed (see Fontaneto et al., 2015 for a review). The combined use of nuclear and mitochondrial DNA markers for the molecular analyses, and their comparison with morphological, ecological, behavioural, and distributional data, is often advocated to search for independent but congruent lines of evidences supporting a given phylogenetic, taxonomical or biogeographical hypothesis; when incongruences among the studied datasets are observed, these should be carefully considered and interpreted (Padial et al., 2010; Schlick-Steiner et al., 2010). Sometimes, incongruences between the results of molecular phylogenetic analyses based on mitochondrial (mtDNA) and nuclear (nuDNA) markers might be observed; this phenomenon, known as "mito-nuclear discordance", is widespread among several animal groups (Toews and Brelsford, 2012) and has profound implications in the assessments of the diversity and the ecology of the studied taxa. Among metazoans, mito-nuclear discordance can be mostly attributed to the retention of ancestral polymorphism between recently-diverged sister taxa ("Incomplete lineage sorting", ILS), or to introgressive hybridization, i.e. the fixation of the introgressed mtDNA of a species in the gene pool of another one due to past hybridization events.

Within the marine species of the west-Palearctic combtooth blenny genus *Salaria* Forsskål, 1775, a sharp decoupling between the mitochondrial DNA diversity pattern and the traditional taxonomy based on morphology, ecology and colour patterning was recently observed (Vecchioni et al., 2019), casting some doubts on the actual species status of the rare Mediterranean endemic species *Salaria basilisca* (Valenciennes, 1836), which might in fact be just a morphotype of the widespread *S. pavo* (Risso, 1810). We have thus sequenced fragments of novel nuclear and mitochondrial markers in specimens from Mediterranean populations of the two species with the explicit aim of exploring the actual diversity of the genus *Salaria* and the possible presence of ILS or introgressive hybridization events confounding its taxonomy.

Moreover, the molecular characterization of *Salaria basilisca*, the type species of the genus, for which no sequences were available to date, contributed to a better understanding of the taxonomy of the genus and of its history of colonization of freshwaters. In fact, to date, contrasting hypotheses about the existence of single or multiple invasion events of inland waters by the genus and about the phylogenetic relationships between freshwater and marine *Salaria* populations have been raised

(see Kosswig, 1967; Perdices et al., 2000; Almada et al., 2009; Hundt et al., 2014), and the complete absence of molecular data regarding one out of the five formally described *Salaria* species precluded from drawing definitive conclusions on these topics.

2. Material and methods

2.1. Sampling

Salaria specimens belonging to the species *S. basilisca*, *S. fluviatilis* (Asso, 1801) and *S. pavo* were collected in selected sampling localities from the central Mediterranean area, i.e. France, Italy and Tunisia (Table 1 and Fig. 1). *Salaria pavo* and *S. fluviatilis* were collected with baited funnel traps or hand nets in intertidal rock pools and in inland waters, respectively. Individuals of *S. basilisca* were collected with baited funnel traps or through trawling from a rowboat. Collected samples were fixed *in situ* in 96% ethanol and identified in laboratory according to Orlando-Bonaca and Lipej (2010), Tiralongo (2015) and Zander (1986). Voucher specimens are deposited in the fish collection of the "Laboratorio di Biologia Evoluzionistica e delle Popolazioni" of the University of Palermo, Italy. *Salaria* spp. voucher specimens were deposited in the collection of the Zoology Section (MZUF), Natural History Museum, University of Florence (Italy) with the collection numbers 17633-17634 MZUF (*S. basilisca*), 17635-17636 MZUF (*S. fluviatilis*) and 17629-17632 and 17637 MZUF (*S. pavo*).

2.2. DNA extraction, amplification and sequencing

DNA extraction was performed on small tissue samples taken from the dorsal fin or the muscles of each specimen. Prior to DNA extraction, fin clips and muscle samples were carefully cleaned and soaked in double-distilled water for 1 hour, and then processed for DNA extraction using the BIORON GmbH "Ron's Tissue DNA Mini Kit" following the manufacturer instructions. Fragments of three mitochondrial markers were amplified using the primers pairs described by Ostellari et al. (1996) (D-Loop), Almada et al. (2005) (12S and 16S). Fragments of the nuclear genes S7 and Rhodopsin were amplified using the primer pairs described by Chow and Hazama (1998) and Lin and Hastings (2013), respectively. Further details on primer pairs and PCR cycling conditions are described in supplementary Table S1.

For all the mitochondrial and nuclear fragments, the PCR mix consisted of 18.9 µl double-distilled water, 2.5 µl Buffer 10X including 25 mM $MgCl_2$ solution, 0.5 µl dNTPs (10 mM of each), 0.9 µl of each primer (10 µM), 0.3 µl Taq Polymerase 5 u/µl and 1 µl of DNA template, for a total volume of 25 µl.

After PCRs, 4 µl of each PCR product were separated by electrophoresis on a 2% of agarose gel at 90 V for 25 min and visualised with a UV Transilluminator. When PCR products showed a clear

and single band of the correct expected length, the whole PCR products were purified using the Exo-SAP purification kit and single-stranded sequenced by Macrogen Inc. (Seoul, South Korea) with an ABI 3130xL sequencer using the same primers used for PCRs.

2.3. Phylogenetic analyses and molecular dating

The quality of the obtained chromatograms was checked through the measurement of their Phred scores (Richterich, 1998). Only sequences with continuous reads of high quality bases (QV > 20) were used. When the forward sequences were not of sufficient quality, the complement/reverse sequences were obtained additionally. Sequences were analysed and manually proofread with the DNA sequencing software Chromas v. 2.6.2 (Technelysium, Pty. Ltd. 1998, Queensland, Australia) and aligned with ClustalX v. 2.1 (Larkin et al., 2007).

In order to investigate the phylogeny and the pattern of molecular diversity of the whole genus *Salaria*, the *Salaria* sequences available on GenBank were included in the analyses in addition to the novel sequences produced in the frame of this study. Accordingly, 155 mitochondrial and 52 nuclear sequences of *S. pavo*, *S. fluviatilis* s.l., *S. economidisi* Kottelat, 2004 and *S. atlantica*, Doadrio, Perea and Yahyaoui, 2011 were downloaded from GenBank and included in the analyses (see Table 1 for their Accession Numbers). No *Salaria basilisca* sequences proved to be available in public repositories. *Parablennius* Miranda-Ribeiro, 1915 is among the most closely related blenniid genera to *Salaria* (Almada et al., 2009; Hundt et al., 2014); accordingly, mitochondrial and nuclear sequences of *Parablennius salensis* Bath, 1990 were downloaded from Genbank to be used as outgroups (see Table 1 for their Accession Numbers).

The incongruence length difference test (ILD; Farris et al., 1995) as implemented in PAUP* (Swofford et al., 1998) was used to test whether the mitochondrial and nuclear fragments can be combined to one dataset. According to Cunningham (1997), if $P > 0.01$, pooling the data improves the phylogenetic accuracy and thus it is justifiable to merge the tested datasets into a single matrix. With $P = 0.01$ this condition was not met, thus the mtDNA and nuDNA data sets were analysed separately to investigate the possible topological incongruence between the gene trees. Conversely, the mtDNA sequences of the three mtDNA markers were concatenated in a single dataset because mitochondrial genes are not independently inherited and their relationships are based upon the same underlying phylogeny. However, in order to account for the different best-fit models suggested for the three mtDNA makers by the Akaike information criterion (AIC; Akaike, 1974), the combined dataset was partitioned, applying the most appropriate model of sequence evolution for each partition (see below).

Accordingly, based on both novel and GenBank sequences, three molecular datasets were composed and analysed: (i) a concatenated "mitochondrial dataset" including 12S, 16S and D-loop sequences of all the species of the genus; (ii) a "S7 nuclear dataset" including all the species of the genus, and (iii) a "Rhodopsin nuclear dataset" including a subset of sequences from *Salaria basilisca*, *Salaria pavo* and *S. fluviatilis* only.

The choice of the best evolutionary model for each dataset was made using Partition Finder v. 1.0.1 (Lanfear et al., 2012) according to the AIC. For the mtDNA dataset, BI and ML analyses were performed setting independent models of nucleotide evolution for the "12S" partition (Kimura 2-parameter model of evolution with gamma-distributed rate variation among sites, K80 + Γ) and the other two partitions "16S" and "D-loop" (Hasegawa–Kishino–Yano model of evolution with a proportion of invariable sites and gamma-distributed rate variation among sites, HKY + Γ + I). For both the nuDNA datasets, BI and ML analyses were performed using a HKY model of sequence evolution.

For both the mtDNA dataset and the two nuclear datasets, Bayesian inference of phylogeny (BI) and Maximum likelihood analysis (ML) were performed as implemented in BEAST v. 1.8.0 (Drummond et al., 2005) and PhyMl v. 3 (Guindon and Gascuel, 2003) software packages. For all the analyses, three independent Markov Chain Monte Carlo (MCMC) analyses of 10^7 steps were performed, sampling every 10,000th generation with the burn-in set at 10^6 generations. The convergence of the runs was assessed with Tracer v. 1.6 (Rambaut et al., 2014) by requiring effective sample size (ESS) values above 200 for all parameters. TreeAnnotator v. 1.8.3 (part of the BEAST package) was used to calculate node support estimates after discarding the first 20% of the trees as burn- in, while keeping the node heights of the highest log clade credibility tree. Nodes' statistical support of BI was evaluated by their posterior probabilities. Branch supports were evaluated by their posterior probabilities in the BI trees and with 1,000 bootstrap replicates in the ML analyses.

In order to date the major cladogenetic events, a lognormal model that relaxes the molecular clock hypothesis and a Yule process prior were used on the mtDNA dataset (Drummond et al., 2006). To calibrate the molecular clock, the split between the freshwater species *S. atlantica* and *S. fluviatilis* was used, assuming they diverged during the late Miocene (5-5.5 MYA), as suggested by Almada et al. (2009).

To investigate the support for a sister-clade relationship between *Salaria pavo* and *S. basilisca*, we compared the unconstrained tree topologies based on the two nuclear datasets with the results from BI and ML inferences where "*S. basilisca*" and "*S. pavo*" were constrained to form reciprocally monophyletic groups. Since some identical mtDNA haplotypes were shared by *Salaria*

individuals morphologically ascribed to *S. basilisca* and *S. pavo* (see below), no constrained trees were built for the mtDNA dataset.

2.4. Species concept and DNA taxonomy

In the frame of this paper, we followed the ‘‘unified species concept’’ (De Queiroz, 2007), thus considering “lineages evolving separately from others” as different species. Accordingly, morphological similarity or identity was not considered *per se* a sufficient evidence for conspecificity, and a DNA taxonomy approach (*sensu* Fontaneto et al., 2015) was implemented.

Following the guidelines of Zhang et al. (2013) and Fontaneto et al. (2015), the identification of the “Molecular Operational Taxonomic Units" (MOTUs)”, i.e. of the putative species, was carried out implementing DNA taxonomy approaches based on different assumptions, i.e. a quantitative approach based on coalescent (“ABGD”) (Puillandre et al., 2012) and a phylogenetic criterion based on branching rates (“PTP”) (Zhang et al., 2011, 2013). Both “ABGD” and “PTP” were implemented through their online interfaces (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html and http://species.h-its.org/ptp/,respectively) using the concatenated mtDNA dataset. Based on empirical data, the number of groups identified by ABGD is considered to generally correspond to the number of species actually present in the studied dataset for a P value of about 0.01 (Puillandre et al., 2012).

Additionally, in order to investigate the plausibility of hybridization or ILS as drivers of the observed mito-nuclear discordance, the statistical framework of Joly et al. (2009) was employed and implemented in JML v. 1.3.0 (Joly, 2012) and described in Papakostas et al. (2016).

2.5. Network analyses

Haplotype networks including all the available *Salaria* spp. sequences were constructed based on subsets of the three datasets using the software Popart v. 1.7 (http://popart.otago.ac.nz) implementing the median-joining network algorithm as suggested by Bandelt et al. (1999).

3. Results

3.1. Samplings

Overall, 32 novel *Salaria* specimens belonging to the species *Salaria basilisca*, *S. fluviatilis* and *S. pavo* were collected and included in the analyses (Table 1). Specimens morphologically identified as *S. basilisca* were collected in Tunisia only, while *S. pavo* specimens were collected from Tunisia, France, and the Italian islands of Sicily and Sardinia. The two novel *S. fluviatilis* specimens included in the analyses were collected from Sicily and Peninsular Italy, respectively, i.e.

from areas where no molecular data pertaining this species were to date available. No specimens belonging to the other *Salaria* species were collected in the frame of this research.

3.2. Phylogenetic analyses and species identification

After having trimmed out the sequence tails which were not present in all the individuals, the alignment of the novel amplified fragments and those downloaded from GenBank led to trimmed aligned fragments of 346-bp (12S), 512 bp (16S), 303 bp (D-Loop), 402 bp (S7), and 629 bp (Rhodopsin), respectively.

The BI and ML trees based on the concatenated mtDNA dataset (Fig. 2) showed a clear separation of a freshwater versus a marine clade of the genus *Salaria*, which are separated by a single cladogenetic event dated at about 5.7 MYA (Table 2 and Fig. S5). Within the freshwater clade, four well-supported clades relating to the species *Salaria atlantica*, *Salaria* cf. *fluviatilis* from Turkey and Israel, *S. economidisi*, and *S. fluviatilis* s.s. are present. The Moroccan species *S. atlantica* is confirmed to be the most divergent taxon within the freshwater clade, being the sister group to a clade including *S. fluviatilis* s.l. and *S. economidisi*. *S. fluviatilis* s.l. is paraphyletic respect to the Greek endemic *S. economidisi*, suggesting that the Israeli and some Turkish populations currently ascribed to *S. fluviatilis*, here referred to as "*Salaria* cf. *fluviatilis*", might in fact deserve species status (see also Almada et al., 2009). Within *S. fluviatilis* s.s., the highest diversity is observed within the Iberian Peninsula, while a single, shallow clade includes the other available sequences from Croatia, Greece, Turkey, peninsular Italy and Sicily. The marine clade splitted in upper Pleistocene in two well-supported subclades (average interclade uncorrected p-distance: 2.6%) including all the studied *S. pavo* and *S. basilisca* specimens. Unexpectedly, whereas one of the marine subclades includes only *Salaria* specimens morphologically ascribed to *S. pavo*, the other one includes specimens morphologically identified as both *S. basilisca* and *S. pavo* (Fig. 2), the latter originating from Tunisia, Sicily (Italy), Spain, Israel and Crete (Greece). Based on the mtDNA dataset, the two marine morphospecies of the genus *Salaria* thus proved to be paraphyletic.

The initial and recursive partitions of the ABGD analysis applied to the concatenated mtDNA dataset converged finding 6 groups of species rank within the ingroup, with prior maximal divergence of intraspecific diversity values ("P") ranging from 0.0010 to 0.0046 (Fig. S1). The six groups highlighted by ABGD correspond to the four freshwater and two marine subclades highlighted in the mtDNA based tree (Fig. 2). PTP analysis estimated the presence of 8 species in the ingroup, i.e. finding five of the six groups highlighted by ABGD analysis, and splitting *S. fluviatilis* s.s. in three groups, as shown in figure S2.

In good accordance to the phylogenetic trees, the mtDNA network shows a clear separation of the marine and freshwater species of the genus, and a grouping of freshwater *Salaria* haplotypes in accordance to the current taxonomical arrangement of the genus. Conversely, two haplotypes (mH4 and mH6, see Fig. 3) proved to be shared by the two *Salaria* morphospecies within the marine clade, and no clear grouping of the observed haplotypes according to the morphology of the sequenced marine specimens could be observed.

Based on the nuclear DNA, although the unconstrained BI and ML analysis of the "S7" dataset failed to support the monophyly of *S. economidisi* respect to *S. fluviatilis* s.s. and the reciprocal monophyly of the two marine *Salaria* species (Fig. S3), the constrained analyses of the same dataset had comparable likelihood values with those of the unconstrained ones (the log files of the analyses are available from the corresponding author on request) thus supporting the sister-species relationship and the reciprocal monophyly of the five *Salaria* currently described morphospecies and of the unnamed *Salaria* cf. *fluviatilis* from Turkey and Israel (Fig. 4). A concordant result was obtained with the "Rhodopsin dataset" (Figs. 5 and S4). The separation of the marine *Salaria* samples in agreement to their morphological identification was also supported by the topologies of the nuclear networks based on both nuDNA datasets (Fig. 6).

To explain the observed mito-nuclear discordance, the null hypothesis that ILS was solely responsible for the pattern observed between *Salaria pavo* and *S. basilisca* was tested based on the "JML" analysis. The alternative hypothesis of hybridization could not be ruled out as the null hypothesis of ILS was rejected with a p-value = 0.1.

4. Discussion

The inclusion of all the currently known *Salaria* species in the present analyses allowed for the first time to draw a comprehensive phylogeny of this blenniid genus. Based on both mitochondrial and nuclear markers, the extant species of the genus *Salaria* are divided in two major clades separated in middle or late Miocene and characterised by markedly different ecology: one is strictly linked to inland waters, the other one is strictly marine (Figs. 2, 4, and 5). In good accordance with Almada et al. (2009), our results thus reject the hypothesis of a polytypic freshwater *Salaria* taxon originating from multiple independent invasions of inland waters by a "*Salaria pavo*-like" marine ancestor (Kosswig, 1967). Conversely, our data suggest the occurrence of a single invasion event of inland waters exerted by a marine-dwelling taxon with a sister-species relationship with the common ancestor of the extant marine species, i.e. *Salaria basilisca* and *S. pavo*; the cladogenetic event originating the freshwater and marine *Salaria* clades took place in the Miocene (see node "a" in Figs. 2 and S5, and Table 2), an epoch in which other Mediterranean fishes experienced

phenomena of allopatric diversification (e.g. Hrbek and Meyer, 2003; Triantafyllidis et al. 2007), and was followed by the allopatric differentiation of the freshwater clade in the four currently known *Salaria* species inhabiting inland waters, and the differentiation of the marine clade in *S. basilisca* and *S. pavo* (see below).

All the analysed datasets show a greater molecular substructuring in the freshwater clade as compared to the marine one. This result is not surprising, since inland water ecosystems are much more fragmented than the marine realm, giving more opportunities for allopatric differentiation events to take place, eventually resulting in vicariance events even in the absence of niche differentiation (Puebla, 2009). In fact, based on the currently available data, the ecology of freshwater *Salaria* species is rather uniform, with a clear preference of all the freshwater species for well-oxygenated waters and rocky or pebbly bottoms both in lentic and lotic habitats. Based on the branching pattern of the mtDNA and nuDNA phylogenetic trees, and on the dating of the cladogenetic events based on the mtDNA dataset, the freshwater *Salaria* species are thus to be considered vicariant species originating from a common ancestor which lived in late Miocene (about 5 MYA, see node "b" in Figs. 2 and S5, and Table 2) in the Atlantic-Mediterranean area and which could spread throughout the circum-Mediterranean inland waters and neighbouring areas during the Messinian salinity crisis. After the re-opening of the Strait of Gibraltar and the re-flooding of the Mediterranean basin, inland water populations remained mostly isolated in different hydrographic basins and experienced a process of allopatric differentiation, although some small- to medium-scale inter-watersheds movements possibly took places during the Pleistocene thanks to the pronounced euryhalinity of the members of the freshwater clade (Plaut, 1998; Plaut and Afik, 2001) and to the presence of intermittent connections between different drainages due to hydrological reorganization associated with climate change and/or orogeny events (e.g. Filipe et al., 2009). The natural history of the freshwater clade brought to a roughly longitudinal separation of the species. *Salaria atlantica*, occurring in the Atlantic watershed of Morocco, is the westernmost species of the clade, followed by *Salaria fluviatilis*, widespread from the Iberian Peninsula to Turkey, and the easternmost species, i.e. *Salaria economidisi*, endemic to a single lake in Greece, and *Salaria* cf. *fluviatilis*, occurring in Israel and Turkey. Both in Greece (*S. economidisi* and *S. fluviatilis*) and Turkey (*S. fluviatilis* and *Salaria* cf. *fluviatilis*) different freshwater *Salaria* species occur sympatrically, albeit not syntopically, highlighting the renowned high biological diversity of the inland waters of these areas (e.g. Griffiths et al., 2004; Reyjol et al., 2007; Smith et al., 2014; Çiçek et al., 2015; Marrone et al., 2017).

Based on both mitochondrial and nuclear datasets, the marine clade consistently showed a structuring in two major subclades (Figs. 2, 4, and 5) whose most recent common ancestor dates

back to upper Pleistocene (see node "e" in Figs. 2 and S5, and Table 2); however, the obtained results highlighted a striking discordance in the phylogenetic signals of the mitochondrial dataset versus the nuclear ones: in sharp contrast with what observed for the freshwater *Salaria* species, which are consistently identifiable based both on morphology and all the implemented molecular markers, the marine species of the genus are separated in two non-congruent clades in the mitochondrial- and the nuclear-based phylogenies. Whereas the two analysed nuDNA datasets show the existence of two well-supported subclades which are in accordance to the morphological identification of the studied specimens (Figs. 4 and 5), one of the two marine subclades reported in the phylogenetic tree based on the concatenated mtDNA dataset (Fig. 2) includes both *S. basilisca* and *S. pavo* specimens identified based on morphology, thus failing to find the two species as reciprocally monophyletic and thus to fulfil one of the properties that have been often adopted as secondary species criterion (see De Queiroz, 2007). Such a result is apparently puzzling, since the two marine species of the genus *Salaria* are sharply and easily distinguishable based on morphology, meristic traits, size, colour patterns, and ecology (Tortonese, 1975; Šoljan, 1975; Tiralongo, 2015; Louisy, 2006); moreover, although their distribution is mostly overlapping, with the distribution range of the rarer *Salaria basilisca* nested within that of the commoner *S. pavo* (Louisy, 2006), as a rule the two species occur allotopically, since *S. basilisca* is linked with seagrass beds of the subtidal zone whereas *S. pavo* is typical of rocky substrates in the intertidal and the first metres of the subtidal zone (Zander, 1986; Tiralongo, 2015).

Such a mitonuclear discordance pattern where the nuDNA-based phylogenies are in accordance with the available morphological, ethological and ecological data but the mtDNA-based ones are not, is already known for both marine and freshwater fishes (e.g. Alvarado Bremer et al., 2005; Koblmüller et al., 2007, 2017) and other taxa (e.g. Nesi et al., 2011, Toews and Brelsford, 2012; Franco et al., 2015; Thielsch et al., 2017) and, among metazoans, it is usually interpreted as due to the retention of ancestral polymorphism or to the presence of introgressive hybridization (Obertegger et al., 2017).

In the present study case, ascribing the absence of reciprocal mitochondrial monophyly between the two marine *Salaria* species to incomplete lineage sorting (ILS) is rather unlikely, due to the sharp and relatively ancient separation of the two well-supported marine subclades, the absence of intermediate haplotypes, and the clear-cut differentiation of the nuclear profiles of *Salaria pavo* and *S. basilisca*. In fact, in presence of ILS, mtDNA is expected to complete the process of lineage sorting faster than nuDNA, thus providing the first evidences of a speciation event (Funk and Omland, 2003), which is exactly the opposite pattern compared to the one observed in the present study case. Moreover, in the case of ILS the presence of shared haplotypes

can be usually observed in both the subclades, i.e. specimens ascribable to *S. basilisca* and *S. pavo* based on morphology and nuDNA profiles should be occurring in both the marine subclades, whereas in the present study case one of the two subclades includes specimens ascribed to the two species, while the other includes only "pure" *S. pavo* ones (Fig. 2).

On the other hand, several independent lines of evidence suggest that the observed mitonuclear discordance between the marine *Salaria* species is ascribable to multiple mtDNA unidirectional introgression events from *S. basilisca* to *S. pavo*. In fact, mtDNA introgression is as a rule asymmetrical (Currat et al., 2008), might take place with phenotypically unrecognizable hybrids (Good et al., 2015), and without a concurrent nuclear DNA introgression (e.g. Ballard and Whitlock, 2004; Nesi et al., 2011; Good et al., 2015), i.e. showing a perfect concordance with the pattern observed in our study case. Moreover, the two marine *Salaria* species are able to hybridize, albeit rarely, in nature (Heymer, 1985), and the results of the JML show that introgressive hybridization event could not be ruled out as a driver of the observed mito-nuclear discordance.

mtDNA introgression is common in areas of secondary contact of closely-related evolutionary lineages, and it occurs almost exclusively from the local to the invading species (Toews and Brelsford, 2012; Currat et al., 2008). Observed unidirectional introgression of "*basilisca*" mtDNA in "*pavo*" specimens, and the breeding phenology of the species of the genus, with territorial males defending nests in crevices and actively courting females (Patzner et al., 1986), suggests that *S. pavo* males might occasionally nest within the bathymetries typically inhabited by *S. basilisca*, accept *S. basilisca* females within their nests and hybridize with them. However, it cannot also be excluded that "parasitic" *Salaria pavo* males, which are known to be more frequent in areas poor of adequate nesting sites as seagrass beds on soft bottoms (Saraiva et al., 2012) might occasionally mimic females in order to approach nesting *S. basilisca* males and fertilize some eggs during spawning events. The possible role of *Salaria pavo* as the "invading species" is also in accordance with its more pronounced euryecy and widespread distribution when compared to those of *S. basilisca*, which make the first species a more suitable candidate "invader" than the second one. The two subclades observed within the marine clade of the genus *Salaria* should be thus interpreted as a "*pavo* mtDNA subclade" and a "*basilisca* mtDNA subclade", with the last subclade including some *Salaria pavo* specimens from Crete (Greece), Israel, Tunisia, Sicily (Italy) and Catalonia (Spain) which are positively identified as *S. pavo* based on morphology, ecology and nuDNA sequences but which belong to the *S. basilisca* mitochondrial subclade due to introgressive hybridization. Some of these sequences were already singled out by Almada et al. (2009) as a peculiar clade including Israeli and Spanish *S. pavo* specimens, and was ascribed by the authors to the persistence of ancient polymorphism or to high levels of gene flow throughout the

Mediterranean. In fact, these specimens are here interpreted as *S. pavo* specimens with introgressed *S. basilisca* mtDNA

The current molecular diversity pattern of the marine clade of the genus *Salaria* is here ascribed to a Pleistocene speciation event, which originated a larger taxon inhabiting seagrass beds on soft bottoms at deeper bathymetries (i.e. *Salaria basilisca*) and a smaller one specialised in inhabiting the rocky substrates of the intertidal and the very first metres of the subtidal zones (i.e. *Salaria pavo*). Both taxa are thermophilous (e.g. Dulčić et al., 2008; Almada et al., 2009) and experienced bottleneck events during the climatic oscillations of the Pleistocene, which are known to have influenced the patterns of molecular diversity of a number of marine organisms inhabiting the Mediterranean Sea (e.g. Segvić-Bubić et al., 2016; Sacco et al., 2017), and possibly caused the relatively low current molecular diversity observed within each of these subclades. This diversity pattern is nowadays partly concealed by the occurrence of introgressive hybridization phenomena between the two taxa, which prevents a sound identification of the specimens based on mtDNA-only sequences and stresses the need of carefully checking the taxonomical and phylogenetic inferences, and the identification of the species itself, when molecular analyses are based on mitochondrial sequences only.

Our results based on an exhaustive taxon coverage allow to clarify the phylogeny of the genus *Salaria* and the taxonomy of its marine clade, albeit urging for prudence when single-marker mtDNA-only analyses are carried out, e.g. when using a DNA barcoding approach, since some individuals would be misidentified if reliance is placed on mtDNA markers only. DNA barcoding is admittedly aimed at species identification only and should not be used for inferring phylogenies (Hebert et al., 2003), but when ILS or introgressive hybridization phenomena occurs, it could not provide reliable results for species identification either (e.g. Nesi et al., 2011; Chapple and Ritchie, 2013; Ermakov et al., 2015; Dupont et al., 2016, and present work). Such a caveat should be bore in mind when biodiversity studies are carried out, and DNA barcode should be considered just one important descriptor in the framework of an integrative species delimitation approach (Sbordoni, 2010).

The updated phylogeny and molecular systematics of the genus *Salaria* here presented stress the need for the realisation of further taxonomical works on the freshwater clade of the genus, which includes at least one taxon of species rank waiting for a morphological characterization and a formal taxonomical description (i.e. the taxon from Israel and Turkey here called *Salaria* cf. *fluviatilis*) and which possibly present some further cryptic diversity yet to be discovered. Conversely, the morphology-based taxonomy of the marine species of the genus is here confirmed, and the existence of two species differing in morphology, ecology and nuclear DNA sequences is

corroborated, whereas the existence of introgressive hybridization phenomena can be misleading when mtDNA sequences only are used for species identification.

Eventually, the carrying out of a wider geographical sampling coverage for both the *Salaria* major clades would allow a better understanding of their phylogeography and evolutionary trajectories, thus allowing also an adequate management and protection of the actual biological diversity of these interesting blenniid taxa.

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Table 1: Origin and GenBank Accession numbers of the analysed specimens. Geographical coordinates are expressed as decimal degrees (Map Datum: WGS84). mtDNA and nuDNA codes refer to figures 3 and 6.

See annexed word file

Table 2: Bayesian estimates (along with the 95% highest posterior density interval) of the divergence times among groups (millions of years ago; MYA) based on the concatenated mtDNA dataset assuming a strict molecular clock of 1.289% (12S) and 1.585% sequence divergence/MY (16S) (Almada et al., 2009). Letters refer to nodes in figure 2. See also supplementary figure 5.

		Bayesian estimates
a.	The most recent common ancestor (mrca) of *Salaria*	5.72 [3.93-8.01]
b.	mrca of the freshwater clade	4.28 [3.19-5.54]
c.	mrca of the "*fluviatilis*", "cf. *fluviatilis*", and "*economidisi*" subclades	2.96 [1.59-4.37]
d.	mrca of the "*fluviatilis*" and "*economidisi*" subclades	1.78 [0.86-2.97]
e.	mrca of the marine clade	1.98 [0.90-3.31]
f.	mrca of marine "*pavo*" subclade	1.44 [0.59-2.50]
g.	mrca of marine "*pavo* and *basilisca*" subclade	1.04 [0.41-1.88]

Figures captions:

Figure 1: Geographic location of the sampled sites. 1: Tabarka; 2: Ghar El Melh; 3: Bizerte lagoon; 4: South lake of Tunis; 5: Sayeda; 6: Chebba; 7: La Louza; 8: Sfax; 9: Gabès; 10: Djerba; 11: Sète; 12: Torre dei Corsari; 13: Palermo; 14: Syracuse; 15: Torrente Frattina; 16: Lake Garda. See Table 1 for the coordinates of the sampled sites and for information on the *Salaria* species collected in each site. Blue circles indicate novel sample sites of *Salaria pavo*, blue squares indicate novel sample sites of *S. basilisca*, blue triangles indicate novel sample sites of *S. fluviatilis*. Green circles and green triangles indicate sample site of *S. pavo* and *S. fluviatilis*, respectively, collected by Almada et al., 2009. Moreover, the green star indicates the sample site of *S. economidisi*, and the green diamond indicates the sample site of *S. atlantica*, both collected by Almada et al. (2009).

Figure 2: Bayesian phylogram (95% majority rule consensus tree) of *Salaria* spp. based on the concatenated mtDNA (12S, 16S and D-loop) dataset. *Parablennius salensis* was used as an outgroup to root the tree. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI) / bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Letters refer to dated nodes as

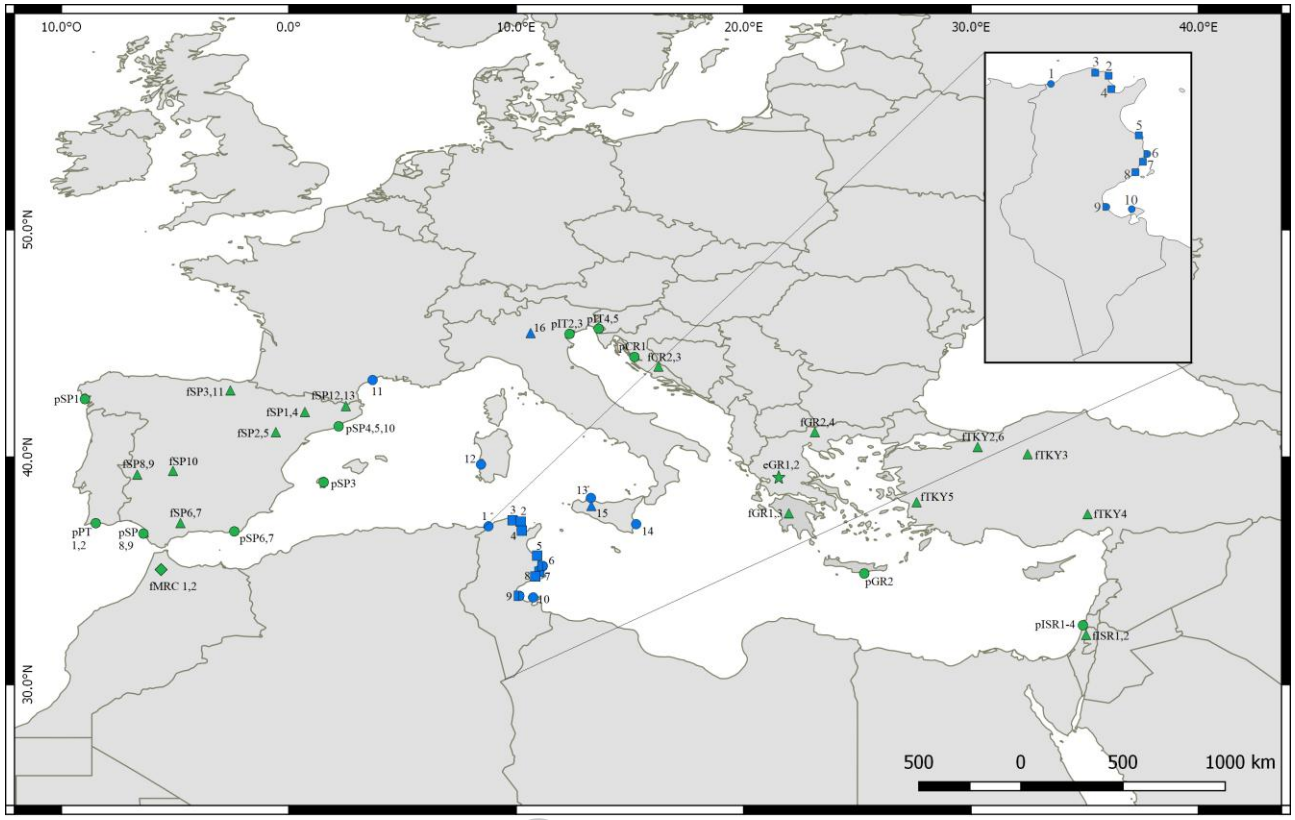
reported in Table 2. Crosshatched rectangles refer to MOTUs as indicated by ABGD (black rectangles) or PTP (grey). Square brackets group the samples according to the current taxonomy of the genus. The analysed specimens are reported using the codes listed in Table 1.

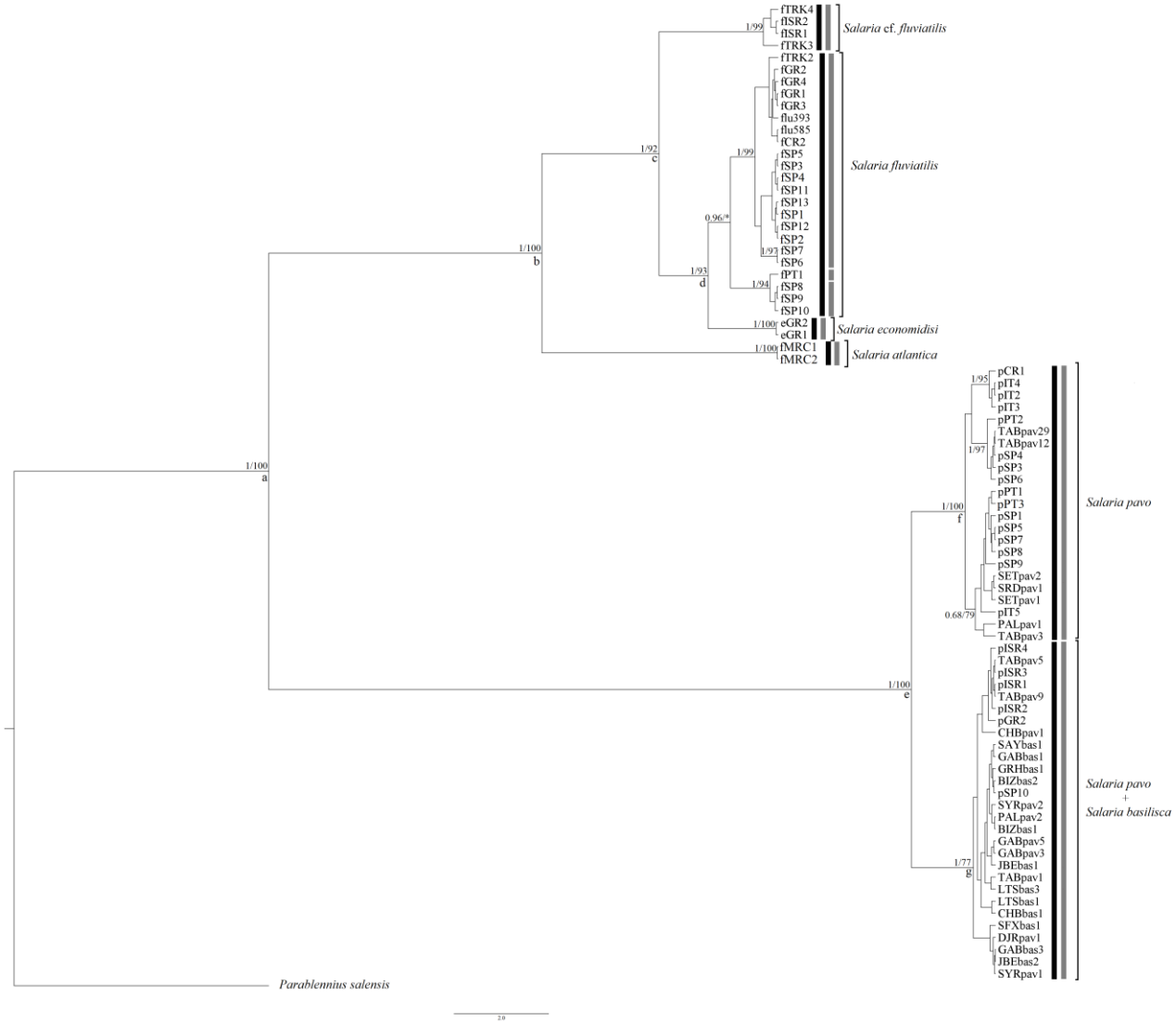
Figure 3: Median-joining haplotype network based on the mtDNA dataset of *Salaria* spp. Dashes indicate substitutions steps. Each circle represents a haplotype and its size is proportional to its frequency. See Table 1 for detailed information on the analysed haplotypes.

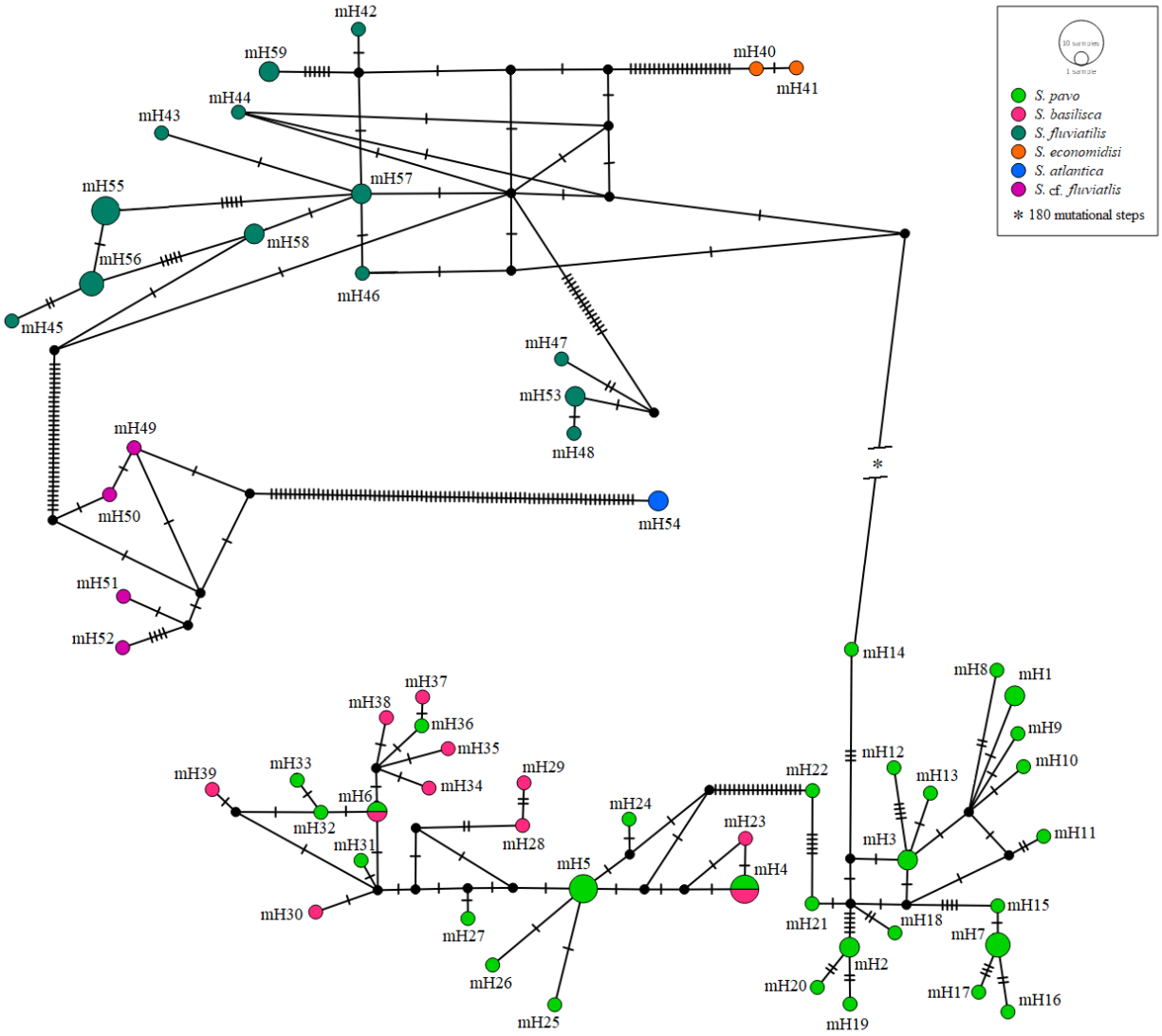
Figure 4: Constrained bayesian phylogram (95% majority rule consensus tree) of *Salaria* spp. based on the S7 nuDNA dataset. *Parablennius salensis* was used as an outgroup to root the tree. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI) / bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Square brackets group the samples according to the current taxonomy of the genus. The analysed specimens are reported using the codes listed in Table 1.

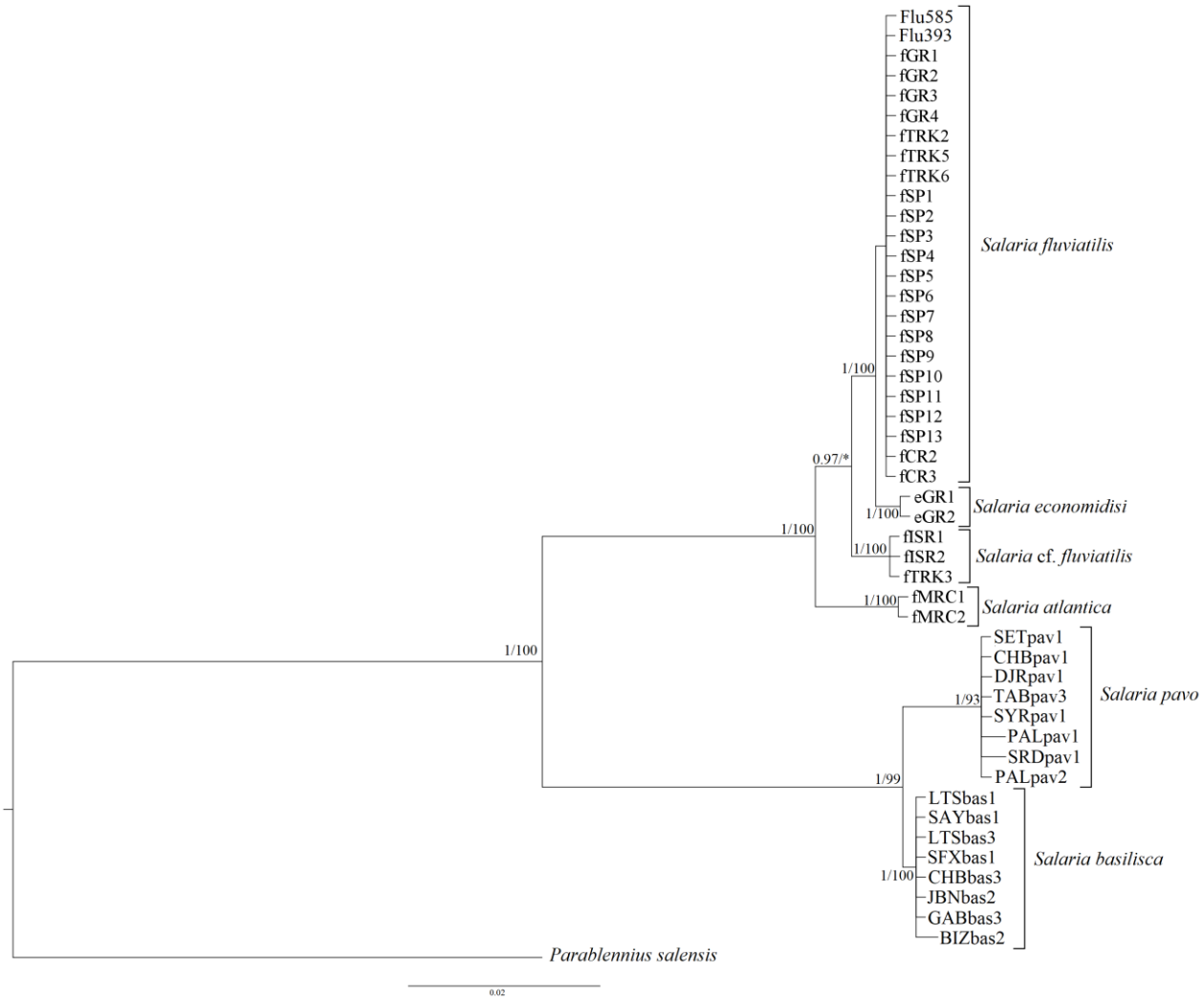
Figure 5: Bayesian phylogram (95% majority rule consensus tree) of *Salaria basilisca* and *S. pavo* based on the Rhodopsin nuDNA dataset. *S. fluviatilis* was used as an outgroup to root the tree. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI) / bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Square brackets group the samples according to the current taxonomy of the genus. The analysed specimens are reported using the codes listed in Table 1.

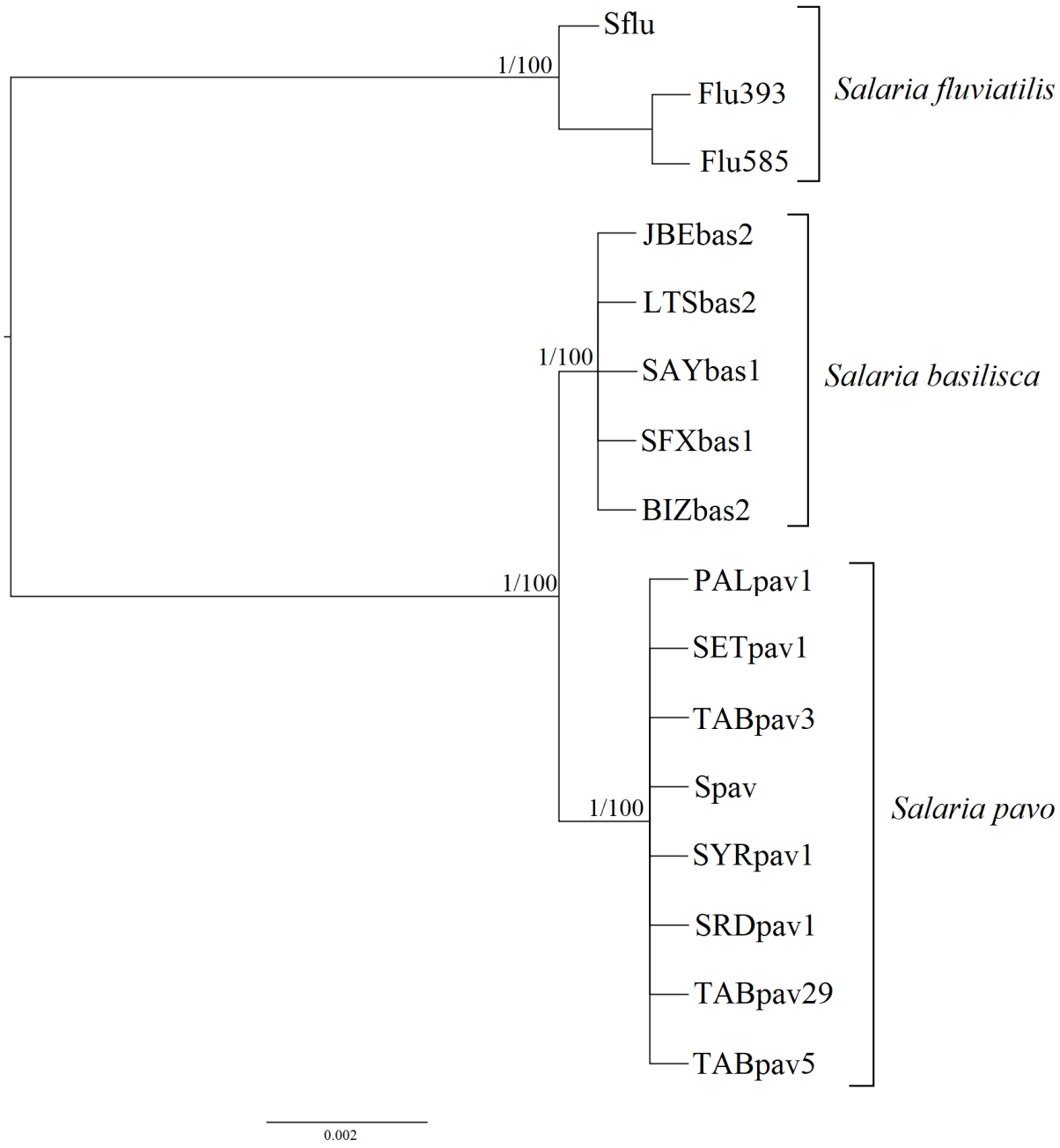
Figure 6: Median-joining haplotype network based on the S7 (a) and Rhodopsin (b) nuDNA datasets of *Salaria* spp. Dashes indicate substitutions steps. Each circle represents a haplotype and its size is proportional to its frequency. See Table 1 for detailed information on the analysed haplotypes.

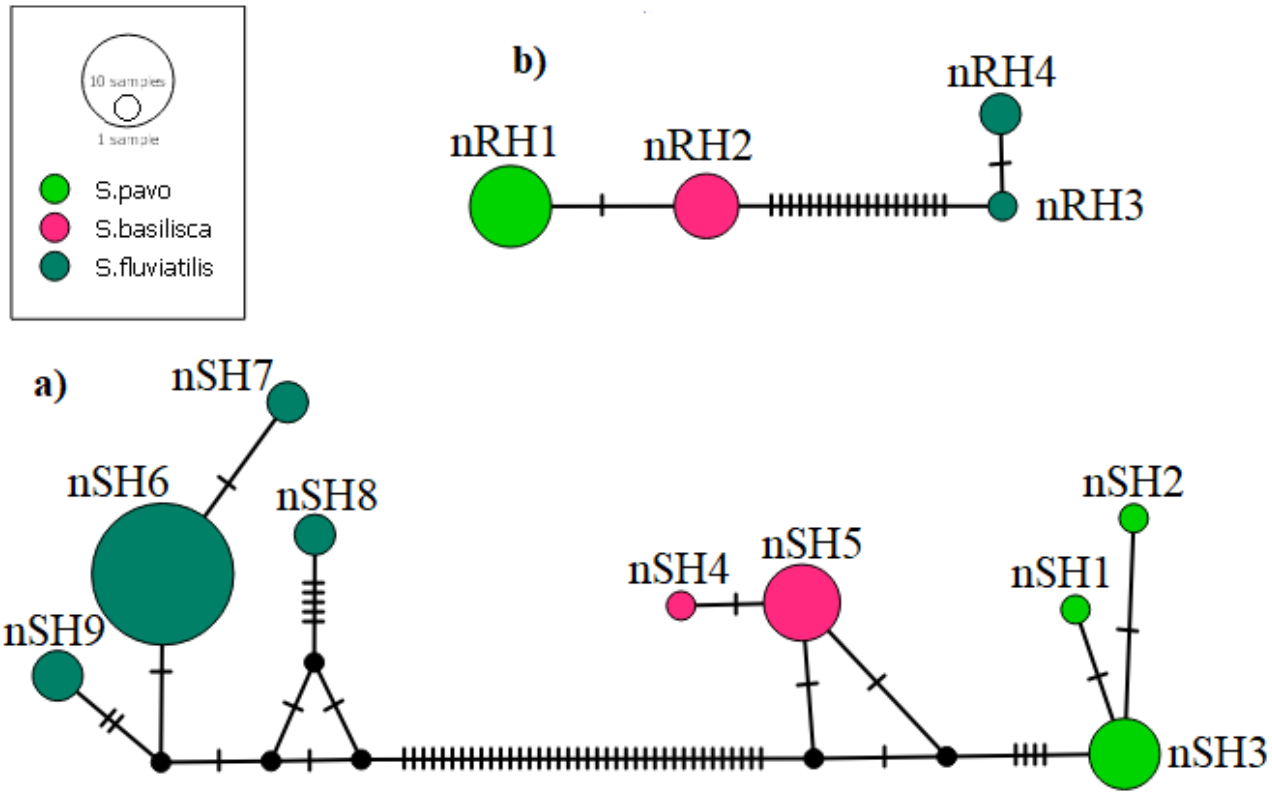










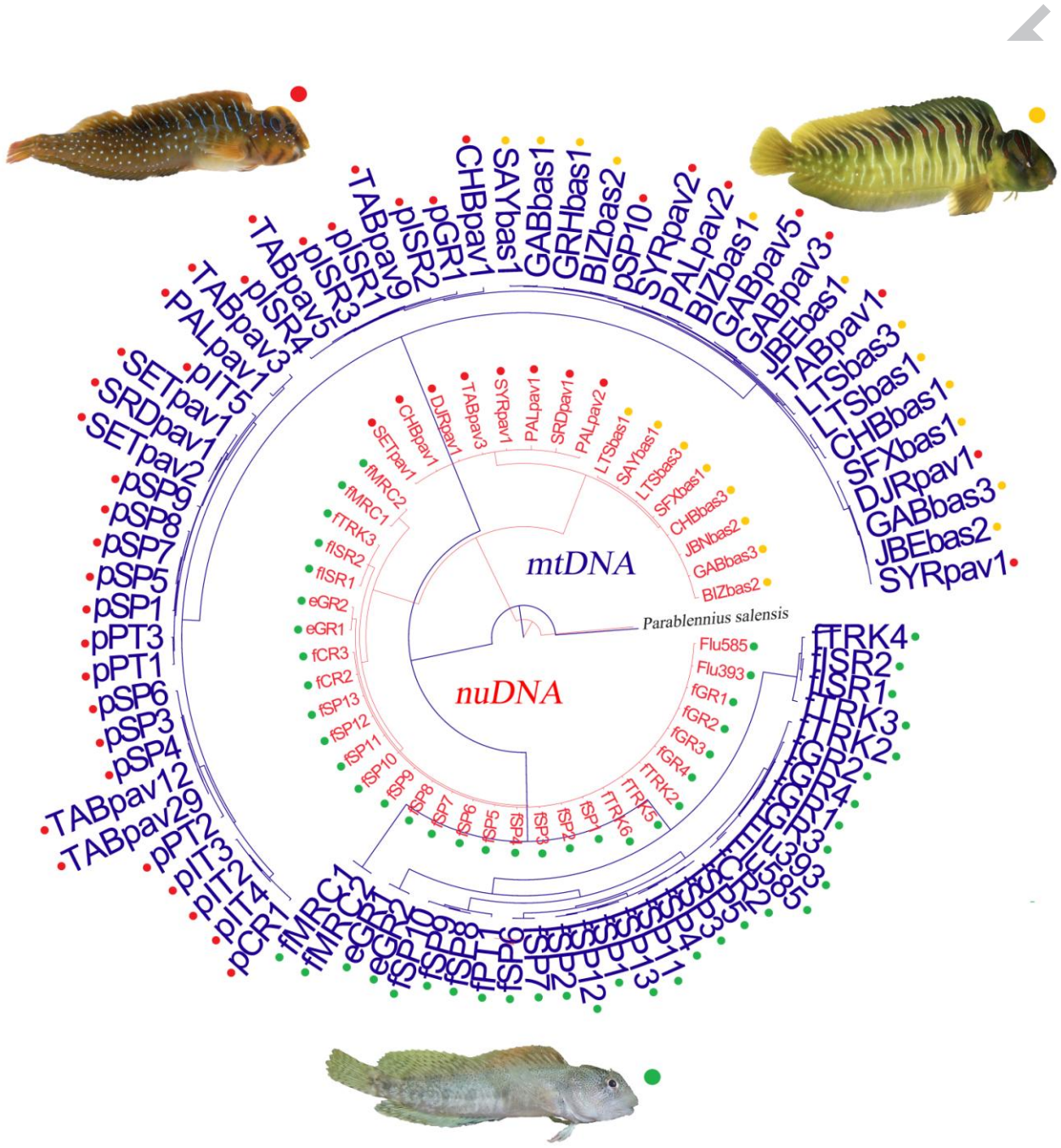


Taxon	12S A.N.	16S A.N.	Dloop A.N.	S7 A.N.	Rhodopsin A.N.	Code	Country	Location	Latitude (N)	Longitude (E)	mtDNA Haplotype	nuDNA Haplotype	Source
S. pavo	MH714485	MH724836	MH715460	-	-	TABpav9	Tunisia	Tabarka	36.95983	8.75366	mH5	-	present work
S. pavo	MH714495	MH724846	MH715470	-	MH715441	TABpav5	Tunisia	Tabarka	36.95983	8.75366	mH5	nRH1	present work
S. pavo	MH714483	MH724834	MH715458	-	-	TABpav1	Tunisia	Tabarka	36.95983	8.75366	mH31	-	present work
S. pavo	MH714486	MH724837	MH715461	-	-	TABpav12	Tunisia	Tabarka	36.95983	8.75366	mH7	-	present work
S. pavo	MH714487	MH724838	MH715462	-	MH715440	TABpav29	Tunisia	Tabarka	36.95983	8.75366	mH7	nRH1	present work
S. pavo	MH714477	MH724828	MH715452	MH715422	MH715435	TABpav3	Tunisia	Tabarka	36.95983	8.75366	mH21	nSH3 nRH1	present work
S. pavo	MH714473	MH724824	MH715448	MH715420	-	CHBpav1	Tunisia	Chebba	35.21145	11.13580	mH27	nSH3	present work
S. pavo	MH714478	MH724829	MH715453	-	-	GABpav3	Tunisia	Gabès	33.90922	10.10645	mH32	-	present work
S. pavo	MH714480	MH724831	MH715455	-	-	GABpav5	Tunisia	Gabès	33.90922	10.10645	mH33	-	present work
S. pavo	MH714474	MH724825	MH715449	MH715421	-	DJRpav1	Tunisia	Djerba	33.85487	10.74219	mH4	nSH3	present work
S. pavo	MH714475	MH724826	MH715450	MH715419	MH715434	SETpav1	France	Sète	43.39255	3.68100	mH3	nSH3 nRH1	present work
S. pavo	MH714484	MH724835	MH715459	-	-	SETpav2	France	Sète	43.39255	3.68100	mH13	-	present work
S. pavo	MH714494	MH724845	MH715469	MH715425	MH715439	SRDpav1	Italy	Torre dei Corsari (Sardinia)	39.68091	8.44775	mH3	nSH1 nRH1	present work
S. pavo	MH714490	MH724841	MH715465	MH715424	MH715431	PALpav1	Italy	Palermo (Sicily)	38.20933	13.28230	mH18	nSH2 nRH1	present work
S. pavo	MH714491	MH724842	MH715466	MH715426	-	PALpav2	Italy	Palermo (Sicily)	38.20933	13.28230	mH6	nSH3	present work
S. pavo	MH714488	MH724839	MH715463	MH715423	MH715436	SYRpav1	Italy	Syracuse (Sicily)	37.05592	15.27153	mH4	nRH1	present work
S. pavo	MH714489	MH724840	MH715464	-	-	SYRpav2	Italy	Syracuse (Sicily)	37.05592	15.27153	mH22	nSH3	present work
S. pavo	FJ465669	FJ465712	FJ465577	FJ465616	-	pPT3	Portugal	Ria Formosa	-	-	mH11	-	Almada et al. 2009
S. pavo	FJ465692	FJ465749	FJ465552	FJ465615	-	pCR1	Croatia	Borovac	-	-	mH19	-	Almada et al. 2009
S. pavo	FJ465668	FJ465721	FJ465559	FJ465619	-	pGR2	Greece	Crete	-	-	mH26	-	Almada et al. 2009
S. pavo	FJ465674	FJ465705	FJ465568	FJ465626	-	pISR1	Israel	-	-	-	mH5	-	Almada et al. 2009
S. pavo	FJ465677	FJ465703	FJ465563	FJ465620	-	pISR2	Israel	-	-	-	mH25	-	Almada et al. 2009
S. pavo	FJ465678	FJ465706	FJ465570	FJ465627	-	pISR3	Israel	-	-	-	mH5	-	Almada et al. 2009
S. pavo	FJ465681	FJ465704	FJ465569	-	-	pISR4	Israel	-	-	-	mH24	-	Almada et al. 2009
S. pavo	FJ465672	FJ465720	FJ465558	FJ465618	-	pIT2	Italy	Chioggia	-	-	mH2	-	Almada et al. 2009
S. pavo	FJ465675	FJ465719	FJ465565	FJ465623	-	pIT3	Italy	Chioggia	-	-	mH20	-	Almada et al. 2009
S. pavo	FJ465679	FJ465713	FJ465561	FJ465632	-	pIT4	Italy	Trieste	-	-	mH2	-	Almada et al. 2009
S. pavo	FJ465680	FJ465714	FJ465571	FJ465635	-	pIT5	Italy	Trieste	-	-	mH14	-	Almada et al. 2009
S. pavo	FJ465666	FJ465717	FJ465574	FJ465636	-	pPT1	Portugal	Olhos de Agua	-	-	mH9	-	Almada et al. 2009
S. pavo	FJ465667	FJ465708	FJ465573	FJ465628	-	pPT2	Portugal	Olhos de Agua	-	-	mH17	-	Almada et al. 2009

Taxon	12S A.N.	16S A.N.	Dloop A.N.	S7 A.N.	Rhodopsin A.N.	Code	Country	Location	Latitude (N)	Longitude (E)	mtDNA Haplotype	nuDNA Haplotype	Source
S. pavo	FJ465671	FJ465715	FJ465579	FJ465613	-	pSP1	Spain	Galiza	-	-	mH10	-	Almada et al. 2009
S. pavo	FJ465676	FJ465709	FJ465562	FJ465622	-	pSP3	Spain	Formentera	-	-	mH15	-	Almada et al. 2009
S. pavo	FJ465685	FJ465710	FJ465555	FJ465614	-	pSP4	Spain	Barcelona	-	-	mH7	-	Almada et al. 2009
S. pavo	FJ465697	FJ465750	FJ465572	FJ465633	-	pSP5	Spain	Barcelona	-	-	mH1	-	Almada et al. 2009
S. pavo	FJ465698	FJ465755	FJ465560	FJ465629	-	pSP6	Spain	Cabo da Gata	-	-	mH16	-	Almada et al. 2009
S. pavo	FJ465699	FJ465751	FJ465576	FJ465630	-	pSP7	Spain	Cabo de Gata	-	-	mH1	-	Almada et al. 2009
S. pavo	FJ465700	FJ465752	FJ465578	FJ465631	-	pSP8	Spain	Cadiz	-	-	mH8	-	Almada et al. 2009
S. pavo	FJ465701	FJ465753	FJ465575	FJ465634	-	pSP9	Spain	Cadiz	-	-	mH12	-	Almada et al. 2009
S. pavo	FJ465702	FJ465754	FJ465557	FJ465617	-	pSP10	Spain	Barcelona	-	-	mH36	-	Almada et al. 2009
S. pavo	-	-	-	-	JQ697370	Spav	Portugal		-	-	-	nRH1	Levy et al. 2013
S. basilisca	MH714470	MH724821	MH715445	-	-	GRHbas1	Tunisia	Ghar El Melh	37.15147	10.17770	mH35	-	present work
S. basilisca	MH714471	MH724822	MH715446	-	-	BIZbas1	Tunisia	Bizerte lagoon	37.22876	9.84539	mH6	-	present work
S. basilisca	MH714482	MH724833	MH715457	MH715418	MH715438	BIZbas2	Tunisia	Bizerte lagoon	37.22876	9.84539	mH37	nSH4 nRH2	present work
S. basilisca	MH714481	MH724832	MH715456	MH715411	-	LTSbas1	Tunisia	South lake of Tunis	36.81581	10.23672	mH29	nSH5	present work
S. basilisca	-	-	-	-	MH715432	LTSbas2	Tunisia	South lake of Tunis	36.81581	10.23672	-	nRH2	present work
S. basilisca	MH714479	MH724830	MH715454	MH715413	-	LTSbas3	Tunisia	South lake of Tunis	36.81581	10.23672	mH30	nSH5	present work
S. basilisca	MH714472	MH724823	MH715447	MH715412	MH715433	SAYbas1	Tunisia	Sayeda	35.67791	10.91640	mH34	nSH5 nRH2	present work
S. basilisca	MH714468	MH724819	MH715443	MH715415	-	CHBbas1	Tunisia	Chebba	35.21145	11.13580	mH28	nSH5	present work
S. basilisca	MH714492	MH724843	MH715467	-	-	JBEbas1	Tunisia	La Louza	35.01936	11.02126	mH39	-	present work
S. basilisca	MH714493	MH724844	MH715468	MH715416	MH715430	JBEbas2	Tunisia	La Louza	35.01936	11.02126	mH4	nSH5 nRH2	present work
S. basilisca	MH714467	MH724818	MH715442	MH715414	MH715437	SFXbas1	Tunisia	Sfax	34.76116	10.84130	mH23	nSH5 nRH2	present work
S. basilisca	MH714469	MH724820	MH715444	-	-	GABbas1	Tunisia	Gabès	33.90618	10.12076	mH38	-	present work
S. basilisca	MH714476	MH724827	MH715451	MH715417	-	GABbas3	Tunisia	Gabès	33.90618	10.12076	mH4	nSH5	present work
S. fluviatilis s.l.	MH714496	MH724847	MH715471	MH715427	MH715428	Flu393	Italy	Torrente Frattina (Sicily)	37.86189	13.30301	mH44	nSH6 nRH4	present work
S. fluviatilis s.l.	MH714497	MH724848	MH715472	MH724808	MH715429	Flu585	Italy	Lake Garda	45.46138	10.63005	mH58	nSH6 nRH4	present work
S. fluviatilis s.l.	FJ465688	FJ465746	FJ465550	FJ465606	-	fCR2	Croatia	-	-	-	mH58	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465689	-	FJ465551	FJ465607	-	fCR3	Croatia	-	-	-	-	-	Almada et al. 2009
S. fluviatilis s.l.	FJ465645	FJ465725	FJ465542	FJ465597	-	fGR1	Greece	River Miras	-	-	mH57	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465646	FJ465738	FJ465539	FJ465603	-	fGR2	Greece	Lake Dojranis	-	-	mH46	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465653	FJ465729	FJ465538	FJ465598	-	fGR3	Greece	River Miras	-	-	mH57	nHS6	Almada et al. 2009

Taxon	12S A.N.	16S A.N.	Dloop A.N.	S7 A.N.	Rhodopsin A.N.	Code	Country	Location	Latitude (N)	Longitude (E)	mtDNA Haplotype	nuDNA Haplotype	Source
S. fluviatilis s.l.	FJ465656	FJ465739	FJ465543	FJ465602	-	fGR4	Greece	Lake Dojranis	-	-	mH43	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465683	FJ465718	FJ465567	FJ465625	-	fISR1	Israel	-	-	-	mH50	nSH9	Almada et al. 2009
S. fluviatilis s.l.	FJ465684	FJ465722	FJ465565	FJ465621	-	fISR2	Israel	-	-	-	mH49	nSH9	Almada et al. 2009
S. fluviatilis s.l.	AY098797	AY098843	AY098865	-	-	fPT1	Portugal	River Guadiana	-	-	mH47	-	Almada et al. 2009
S. fluviatilis s.l.	FJ465648	FJ465741	FJ465521	FJ465604	-	fSP1	Spain	River Noguera-Pallaresa	-	-	mH45	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465649	FJ465744	FJ465533	FJ465596	-	fSP2	Spain	River Matarraña	-	-	mH56	-	Almada et al. 2009
S. fluviatilis s.l.	FJ465650	FJ465730	FJ465528	FJ465594	-	fSP3	Spain	Lake Calahorra	-	-	mH55	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465651	FJ465745	FJ465532	FJ465605	-	fSP4	Spain	River Noguera-Pallaresa	-	-	mH55	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465652	FJ465740	FJ465522	FJ465595	-	fSP5	Spain	River Matarraña	-	-	mH55	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465654	FJ465724	FJ465529	FJ465587	-	fSP6	Spain	River Verde	-	-	mH59	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465655	FJ465743	FJ465524	FJ465586	-	fSP7	Spain	River Verde	-	-	mH59	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465660	FJ465734	FJ465525	FJ465590	-	fSP8	Spain	River Zújar	-	-	mH53	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465661	FJ465732	FJ465531	FJ465589	-	fSP9	Spain	River Zujar	-	-	mH53	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465662	FJ465728	FJ465530	FJ465588	-	fSP10	Spain	River Esteras	-	-	mH48	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465665	FJ465726	FJ465523	FJ465593	-	fSP11	Spain	Lake Calahorra	-	-	mH55	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465690	FJ465747	FJ465556	FJ465638	-	fSP12	Spain	Lake Banõles	-	-	mH56	-	Almada et al. 2009
S. fluviatilis s.l.	FJ465691	FJ465748	FJ465566	FJ465637	-	fSP13	Spain	Lake Banõles	-	-	mH56	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465657	FJ465731	FJ465535	FJ465610	-	fTRK2	Turkey	Lake Iznik	-	-	mH42	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465658	FJ465727	FJ465536	FJ465611	-	fTRK3	Turkey	Ilica	-	-	mH52	nSH9	Almada et al. 2009
S. fluviatilis s.l.	FJ465659	FJ465723	FJ465537	-	-	fTRK4	Turkey	River Catk t	-	-	mH51	nSH6	Almada et al. 2009
S. fluviatilis s.l.	FJ465687	-	FJ465549	FJ465585	-	fTRK5	Turkey	River Tahtal	-	-	-	-	Almada et al. 2009
S. fluviatilis s.l.	-	-	FJ465534	FJ465612	-	fTRK6	Turkey	Stream Çak rca	-	-	-	-	Almada et al. 2009
S. fluviatilis s.l.	-	-	-	-	JQ697369	Sflu	Portugal	-	-	-	-	nRH3	Levy et al. 2013
S. atlantica	FJ465663	FJ465736	FJ465527	FJ465591	-	fMRC1	Morocco	River Overrha	-	-	mH54	nSH8	Almada et al. 2009
S. atlantica	FJ465664	FJ465737	FJ465526	FJ465592	-	fMRC2	Morocco	River Overrha	-	-	mH54	nSH8	Almada et al. 2009
S. economidisi	FJ465643	FJ465733	FJ465540	FJ465600	-	eGR1	Greece	Lake Trichonis	-	-	mH40	nSH7	Almada et al. 2009
S. economidisi	FJ465644	FJ465735	FJ465541	FJ465599	-	eGR2	Greece	Lake Trichonis	-	-	mH41	nSH7	Almada et al. 2009
Parablennius salensis	AY098789	AY098836	AY098863	FJ465581	-	-	-	-	-	-	-	-	Almada et al. 2009

Graphical abstract



Highlights

- *Salaria* marine and freshwater species belong to two independent clades
- Freshwater species are vicariant taxa with concordant mtDNA and nuDNA phylogenies
- Lack of reciprocal mtDNA monophyly is observed for the marine species of the genus
- The observed mito-nuclear discordance is due to introgressive hybridization
- Prudence is urged when implementing DNA barcoding approaches to fish identification