New insights into the phylogeography and evolutionary history of two Mediterranean clingfish genera (Teleostei, Gobiesocidae)

Maximilian Wagner, BSc

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Priv.-Doz. Mag. Dr.rer.nat Koblmüller Stephan

Karl-Franzens Universität Graz

Institut für Zoologie

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“I have had the privilege of working on a valuable and beautiful group of fishes. However, their value is not economic but biological and their beauty lies in their marvellous adaption to their habitat.”

John C. Briggs

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Summary

Throughout the Mediterranean Sea, the clingfish genera *Lepadogaster* and *Gouania* (Teleostei, Gobiesocidae) are found in the interstitial of pebble and boulder beaches. Even though these fishes can reach fairly high abundances, the overall knowledge about the two genera is still very poor. Hence, this thesis should provide novel insights into diversity, evolution and distribution of these two clingfish taxa.

The first part of this work addresses phylogenetic and biogeographic implications in the clingfish species *Gouania willdenowi* (Risso 1810), the sole representative of the monotypic genus *Gouania*. Endemic to the Mediterranean Sea hardly anything is known about its biology, behaviour, phylogeny or taxonomy of this species. DNA-barcoding, multi-locus species tree analysis, as well as geometric morphometrics and MicroCT imaging across a pan-Mediterranean sample, revealed unexpected results regarding evolutionary history and species diversity in this genus. Molecular data suggests that the genus underwent a thus far unrecognized Pleistocene radiation into several fairly divergent species. Moreover, whereas little diversity is seen in the western basin, convergently evolved syntopic species pairs are found throughout the eastern basin and the Adriatic Sea. Apparently, the extremely low active dispersal ability of adults implies that larval drift strongly constrains gene flow among species in different Mediterranean basins.

The second part of the thesis reports on a significant extension of the distribution range of the species *Lepadogaster purpurea*. A combined approach of molecular and classical morphometrics on newly collected material from the eastern Mediterranean basin showed that the geographic distribution of this fish has been underestimated in the literature. Based on already published results (Wagner et al. 2017), preliminary insights into phylogeographical structure of *L. lepadogaster* and *L. purpurea* indicate marked differences between these two species.

Zusammenfassung

Die beiden Schildfischgattungen *Gouania* und *Lepadogaster* (Teleostei, Gobiesocidae) sind typische Bewohner von Kies- und Geröllstränden entlang der gesamten Mittelmeerküste. Trotz hoher Individuendichten im Lebensraum ist unser Wissen über diese Fischarten dennoch stark begrenzt. Das Ziel dieser Arbeit ist es Wissenslücken, welche vor allem die Diversität und Evolution dieser Gattungen im Mittelmeer betreffen, zu füllen.

Der erste Teil der Arbeit beschäftigt sich mit der monotypischen Gattung *Gouania* und der dazugehörigen endemischen Art *Gouania willdenowi* (Risso 1810). Aufgrund ihrer versteckten Lebensweise, im Zwischenraum von Kiesstränden, bekam sie nur wenig Aufmerksamkeit in wissenschaftlichen Studien und zählt damit sicher zu den am wenigsten erforschten Schildfischen überhaupt. Durch die Kombination aus molekularen, geometrisch morphometrischen Methoden, sowie durch MicroCT Aufnahmen gelang es bemerkenswerte Einblicke in die Diversität und Evolution dieser Art zu bekommen. Die Ergebnisse deuten auf eine bislang unbekannte Radiation im Pleistozän hin, aus der sich mehrere hoch divergente Arten entwickelten. Des Weiteren, ergaben morphologische Untersuchungen das Vorhandensein von zwei konvergent entstandenen Ökomorphen in der Adria und im östlichen Mittelmeerbecken. Ein Grund für den reduzierten Genfluss in den verschiedenen Becken könnte die kurze pelagische Larvendauer und die limitierte aktive Ausbreitungsfähigkeit adulter Tiere sein.

Der zweite Teil der Arbeit beschäftigt sich mit der Gattung Lepadogaster, genauer gesagt mit den Schwesterarten *L. lepadogaster* und *L. purpurea*. Im Zuge meiner Sammelaktivitäten konnte die Verbreitung von *L. purpurea* mit Hilfe von molekularen und morphologischen Daten signifikant erweitert werden. Basierend auf diesen bereits publizierten Daten (Wagner et al. 2017) gebe ich in dieser Arbeit vorläufige phylogeographische Einblicke in diese beiden Schwesterntaxa und zeige, dass sich diese stark voneinander unterscheiden.

I Introduction

The family of clingfish (Gobiesocidae, Gobiesociformes), generally small cryptobenthic fishes, is distributed worldwide in temperate and tropical waters (Briggs 1955). Apart from the typical adaptions to a benthic lifestyle, such as a compressed body, a lack of scales or swim bladders, a key feature of their evolution is a unique trait, a thoracic adhesive disc, which, during larval metamorphosis, is developed from parts of their ventral and pectoral fins (Allen 1984). The disc bears numerous papillae, which are important characters for species delimination, also among closely related species and are responsible for the sucking capacity (Briggs 1955; Wainwright et al. 2013). These papillae are full of fine structures, microvilli, comparable to the hairs of geckos or salticid spiders that allow the fish to reversibly cling on preferably rough surfaces without deprivation of the sucking force (Pennisi 2012; Wainwright et al. 2013). Hence, it is not very surprising that this unique way of adherence, enabled them to occupy a variety of extreme habitats such as of fast flowing freshwater streams (Guzmán et al. 2001; Conway et al. 2017a) or exposed environments like the rocky intertidal zone (Ditsche et al. 2014).

Most of the members of the clingfish family inhabit coastal marine habitats and sometimes occupy highly specialized niches such as the leafs of seagrass meadows, the spines of sea urchins or occur in the interstices of pebble and boulder beaches (Patzner 1999a, b; Hofrichter and Patzner 2000). Whereas some species can be found in the deep sea, down to a depth of 337 m (Hutchings 1991; Moore et al. 2012; Sparks and Gruber 2012), others inhabit the intertidal zone and evolved passive and active amphibious behaviours (Ebeling et al. 1970; Bilecenoğlu 2015). Apart from that, cleaning behaviour has been observed (Weitzmann and Mercader 2012; Fricke et al. 2015) and some carry venomous glands on the subopercle (Conway et al. 2014).

Even though the importance of small cryptobenthic fishes for the marine environment has been recognized by a variety of authors (e.g. Ackerman and Bellwood 2000; Depczynski and Bellwood 2003), the overall knowledge about the taxonomy, biology, ecology or evolution of cryptobenthic fishes, including the clingfishes, is still very poor. Thus far, according to the “Eschmeyer’s Catalog of Fishes” (Eschmeyer et al. 2006, visited: 04.01.2017) 165 species in 48 genera of Gobiesocidae have been described worldwide, but, despite the growing number of newly described species in the last few years (e.g. Fricke et al. 2010;

Conway et al. 2014; Fricke et al. 2015; Bilecenoğlu et al. 2017; Conway et al. 2017b; Fricke and Wirtz 2017), a complete species inventory still will take a lot of effort. A major taxonomic revision seems difficult, mainly because of poor species descriptions in the past that led to high numbers of synonyms (e.g. Henriques et al. 2002) and due to a low level of taxonomic expertise that still underestimates or overlooks very abundant taxa (Wagner et al. 2017). In addition to that, their cryptic behaviour precludes efficient sampling (Willis 2001; Brandl et al. 2011) and makes in vivo observations without anaesthesia for some taxa almost impossible.

Despite the advantages of molecular methods, such as DNA-Barcoding, to aid in describing and delimiting species and populations (Hebert et al. 2003; Ward et al. 2005), it has rarely been applied to clingfish (e.g. Hickerson and Ross 2001; Henriques et al. 2002; Almada et al. 2008; Conway et al. 2014, 2017a; Bilecenoğlu et al. 2017). Some recent species descriptions even completely resign to use molecular methods and are only based on a couple of morphological traits (Fricke et al. 2015; Fricke and Wirtz 2017). Since there is no doubt that the combination of morphological, molecular as well as ecological and behavioural methods will increase our understanding of clingfish evolution and systematics (Wahlberg et al. 2005), a combined approach employing different methods should fill knowledge gaps in this fish family (Williams and Tyler 2003; Conway et al. 2017a).

As a result, until no deeper understanding is achieved, some authors propagate to use the “old” nine subfamily classification by Briggs (1955), in order to avoid confusion during the ongoing taxonomic revision of this group (Conway et al. 2017a).

Mediterranean clingfishes

The Mediterranean clingfish fauna currently includes nine valid species: *Gouania willdenowi* (Risso, 1810), *Lepadogaster lepadogaster* (Bonnaterre, 1788), *Lepadogaster purpurea* (Bonnaterre, 1788*)*, *Diplecogaster bimaculata* (Bonnaterre, 1788), *Diplecogaster umutturali* (Bilecenoğlu et al. 2017), *Apletodon dentatus* (Facciola, 1887), *Apletodon incognitus* (Hofrichter and Patzner, 1997), *Mirbelia candolii* (Risso, 1810) and *Opeatogenys gracilis* (Canestrini, 1964). According to Briggs (1995), all members of the Mediterranean clingfish fauna belong to the subfamily Lepadogastrinae which is characterised by a double sucking disc, an attached gill membrane to the Isthmus and 3.5 gills. However, the status of this subfamily is highly controversial and will need major revision in the future.

The habitat preferences of Mediterranean clingfishes have been investigated thoroughly in the past (Fig. 1). *Gouania willdenowi*, *L. purpurea*, *L. lepadogaster* and *M. candolii* prefer pebble beaches, rocky boulder fields and crevices of larger rocks. *D. bimaculata* can be found on sandy substrate, close to seagrass beds and in empty shells, where they even reproduce (Hofrichter 1995; Brandl et al. 2011). *Apletodon incognitus* is highly adapted to a life in-between the spines of sea urchins (e.g. *Arbacia lixula, Sphaerechinus granularis, and Paracentrotus lividus*), its sister species *A. dentatus* prefers *Cystoseira* beds, but can also be found among sea urchin spines (Gonçalves et al. 2002). One of the most camouflaged Mediterranean clingfish species is *O. gracilis*, which lives exclusively on leaves of the seagrass *Posidonia oceanica* (Hofrichter and Patzner 2000).

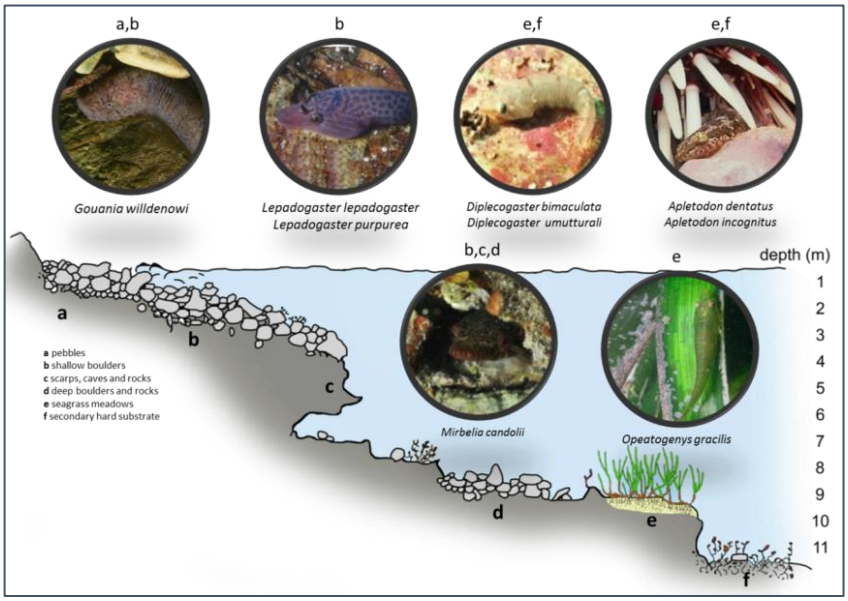

Fig. 1 The Mediterranean clingfish fauna and microhabitat preferences of single species. Drawing changed after Hofrichter (1993) p. 33[1].

Even though some authors took great efforts to study various aspects of the European clingfish fauna, our overall knowledge about is still very poor. Whereas some studies worked on ontogenetic and behavioural questions (Gonçalves et al. 1996, 1998, 2002; Faria and Goṇalves 2010; Tojeira et al. 2012), others mainly tackled their ecology and biology (Hofrichter 1995; Patzner 1999a; Hofrichter and Patzner 2000; Kovačić et al. 2012). Again others focussed on population structure and discussed biogeographical questions (Klein 2016; Klein et al. 2016; Wagner et al. 2017), or aimed at establishing more efficient sampling methods (e.g., Brandl et al. 2011).

[1] Hofrichter R (1993) Beitrag zur Kenntnis der mediterranen Schildfische (Teleostei, Gobiesocidae) mit besonderer Berücksichtigung der Fortpflanzung von *Lepadogaster lepadogaster.* Diploma-Thesis.

II Aims of this work

This work should provide novel insights into diversity, evolution and distribution of some selected European clingfish taxa, and thus contributed to slowly, but steadily, increasing knowledge of this charismatic group of fishes. In the following, the thesis will be split into two subchapters, the first one targeting the genus *Gouania* Nardo, 1833, the second one focusing on the genus *Lepadogaster* Gouan, 1770.

PART I

The genus *Gouania* (Gobiesocidae): a thus far overlooked radiation of cryptobenthic fishes in the Mediterranean Sea.

The morphological and genetic investigation of a pan Mediterranean sample of the genus *Gouania* reveals interesting results according to the diversity and evolution of this genus. The aim of this part is to deliver a new promising model system for studying evolutionary processes and underlying forces, such as oceanic currents, in marine environments.

PART II

First phylogeographic insights into the genus *Lepadogaster* (Gobiesocidae) (Based on new records by Wagner et al. 2017)

Based on a combined approach of molecular and morphometric tools I provide evidence that the distribution of this fish species has been underestimated in the literature. Based on these results – published in Wagner et al. 2017 (see Supplementary material for Part II) – the second part of this thesis will give preliminary insights into phylogeographic differences between the sister species *Lepadogaster lepadogaster* and *L. purpurea*.

PART I

The genus *Gouania* (Gobiesocidae): a thus far overlooked radiation and convergent evolution of cryptobenthic fishes in the Mediterranean Sea.

Gouania willdenowi (Risso, 1810)

1.1 Introduction

The blunt snouted clingfish, *Gouania willdenowi* (Risso 1810), is the sole representative of the genus *Gouania* Nardo, 1833 and is endemic to the Mediterranean Sea. With its wormlike body, small eyes, rudimentary dorsal and anal fins it is perfectly adapted to a life in the interstices of pebble beaches (Hofrichter and Patzner 2000) and gives the species an overall unique appearance among all other members of the subfamily Lepadogastrinae (Hofrichter 1995). Even though *G. willdenowi* reaches high abundances (up to 24 individuals per m^2) in suitable habitat (Hofrichter and Patzner 2000), most of the current knowledge, except for those of Hofrichter (1995) and his subsequent work (Hofrichter and Patzner 2000), seems to have accumulated either coincidentally (Bilecenoğlu 2015; Brandl et al. 2011) or in course of biodiversity assessments (Kovačić 1998). Hence, it is greatly underrepresentated compared to the huge amount of scientific work available for its sister genus *Lepadogaster* (e.g. Gonçalves et al. 1996; Gonçalves et al. 1998; Henriques et al. 2002; Almada et al. 2008; Faria and Goṇalves 2010; Tojeira et al. 2012; Klein et al. 2016; Wagner et al. 2017). It is for sure its cryptic lifestyle that makes research difficult, even though it can be found in almost every suitable habitat – also in strongly frequented beaches (Wagner unpubl.) – from 2 m depth up to the intertidal zone (Hofrichter and Patzner 2000).

Whereas Hofrichter and Patzner (2000) hesitated to call *G. willdenowi* an amphibious species, more recently Bilecenoğlu (2015) described for the first time passive amphibious emergence behaviour and showed that the fish can survive for two hours exposed to the surface without being negatively affected. This sounds essential, considering the species' occurrence in the intertidal zone that brings along changes in water availability caused by tidal variation. Also, own observations from the Adriatic Sea showed that, during low-tide, individuals can be found outside the water in deeper layers of pebbles (compare with Hofrichter and Patzner 2000).

So far, only little is known about the biology of *G. willdenowi*. In general, Hofrichter (1995) assumes that all Mediterranean clingfishes show high similarities in their spawning behaviour (paternal nest care) and spawning period (mainly springtime). Furthermore, he for the first time, managed to find nests of this species in Messina, underneath smooth surfaced boulders in shallow water. Apart from that, he redescribed – after Facciola (1887) – a conspicuous sexual dimorphism whereby males tend to have finger like appendices on the

sucking disc, that are well supplied with blood. Even though this trait is very exclusive among all members of the subfamily Lepadogastrinae, the role of it is still unclear and surprisingly, it has been ignored by many authors such as Briggs (1995), who intensively worked on the clingfish family (Hofrichter 1995).

Concerning the phylogenetic background or evolutionary history close to nothing is known. Almada et al. (2008) shows that *Gouania* is the sister genus of *Lepadogaster*, but the phylogeny is only based on two rDNA markers and includes one single *Gouania* specimen from the Adriatic Sea. No bio- or phylogeographic studies have been conducted so far, even though, the species' distribution all across the Mediterranean Sea – from Israel to southeastern Spain with some records from Algeria and Syria (Briggs 1986; Hofrichter 1995) – as well as its peculiar lifestyle and planktonic larval phase would make it a highly interesting study system (especially with regards to potential phylogeographic structure witin the species).

As a result, the monotypic genus *Gouania* is for sure one of the least investigated European clingfish species. Thus, for this study, a pan-Mediterranean sample of this genus was investigated. A combined approach of molecular as well as classical- and modern-morphometrical methods gives insights into the species diversity and sheds a light on the underlying evolutionary factors.

1.2 Material and Methods

1.2.1 Sampling

Sampling was conducted during 2014 - 2016 across the Mediterranean Sea (Fig. 2A). A detailed list including coordinates is given in *Supplementary Table 1*. Specimens were primarily collected with a simple bucket at pebble beaches or boulder fields from the waterline down to one meter depth (Fig. 2B). Aquarium nets were used to collect specimens from deeper water, by turning stones. After collecting, fishes were anaesthetised with MS-222. Standardized pictures were made from the lateral and dorsal side of the fish using a Nikon DSLR camera combined with a 105 mm 2.8 Macro-Lens. Following this, specimens were either fixed in > 95 % Ethanol or Formalin 7 %. However, for all specimens fixed in Formalin, fin clips were stored in ethanol for subsequent DNA extraction.

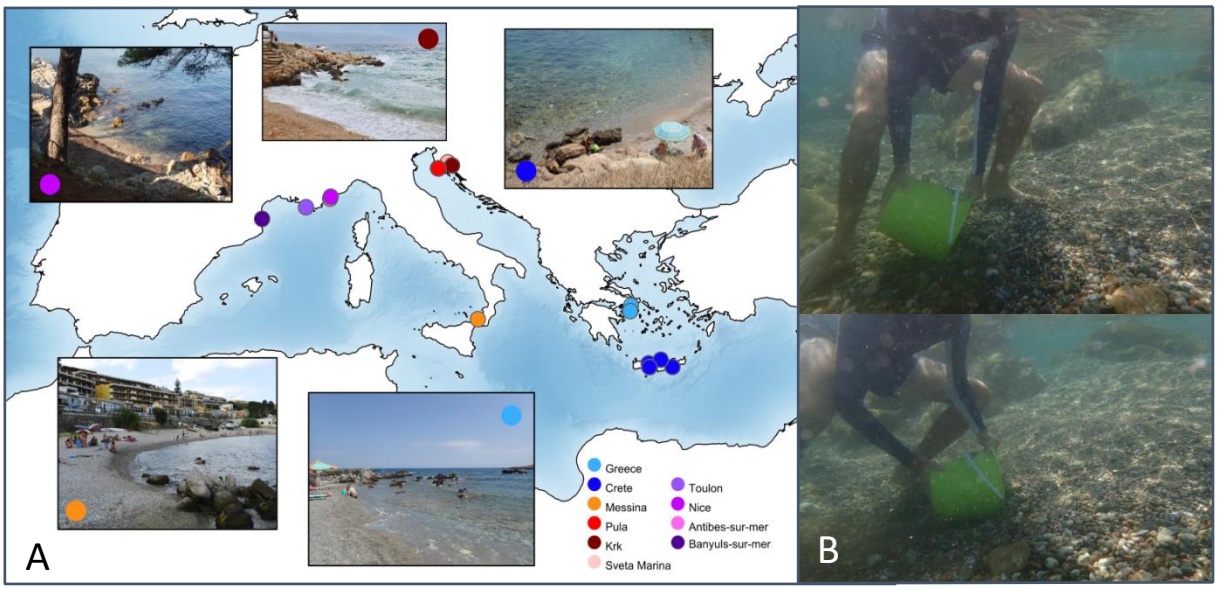

Fig. 2 Sampling localities (**A**) and sampling method (**B**): A detailed list of sampling sites is shown in the Appendix *Supplementary Table 1*. The sampling map was constructed in SimpleMappr (Shorthouse 2010).

1.2.2 DNA extraction, amplification and sequencing

DNA was extracted from fin or muscle tissue using the rapid Chelex protocol (Richlen and Barber 2005). In total 10 genetic markers were amplified according to the protocols in Duftner et al. 2005 and Li et al. 2007. A detailed list including primer pairs, annealing temperatures and number of amplification cycles for each marker is given in *Table 1.* DNA

fragments were purified with SephadexTM G-50 (GE Healthcare) and visualized on an ABI 3130xl capillary sequencer (Applied Biosystems).

In a first step, DNA barcodes based on the mtDNA COI gene (Hebert et al. 2003) of 97 individuals of the genus *Gouania* as well as another 11 specimens from the genus *Lepadogaster* (incl. *L. purpurea* and *L. lepadogaster*) were generated. In addition, following Li et al. 2007, nine nDNA markers (Table 1) were sequenced for 37 individuals of *Gouania* and two outgroup specimens, *L. lepadogaster* and *L. purpurea*.

1.2.3 Sequence alignment and phylogenetic analysis

Sequences were aligned in MEGA 7.0 (Kumar et al. 2016) using MUSCLE (Edgar 2004). Aligned sequences were first tested for "Best fitting substitution model" in MEGA, employing the Bayesian Information Criterion (BIC). The six best models, according to the analysis in MEGA, are shown in *Table 1*. In a first step, a maximum likelihood (ML) based phylogenetic analysis using RAxML-HPC version 8 (Stamatakis 2014) was conducted for the COI gene using GTR-GAMMA model and 10 000 Bootstraps (BS), as well as Bayesian inference (BI) approach in MrBayes v.3.2.6 (Ronquist et al. 2012), which was run for 50 million generations and a sampling frequency of 10.000. Apart from that, mean net distances between clades were calculated in MEGA using Kimura-2-paramter model and maximum-parsimony networks were inferred for COI-gene sequences in PopArt (Leigh and Bryant 2015).

In a second step the genetic data including all genes was concatenated into two major data sets: (1) including only nDNA markers and (2) a combined dataset of mtDNA and nDNA markers (Table 1). ML and BI phylogenetic analyses were conducted for both data sets. Again, ML was run in RAxML for 1000 BS replications and BI analysis was run in MrBayes starting from a random tree over 20 million generations with a sampling frequency of 10.000, employing the best fitting model for each gene (Table 1).

Furthermore, a Species Tree was inferred using the StarBeast2 (*BEAST) package (Heled and Drummond 2010) implemented in BEAST v.2.4.7 (Bouckaert et al. 2014). All prior information was set in BEAUti. Following settings were made: Population model: "Analytical Population Size Integration", gene ploidy according to genetic marker, best fitting substitution models according to the model test in MEGA (Table 1). To obtain absolute divergence times, clock rates inferred by Conway et al. (2017a); based in part on Near et al.

(2013) for five of the ten markers were employed. Different clock rates and models are shown in *Table 1*.

Chain convergence and stationarity was assessed in Tracer 1.6 (Rambaut and Drummond 2009). In all of the BI analyses, effective sample sizes (ESS) exceeded a value of 200, implying an accurate representation of the posterior distribution (Kuhner 2009). COI-Barcoding and concatenated trees were visualized in FigTree v1.4.2. The species tree was created in DensiTree2 (Bouckaert and Heled 2014).

Different clades were defined as followed: more than 3 % divergence on mtDNA and – for distinguishing syntopically occurring genetic lineages – occurrence of star-shaped pigmentation around the eyes with asterisks ("*") or with a tilde ("~").

1.2.4 Geometric morphometrics and classical morphometrics

Based on photographs of life specimens (see 1.2.1) a 2D geometric morphometric (GM) approach was used to infer potential shape differences among genetic lineages. GM analyses are based on the use of homologous points, so called landmarks that can be described by Cartesian coordinates by a certain position of a biological trait (Bookstein 1991). However, *Gouania* lacks many morphological traits (e.g. dorsal fins, anal fins), which are usually used for GM analysis in other Actinopterygians, such as cichlids or cryptobenthic fishes (e.g. Clabaut et al. 2007; Herler et al. 2010; Untersteggaber et al. 2014). Hence, a new set of 10 and 8 landmarks was developed for the dorsal and lateral site respectively for this fish genus. In addition, a set of 21 and 20 semi-landmarks for both sides were used to quantify differences in curved head and tail regions of the fishes. A detailed description of all homologous landmarks and semi-landmarks is shown in *Fig. 3* and in the *Appendix Part I*.

After randomizing images in tpsUtil 1.6 (Rohlf 2015), 143 and 134 individuals were digitized with pixel-based landmarks from dorsal and lateral images, respectively, in tpsDig 2.26 (Rohlf 2016a). For preventing unwanted photographic artefacts (such as irregular bending of the fish) the "Unbending specimens" function in tpsUtil was used (Haas 2011) for the dorsal landmark configuration. Therefore, the semi-landmarks 19 – 21 functioned as an "unbending-axe". Following this, the tps-files were aligned in tpsRelw 1.65 (Rohlf 2016b).

Table 1 Primer compositions, annealing temperatures, numbers of cycles, 6 best substitution models and length of amplified fragment (bp), clock rates (if known) for all genetic markers used in this study. Table was changed after Li et al. 2007.

Gene*	Primers sequences	Annealing	# cycles	Models**	bp	Rate***/model
zic1						
zic1_F9	5' GGACGCAGGACCGCARTAYC 3'	55-57 °C	32-35	1st T92, 2nd T92+G, 3rd T92+I, 4th HKY, 5th K2, 6th JC;	741	4.8855E-4/strict
zic1_R967	5' CTGTGTGTGTCCTTTTGTGRATYTT 3'					
myh6						
myh6_F459	5' CATMTTYTCCATCTCAGATAATGC 3'	51 °C	35	1st JC, 2nd K2, 3rd JC+I, 4th JC+G, 5th T92, 6th K2+I;	697	0.0020889/strict
myh6_R1325	5' ATTCTCACCACCATCCAGTTGAA 3'					
RYR3						
RYR3_F15	5' GGAACTATYGGTAAGCARATGG 3'	52 °C	36	1st K2, 2nd T92, 3rd K2+I, 4th K2+G, 5th T92+I, 6th JC;	735	N.A.
RYR3_R968	5' TGGAAGAAKCCAAAKATGATGC 3'					
tbr1						
tbr1_F86	5' GCCATGMCTGGYTCTTTCCT 3'	51 °C	39	1st T92, 2nd T92+I, 3rd T92+G, 4th HKY, 5th JC, 6th T92+G+I;	534	N.A.
tbr1_R811	5' GGAGCAGTTTTTCTCRCATTC 3'					
ENC1						
ENC1_F85	5' GACATGCTGGAGTTTCAGGA 3'	53 °C	35	1st K2, 2nd T92, 3rd K2+I, 4th K2+G, 5th T92+I, 6th T92+G;	688	N.A.
ENC1_R982	5' ACTTGTTRGCMACTGGGTCAAA 3'					
Glyt						
Glyt_F577	5' ACATGGTACCAGTATGGCTTTGT 3'	50-51 °C	39-40	1st K2, 2nd T92, 3rd JC, 4th K2+I, 5th K2+G, 6th T92+I;	651	0.0025593/strict
Glyt_R1464	5' GTAAGGCATATASGTGTTCTCTCC 3'					
SH3PX3						
SH3PX3_F461	5' GTATGGTSGGCAGGAACYTGAA 3'	48 °C	42	1st JC, 2nd K2, 3rd JC+I, 4th JC+G, 5th T92, 6th K2+I;	564	0.0013301/strict
SH3PX3_R1303	5' CAAACAKCTCYCCGATGTTCTC 3'					
plagl2 ****						
plagl2_F9	*5' CCACACACTCYCCACAGAA 3'*	54 °C	37	1st T92, 2nd T92+I, 3rd T92+G, 4th K2, 5th HKY, 6th HKY+I;	617	N.A.
plagl2_R930	5' TTCTCAAGCAGGTATGAGGTAGA 3'					
plagl2_R920	*5' GGTATGAGGTAGATCCSAGCTG 3'*					
sreb2						
sreb2_F10	5'ATGGCGAACTAYAGCCATGC 3'	54 °C	37	1st K2, 2nd JC, 3rd T92, 4th HKY, 5th K2+I, 6th K2+G;	731	N.A.
sreb2_R1094	5' CTGGATTTTCTGCAGTASAGGAG 3'					
COI						
FishF1	5' TCAACCAACCACAAAGACATTGGCAC 3'	52 °C	35	1st TN93+I, 2nd HKY+I, 3rd TN93+G+I, 4th HKY+G+I, 5th K2+I, 6th K2+G+I;	610	0.0323/uncor. log. norm.
FishR1	5' TAGACTTCTGGGTGGCCAAAGAATCA 3'					

*Gene markers are named following annotations in ENSEMBLE. zic1, zic family member 1; myh6, myosin, heavy polypeptide 6; RYR3 (si:ch211- 189g6.1), novel protein similar to vertebrate ryanodine receptor 3; Ptr (si:ch211-105n9.1), hypothetical protein LOC564097; tbr1, T-box brain 1; ENC1(559445 Entrezgene), similar to ectodermal-neural cortex 1; Glyt (zgc:112079), glycosyltransferase; SH3PX3, similar to SH3 and PX domain containing 3 gene; plagl2, pleiomorphic adenoma gene-like 2; sreb2, Super conserved receptor expressed in brain 2.
**Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor; +I: assuming invariable sites; +G: discrete Gamma distribution
***Clock rates per millions of years (Conway et al. 2017a & Near et al. 2013)
****For *Lepadogaster* plagl2_F9 und plagl2_R920 -> 56,5 °C – 45 cycles

General Procrustes Analysis (Rohlf and Slice 1990) and Principal component analysis (PCA) were conducted MorphoJ (Klingenberg 2011). Because PC2 mainly represented a major photographic artefact, it was excluded for the analysis of the dorsal landmark configuration. Allometry, usually affecting the first principle component of single species or populations (Klingenberg 1998), had no major impact on shape differences (Fig. 9).

Additionally, from all vouchers standard length (SL) and horizontal diameter of the eye was measured from pictures in tpsDig. Calculations and Boxplots were visualized in RStudio.

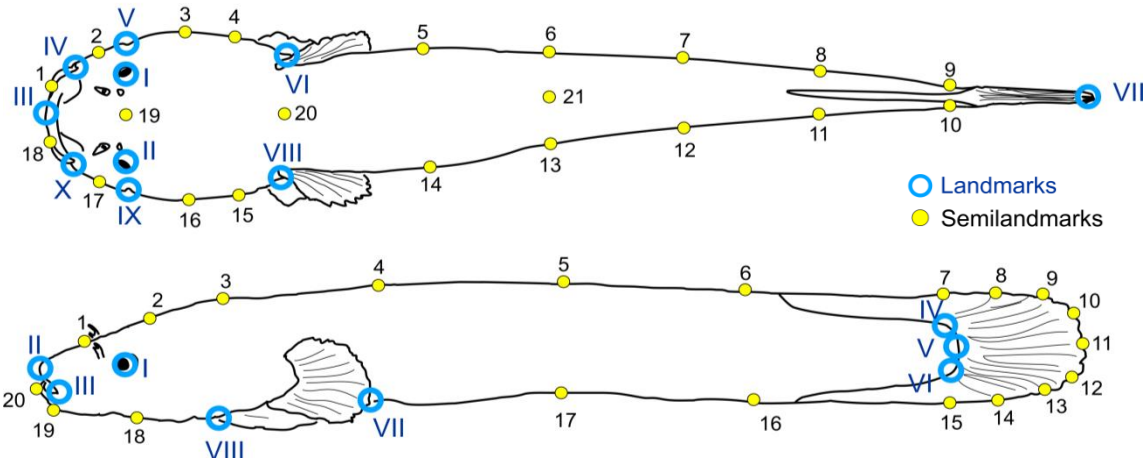

Fig. 3 Landmark (blue circles) and semi-landmark (yellow blotches) configuration used for this study. A detailed description for each landmark can be found in the Appendix.

1.2.5 Micro-CT Imaging and image processing

Despite the geometric morphometric approach, micro-computer-tomography (μCT) was used to obtain pictures of the osteology of the fishes with the major aim of counting number of vertebrae. All scanned specimens were fixed in 7 % formalin. Scanning was conducted at the University of Graz. Fish were fixed in a 15 mm diameter tube including formalin and scanned with 1600 – 3000 slices every 15 to 20 μm.

Raw tiff-files were cropped and stacked in FIJI 1.0 and imported into drishtiimport. 3D modelling was conducted in Drishti v.2.6.4 (Limaye 2012). From 3D models (see Supplementary Fig. 1) vertebrae were counted and differences between genetic lineages were visualized by means of a bubble diagram in Excel v.15.40 (Fig. 8B).

1.3 Results

1.3.1 DNA barcoding

DNA barcoding of more than 90 individuals revealed high cryptic diversity in the genus *Gouania*. In total, 6 highly distinct clades emerged after analysing a 560 bp long fragment of the mtDNA COI gene (Fig. 4). Apart from the originally described *Gouania willdenowi* (Risso, 1810), which is distributed throughout the western Mediterranean, from Messina to Banyuls-sur-Mer, another 2 clades were detected in the Northern Adriatic Sea (Pula, Krk, Sveta Marina) and again 3 more clades were found in the eastern basin (peninsula of Attica and Crete) (see Fig. 7A). All in all, high posterior probabilities of the MrBayes analysis support these clades (Fig. 4B). On the contrary, bootstrap support (BS) is generally lower for the RaxML-HPC tree, especially for the split between "Croatia~" and "West" (Fig. 4A). Nevertheless, both methods, ML and BI, revealed similar topologies based on COI sequence data.

Estimations on net evolutionary divergences between these 6 groups (Table 2) yielded surprisingly high genetic distances. Interestingly, the degree of divergence does not reflect the geographical distributions of these clades. Hence, the sympatric lineages in the Adriatic Sea and in the eastern basin (around Greece) achieve genetic distances of 13.96 % and 14.32 %, respectively. On the other hand, the net divergence of allopatric sister lineages "Croatia*" and "Greece*" is only 9.41 %. While mainland populations of "Greece*" are 3.24 % different from their relatives on Crete ("Crete*"), the genetic distance between "Greece~" and "Crete~" was much smaller. The highest net divergence of 15.10 % was detected between the lineages "West*" and "Greece~".

Intragroup distances vary from 0.0 % ("Croatia~") to 1.6 % ("Greece~") and maximum parsimonious networks (Fig. 5; Supplementary Fig. 3) highlight geographic population structure. According to this, almost no genetic structure can be seen within clades of the western basin and the Adriatic Sea. In contrary to that, the Greek lineages show high geographic structure leading to major splits of mainland versus island populations in the clade "Greece~" and the complex "Greece*/Crete*". Furthermore, with the exception of one individual, the clade "Greece~" shows a clear phylogeographic structure between the northern and southern coast of Crete.

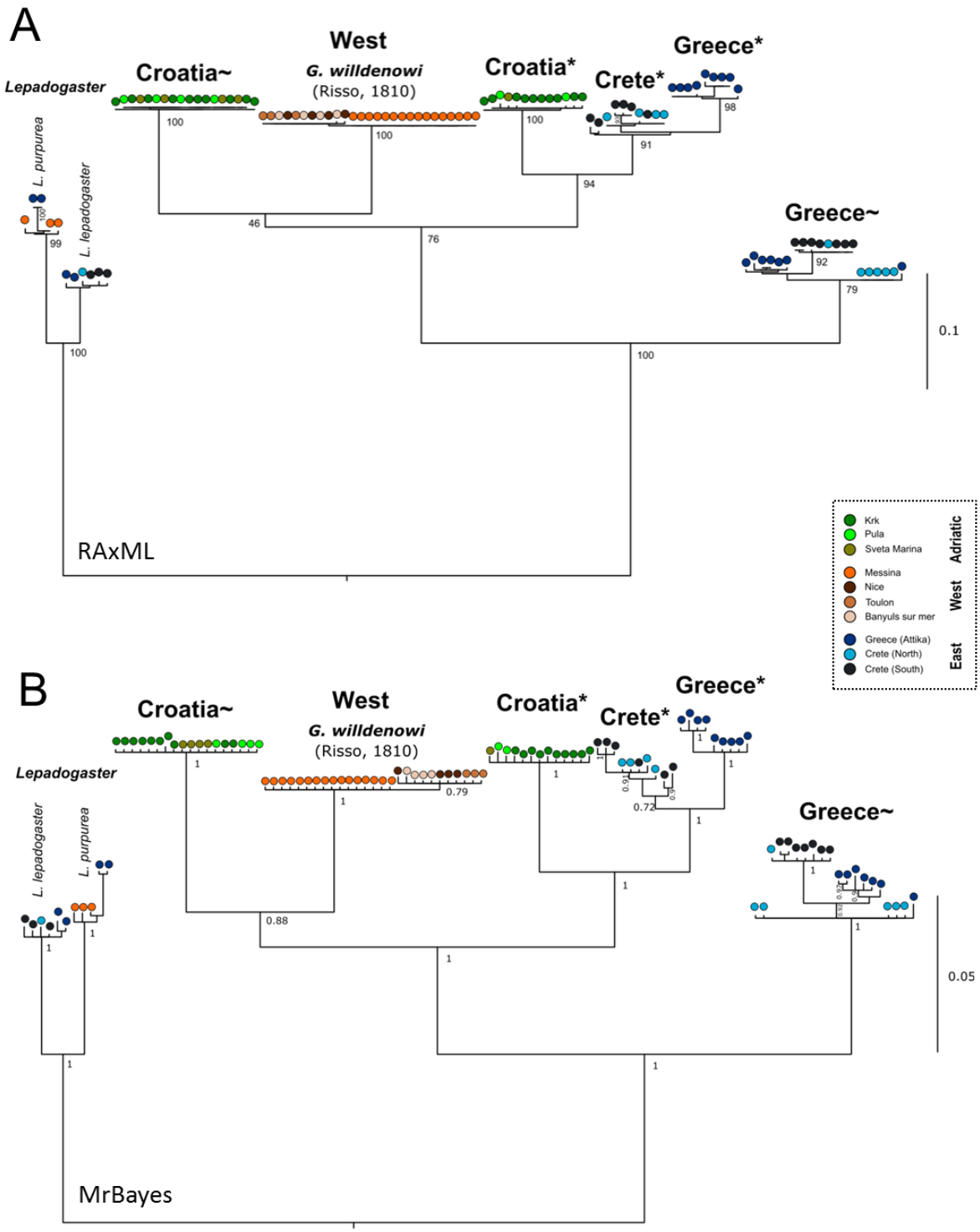

Fig. 4 COI barcoding trees based on RAxML (A) and MrBayes (B) analysis. Each blotch represents one individual barcode. Genetic clusters (> 3 % interspecific divergence) were named according to their geographical distributions and morphological traits (* and ~). Posterior probabilities and bootstrap values larger than 0.7 or 70 %, respectively, are shown.

Table 2 Estimates of net evolutionary divergence between groups of sequences. Standard error estimate(s) are shown above the diagonal. Diagonal values show intraspecific mean distances. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The analysis involved 108 nucleotide sequences. All positions with less than 95% site coverage were eliminated. Colours indicate the relative level of divergences (green: high, yellow-orange: intermediate, red: low).

	Croatia~	Croatia*	*G. will.*	Greece~	Greece*	Crete*
Croatia~	**0.00**	1,72	1,46	1,76	1,63	1,66
Croatia*	13,96	**0.20**	1,57	1,69	1,33	1,20
G. will.	11,31	12,95	**0.10**	1,68	1,50	1,51
Greece~	14,40	14,83	15,10	**1.60**	1,70	1,65
Greece*	12,90	9,41	11,78	14,32	**0.80**	0,68
Crete*	12,85	7,85	11,94	13,27	3,24	**1.00**

SE [%]

Pairwise divergence [%]

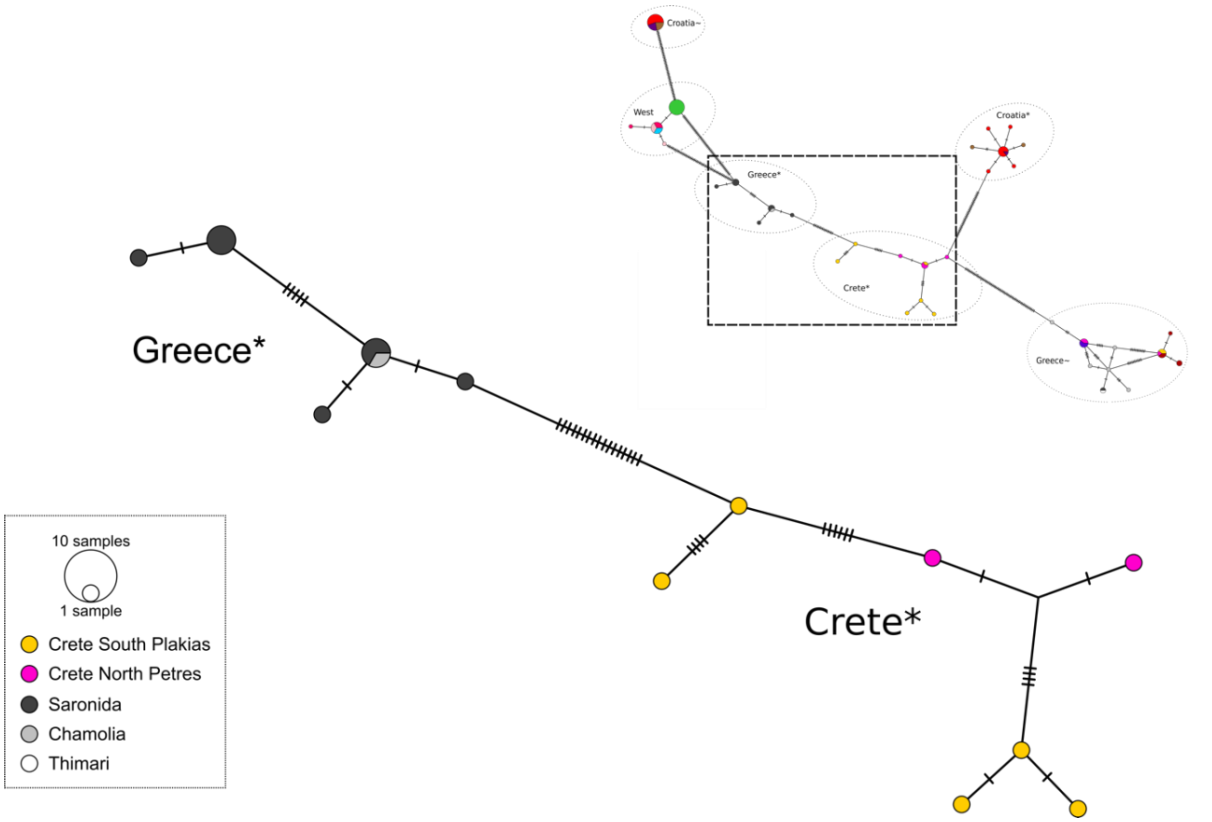

Fig. 5 Maximum parsimonious networks representing the clades Greece* and Crete*. In the right corner the network for the whole genus *Gouania* is shown (see Supplementary Fig. 3).

1.3.2 Concatenated data sets and Species Tree Analysis

Phylogenetic analysis of the concatenated datasets (Fig. 6) – including nine nuclear and one mitochondrial marker – reveals similar results as the Barcoding tree (Fig. 4). However, the inclusion of the mtDNA COI gene has major impacts on the resolution of the tree. As can expected for a "fast" marker, the inclusion of COI in the analysis produced a well resolved tree also among closely related lineages (Fig. 6B). In contrast, if the dataset is reduced to include only nDNA markers, the "Crete*/Greece*/Croatia*" complex cannot be resolved very well (Fig. 6B).

Different statistical methods (MrBayes vs. RaxML) slightly influence tree topology and show differences in respect to split support. Generally, MrBayes analysis obtains higher support for major splits and the inclusion of the mtDNA for the RaxML analysis (Fig. 6B-right) delivers a different topology than the Bayesian Inference (Fig. 6B-left) approach, but these differences only concern poorly supported nodes.

The species tree analysis (*BEAST) (Fig. 7) is consistent with the topology of concatenated datasets (Fig. 6) and it gives insights into coalescence events. According to this, the major split between *Gouania* and its sister genus *Lepadogaster* happened around 3 million years ago, at the Pliocene-Pleistocene boarder. The diversification in the genus *Gouania* can be dated one to two million years ago, parallel to the split of the genus *Lepadogaster* into *L. lepadogaster* and *L. purpurea*. A second radiation event, around 200.000 years ago, divided the clades "Greece*", "Crete*" and "Croatia*". Alternative topologies of the DensiTree (pink, green) have low nodal support and seem to be the product of the influence of single genes (e.g. COI).

1.3.3 First morphological impression

Overall body colouration changes across geographical distributions (Fig. 7). Specimens found in the Adriatic Sea have a lighter body colouration (compare with Hofrichter and Patzner 2000) than individuals from the western Mediterranean Sea and the eastern basin, which are more pigmented and sometimes show a striped pattern. The darkest individuals were found on Crete ("Crete*"). Star-like pigmentation around the eyes has been found in specimens from the western Mediterranean ("West*"), eastern Mediterranean ("Crete*",

"Greece*") and the Adriatic Sea ("Croatia*"). Furthermore, all genetic lineages differ significantly in the eye-size to standard length ratio, whereby "West*", "Crete*/Greece*" and "Croatia*" have larger eyes (Fig. 8A; Supplementary Fig. 4).

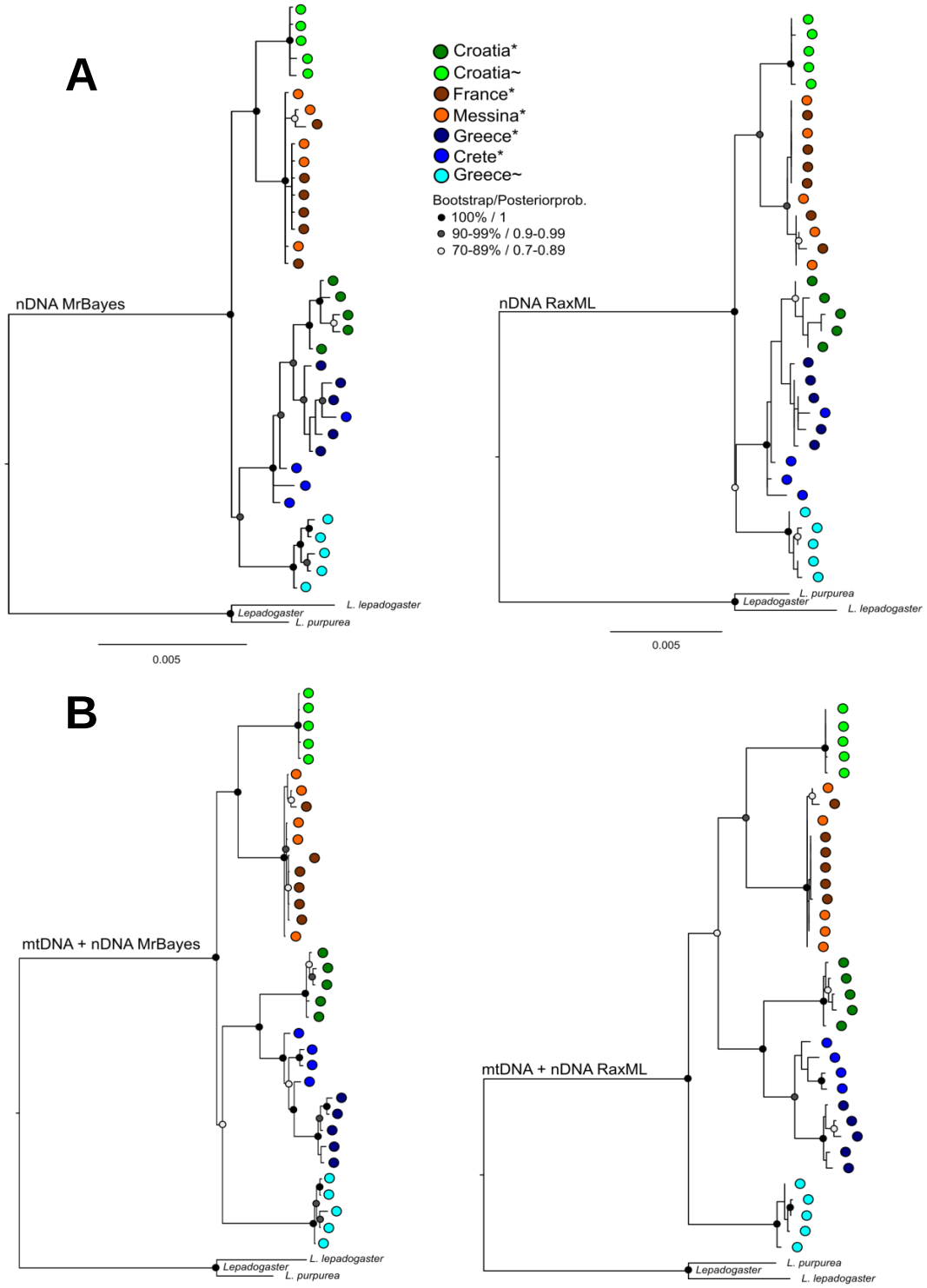

Fig. 6 Concatenated datasets investigated with different statistical methods (MrBayes, RaxML). (A) Exclusion of the mtDNA COI marker leads to a length of 5957 bp. (B) Inclusion of the mtDNA marker results in a 6567 bp long dataset. Each dot represents one individual. Posterior probabilities and bootstrap values larger than 0.7 or 70 %, respectively, are shown.

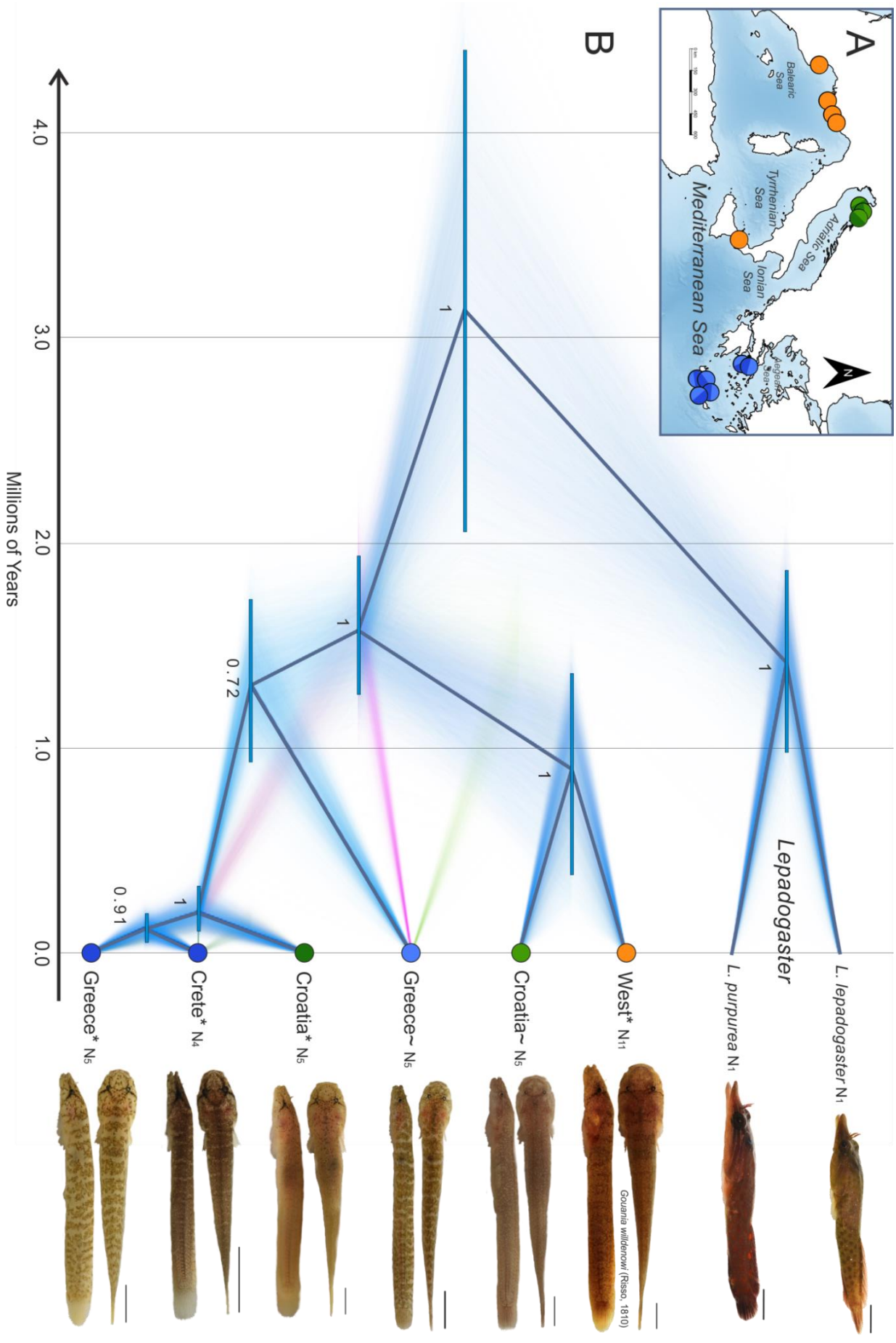

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Fig. 7 Distribution map of genetic lineages (**A**) and DensiTree visualization of the Species Tree analysis (**B**) for the genus *Gouania*. (**A**) Each coloured dot of the distribution map represents one clade of the Species Tree topology. The clades "Crete*" and "Crete*" share the same blotch colour. (**B**) DensiTree enables to show alternative tree topologies: most common tree topology is light-blue, the second most common is pink, the third most common is light-green and the others are dark green. Blue bars represent high-posterior-density intervals and posterior probabilities are given below branches. Furthermore, a time scale is given in 1.0 million years steps. Additional images, including a 5 mm scale (black) are also shown for each species.

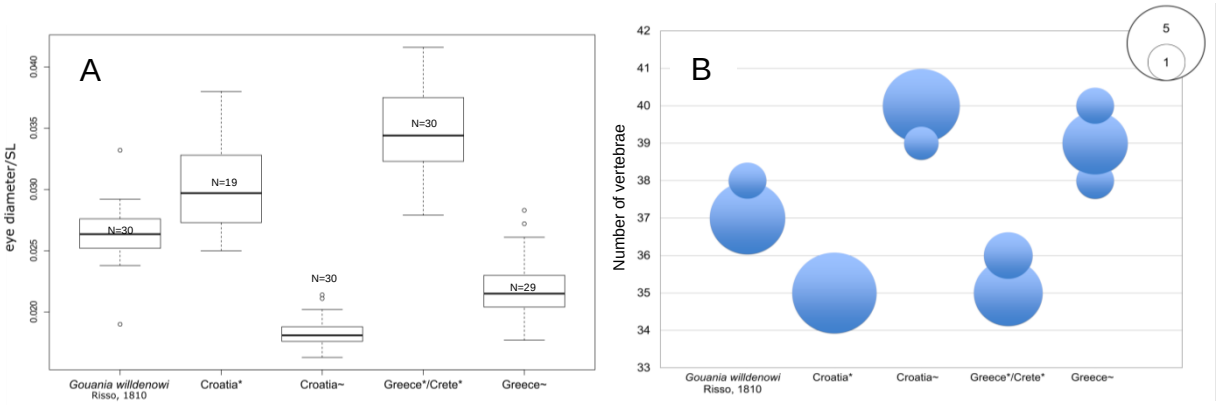

Fig. 8 Measurements and meristics (**A**) Ratio of horizontal diameter of the eye and Standard length compared for all genetic lineages (**B**) Bubble diagram showing different number of vertebrae for the different genetic lineages. Screenshots of all 3D-models are appended to the Supplementary material.

1.3.4 Geometric Morphometrics and Micro-CT scanning

The Principal component analysis (PCA) of the geometric morphometric approach resulted in two major clusters, respectively, from the dorsal (Fig. 9A) and lateral (Fig. 9B) side of the fish-images. PC-1 of dorsal and lateral images explains almost 80 % and 76 % of the variance in overall body-shape, respectively. According to this, the genetic lineages "Croatia~" and "Greece~" cluster and represent an elongated wormlike shape, compressed in the head and tail region. In contrast, an overall bulkier shape with an enlarged head can be found in specimens of the lineages "Greece*/Crete*", "Croatia*" and "West*". The ordinate is represented by PC-3 and PC-2 and only shows slight shape differences of the head and caudal area.

MicroCT-imaging produced interesting insights about the osteological differences between genetic clusters. Counts of vertebrae significantly differ between lineages (Fig. 8B). The highest number of vertebrae (around 39 – 40) was found in clades "Croatia~" and "Greece~". The "Croatia*" and individuals of "Greece*/Crete*" complex have around 35 to 36 vertebrae. An intermediate count of vertebrae can be found in "West*" (37 – 38 vertebrae).

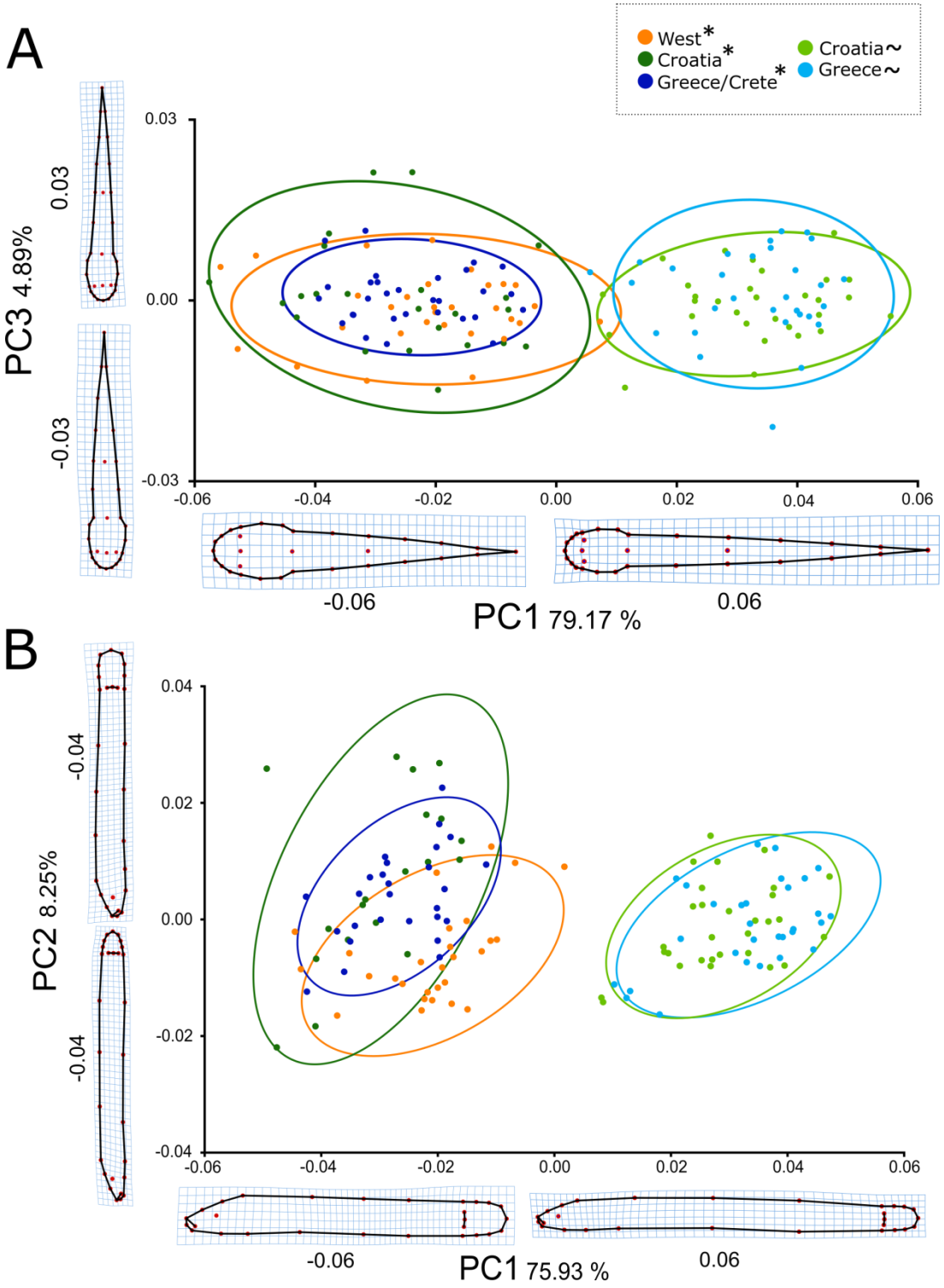

Fig. 9 Principal component analysis (PCA) of the Geometric-Morphometric (GEMO) investigation for the dorsal (A) and lateral (B) site. Each dot represents one individual. Specimens are classified in different colours according to genetic (> 3 % divergence), morphological (star-like pigmentation or wormlike body) and geographical clusters. Apart from that, confidences-ellipses support clusters with a probability of 0.9. Proportions of variances explained by the shift of the PC-axis are given beside and below each axis. Furthermore, deformation-grids (including PC-scores) are also shown to see major shape difference along a shift of the PC-axis.

1.4 Discussion

1.4.1 Barcoding reveals cryptic diversity

Despite the advantages of DNA barcoding as a cheap and comprehensive method for delimiting species (Hebert and Gregory 2005), it has its limits especially in taxa that underwent recent radiations, frequently hybridize or have nuclear mitochondrial pseudogenes (Moritz and Cicero 2004). However, DNA barcoding revealed a thus far unknown radiation in the genus *Gouania*, with six highly diverged mitochondrial lineages (Fig. 4). Recent studies on other clingfish genera, also found cryptic diversity. For example, Conway et al. (2014) describe cryptic diversity in the new world clingfish genus *Acyrtus*, and Conway et al. (2017b) found a new species of *Trachelochismus* from New Zealand also based on COI gene comparisons. Cryptic diversity as such, however, was detected in clingfishes also without using molecular methods and findings were only based on traditional morphometric methods (Williams and Tyler 2003). Nonetheless, the question must be raised if it makes sense considering DNA barcoding as a very basic and cheap pre-screening tool for species identification in fishes (Ward et al. 2005).

The prerequisite for a successful DNA barcoding approach is the concept of the "DNA barcoding gap", meaning higher interspecific than intraspecific distance (Wiemers and Fiedler 2007). The overall mean genetic distance on the COI gene in the genus *Gouania* for the given dataset is around 10 %, and, hence, much higher than the distance within single lineages, which varies from 0.0 % and 1.6 % (Table 2). These large distances between genetic clusters could be a sign of cryptic undescribed species.

Whereas the general view of "what makes a species?" is still a very philosophical and arbitrary one (see Zachos 2016), quantifying "species" based on DNA differences of single genes is common (Hebert et al. 2004). However, defining a minimum threshold of divergence is difficult and still very inconsistent. The "Barcode of Life Data System" (BOLD) suggests a minimum divergence of 3 % for discussion species levels (Ratnasingham and Hebert 2007). In clingfishes, Conway et al. (2014) describe a new clingfish species of the genus *Acyrtus,* based on a genetic COI distance of 8.4 %. Assuming a 3 % threshold of divergence for *Gouania* could elevate the species number from one to five simply on the knowledge based on this gene.

Overall, the radiation of genus *Gouania* is a wonderful example of how cryptic diversity can be detected based on mtDNA COI barcodes. However, determining species simply based on COI sequences is often not enough and faces problems (see 1.4.2). Hence, species delimitation can be enhanced by the use of traditional morphological methods, which are – against a widespread view – still the prerequisite for understanding and describing the immense diversity on earth (Ajmal et al. 2014).

1.4.2 Phylogenetic methods

Even though there is a broad consensus that trees based on single genes do not reflect the phylogenies based on a set of multiple genes (e.g. Pamilo and Nei 1988; Doyle 1992), the results based on multilocus analysis (Fig. 6, 7) revealed similar results like the DNA barcoding approach. Major reasons for the discrepancy gene trees versus species trees can be explained by horizontal gen transfer, lineage sorting or gen-duplication and extinction events (Maddison 1997), whereas for the underlying data set differential lineage sorting is of major relevancy.

For clingfishes, only a few studies have been conducted based on multilocus phylogenies and most of them simply include Bayesian inference methods on concatenated datasets (e.g. BEAST) (Conway et al. 2017a). Since there is an inconsistency between phylogenies based on concatenated datasets compared to multispecies coalescence based methods such as *BEAST (Fig. 7), which are generally more accurate for short branch lengths (Lambert et al. 2015), a comparative approach of both statistical methods for small datasets should always be conducted.

In this study, the results of the different multilocus phylogenetic methods, concatenation (MrBayes, RAxML) and multi-species coalescence (*BEAST), are more or less congruent (Fig. 6, 7). However, concerning the concatenated dataset one major difference can be observed. The exclusion of mtDNA markers (COI) for the analysis yields a comparatively poorly resolved tree, meaning that especially the young split of the lineages “Greece*”, “Croatia*” and “Crete*” cannot be resolved very well simply based on nDNA markers only (Fig. 6A). Owing to the fact that changes in the mitochondrial genome are four times more likely compared to genomic DNA (Moore 1995), the impact of mtDNA on the analysis is quite high. Nevertheless, it seems surprising that the amplified highly conserved

single-copy nuclear DNA genes – originally designed for large scale phylogenies across ray-finned fish orders (Li et al. 2007) – do resolve the genus *Gouania* very well, with the exception of very recent splits.

In a nutshell, the combined approach of different mtDNA and nDNA markers leads to high resolved and well supported phylogenies. Apart from that, a comparative statistical analysis, using multiple methods, is necessary in order to prevent a flawed and artefactual interpretation of data.

1.4.3 Pleistocene radiation

Dating evolutionary events and calibrating trees has become increasingly important with the high accessibility and use of molecular data (Forest 2009). However, secondary calibrations depend on fossils findings, which are scarcely found in some phyla and afflicted with uncertainties according to their age or taxonomic placements (Forest 2009). For the family Gobiesocidae only one fossil record, a putative *Apletodon* species, from the middle Miocene is available (Schwarzhans et al. 2017). Since the authors hesitate to determine their finding on species level, its use as calibration point for phylogenetic analyses of the Gobiesocidae is almost impossible.

To circumvent the lack of clingfish fossils, recent work by Conway et al. (2017a) used secondary calibration points based on the large scale phylogenetic study by Near et al. (2013). As a result, it was possible to employ molecular clock rates for 5 of the 10 genes in this study (Table 1). According to this, the onset of the radiation of the genus *Gouania* can be estimated around 1 – 2 million years ago, in the Pleistocene, and was followed by a second small radiation around 100.000 – 200.000 years ago (Fig. 7). On first glance, the age of this radiation seems very young considering the high interspecific divergences on COI gene (Table 2). However, clingfishes emerge with long branches in large scale phylogenetic analyses (Wainwright et al. 2012; Near et al. 2013), which implies a fast rate of evolution. Furthermore, if we only consider COI rates (Table 2) for the analysis the age of the radiation would be pushed only around 500.000 years further into the past.

The Pleistocene as such, initiated through an extreme drop of sea level, had major effects on the marine environment (Ludt and Rocha 2015). Whereas there is evidence that drastic changes, initiating this period, resulted in rapid speciation and great diversification of

several marine taxa (Jackson and Sheldon 1994), others went extinct, like most of the marine megafauna (Pimiento et al. 2017). However, whether these major Pleistocene environmental changes affected the radiation of the genus *Gouania* is difficult to discuss, unless we gain more detailed knowledge about the palaeoenvironmental and genetic (e.g. bottlenecks) situation in the Mediterranean Sea during this time.

1.4.4 Larval drift underpins allopatry

Especially in taxa with limited dispersal capacity, such as benthic marine fishes, allopatric speciation plays a major role for explaining their diversification. However, the homogeneity of the marine water body enables such taxa to disperse passively over long distances through a larval stage which can last several weeks to months (Macpherson and Raventos 2005). Thus, gene flow among distant populations supresses evolutionary processes, and leads to the general conclusion that structure within such population is scarce (Weersing and Toonen 2009). The genus *Gouania*, has a pelagic larval duration (PLD) of approximately 13 days and a drifting range of almost 3000 km, which is rather short compared to other cryptobenthic fish families such as triple-fin blennies (up to 53 days) (Macpherson and Raventos 2005). Since the concordance of PLD and population connectivity is not always given (Weersing and Toonen 2009) – e.g. long PLD can also result in high genetic structure (Bowen et al. 2006) – the interpretation of PLDs as an evolutionary driver per se is difficult and unsatisfactorily.

On the other hand, larval behaviour and adult ecology can highly influence larval retention and recruitment (Hellberg 2009). Thus, ontogenetic behavioural studies can shed light on future dispersal capacities. For clingfishes this has been very well studied by Faria and Gonçalves (2010) who compared two species of the genus *Lepadogaster* and found that early swimming capacity and behaviour majorly constrain the capacity of offshore drifting. Since *Gouania* shows high similarities with *Lepadogaster* concerning its breeding behaviour (demersal breeding) and can even occur in sympatry (Hofrichter 1995), it is to be expected that larval behaviour and dispersal capacity of these genera are comparable. Macpherson and Raventos (2005) define *G. willdenowi* as an inshore drifter, meaning that the maximum distance from shore is less than 1 mile, which prevents the species from reaching large oceanic current systems. Klein et al. (2016) encountered strong separations of *Lepadogaster*

lepadogaster populations in the Atlantic, due to high levels of self-recruitment and short PLD. As a result, short PLD, patchy distribution of suitable habitats and limited oceanographic transport in the genus *Gouania* could constrain dispersal distance and lead to high levels of larval retention, recruitment and separation of populations in different Mediterranean basins (Fig. 7A).

Furthermore, the Mediterranean Sea is embedded in lots of annually and seasonally changing circulation systems (Pinardi and Mosetti 2000), that can influence gene flow among populations at a temporal (Selkoe et al. 2014) and geographical scale (e.g. Galarza et al. 2009; Schunter et al. 2011; Koblmüller et al. 2015). Small local current systems – such as the Eastern Cretan gyre – can also separate populations (Gilg and Hilbish 2008) and could be a potential explanation for the high mtDNA divergences (3 %) between fishes from the Greek mainland ("Greece*") and Crete ("Crete*"). On the contrary, the syntopic lineage "Greece~" does not match this pattern and only yields intraspecific divergences of 1.6 % across continental and island populations. However, structure between the northern and southern coast of Crete can also be seen in this lineage.

To sum up, as our knowledge on palaeocirculation aspects of the Mediterranean Sea is limited, a meaningful interpretation of the radiation of the genus *Gouania* on the basis of today's current systems is questionable and flawed. Further whole genome analyses and coalescence studies, however, could increase our understanding of the early stages of the radiation and gain insights into evolutionary factors and processes underlying their diversification (Hellberg 2009). A combined approach of genetic/genomic approaches with behavioural, ecological and morphological studies would be indispensable in order to understand factors that led to this stunning radiation of cryptobenthic fishes.

1.4.5 Convergent evolution

The genus *Gouania* is perfectly adapted to a life in the interstices of pebble beaches and small boulders by having small eyes, a blunt snout and an elongated wormlike body shape (Hofrichter and Patzner 2000). However, the results of this thesis show that the species is not as monomorphic as previously thought. Principal Component Analysis of the lateral and dorsal geometric morphometric data produced two highly divergent morphological clusters, with major differences in the overall body shape (Fig. 9). As a result,

the investigated specimens can be divided into two major morphs, those that are gracile and elongated, found in the Adriatic, Greece and the Western Mediterranean and those that are stockier and bulky, also found in the Adriatic and in Greece. Clustering of these morphs based on their phylogenetic background (Fig. 7) is incongruent with the phylogenetic patterns and indicates that these body-shapes must have evolved repeatedly. Furthermore, it looks like the compression of the body in the stocky morph is due to a reduction of the number of vertebrae (Fig. 8B).

Whereas convergent evolution is widespread across the whole tree of life (Stayton 2008) – even in clingfishes (Briggs 1969) – the underlying reasons are highly controversial (Losos 2011). However, there is no doubt that similar environmental pressures selecting against (or for) similar environmental adaptations lead to stabilized ecomorphs within certain ecological niches (Endler 1982; Rundle et al. 2000; Donley et al. 2004; Gleiss et al. 2011; Muschick et al. 2012; Ingram and Kai 2014). For *Gouania*, who lives stenoeciously in pebble and boulder beaches, a major environmental pressure could be interstitial space and space competition. Similar to the habitat choice and shape of coral-associated fishes, that can be influenced by their host coral (Untersteggaber et al. 2014; Wehrberger and Herler 2014), the size of pebble and boulders could influence the shape and occurrence of different *Gouania* species. As a result, individuals with a more elongated and wormlike body might reach deeper layers of small pebbles with narrow interstitial space, whereas a bulkier one might have advantages in layers of larger pebbles and boulders. The significantly smaller eyes of the wormlike morphotype (Fig. 8A) could be an adaptation to light-poor environments, such as deep layers of pebbles or cobbles with narrow interstitial space. In the absence of light, reduction or even loss of sight and pigmentation is very common in cave living animals and sometimes is determined by genetic factors (Arendt and Reznick 2008).

Since two species usually do not occupy identical niches (Gause 1934, but see Muschick et al. 2012), the independently evolved sympatric species pairs, in the eastern Mediterranean basin and the Adriatic Sea, might be explained by disruptive selective pressures on ancient intermediate phenotypes, that enhanced selection against certain adaptations to extreme micro-niches and habitats (Rueffler et al. 2006). Interestingly, the originally described and phylogenetically old (Fig. 7) *G. willdenowi* emerges as an intermediate ecotype in terms of eye size, number of vertebrae and also (but to a lesser degree) body shape (Fig. 8, 9). Owing to the fact that this lineage has no direct intrageneric

sympatric competitor, the absence of disruptive selective pressures could explain the monotypic occurrence in the Western Mediterranean Sea.

If all that was true, we would expect a significant separation of sympatric ecotypes according to microhabitats (pebbles, boulders) and probable niche overlap of different developmental stages. Whereas first experiences in the field highly underpin these assumptions, further ecological investigations will be important to fully understand evolutionary pathways leading to this convergence of “ecotypes” in the genus *Gouania*.

Lepadogaster lepadogaster
(Bonnaterre, 1788)

PART II

First phylogeographic insights into the genus *Lepadogaster* (Gobiesocidae)

(Based on new records by Wagner et al. 2017)

Lepadogaster purpurea
(Bonnaterre, 1788)

2.1 Introduction

Of all Mediterranean clingfishes the genus *Lepadogaster* Gouan, 1770 is for sure the best investigated one. This is not very surprising considering its occurrence in very shallow water and high abundances in the interstices of boulder beaches (Hofrichter and Patzner 2000). However, the taxonomic background of this species was long unclear and a challenge for taxonomists. Originally, Briggs (1955) recognized three valid species *L. Lepadogaster*, *L. zebrina* and *L. candolii*, whereas the species *L. Lepadogaster* was divided into two subspecies *L. l. Lepadogaster* and *L. l. purpurea* which was also acknowledged by Hofrichter (1995). However, molecular and morphological analysis by Henriques et al. (2002) synonymised *L. zebrina* as a local population of *L. Lepadogaster* from Madeira and reduced the genus to the sympatric species pair *L. lepadogaster* and *L. purpurea*. Furthermore, Almada et al. (2008) separated *L. candolii* into the genus *Mirbelia* based on genetical, morphological and behavioural evidence.

Despite the clear genetic difference, the two species can be distinguished by a variety of morphological, behavioural and ecological traits such as the size and number of papillae on the sucking disc, size of head-marks and eyespots, different body colouration, the length of snout and interorbital distance, microhabitat preferences and breeding seasons (Henriques et al. 2002) as well as larval development and behaviour (Faria and Goṇalves 2010; Tojeira et al. 2012). *Lepadogaster lepadogaster* is a spring spawner (May – July) that can be encountered mainly in boulder fields and pebbles down to a depth of 0.5 to 1 m (Patzner 1999; Henriques et al. 2002). On the other hand, *L. purpurea* mainly prefers larger boulders and is a winter-spawner (from October to April) (Henriques et al. 2002).

In the course of this thesis, individuals of the species *L. purpurea* were found in Sicily, Croatia and Greece (Wagner et al. 2017 – see Appendix Part II) and significantly extending the original distribution range as described by Henriques et al. (2002). In the following chapter, preliminary findings on phylogeographic patterns in *L. purpurea* and its sister *species L. lepadogaster* will be discussed.

2.2 Material and Methods

Statistical parsimony networks (Templeton et al. 1992) of both *L. lepadogaster* and *L. purpurea* were inferred in PopArt 1.7 (Leigh and Bryant 2015) from the 12S rDNA data published in Wagner et al. (2017). Because of the small number of available COI sequences and insufficient geographic coverage no haplotype networks were inferred from this gene.

2.3 Results

The statistical parsimony networks based on the 12S rDNA data indicate a clear phylogeographic substructuring in *L. lepadogaster*, with three distinct clusters, corresponding to the European Atlantic coast, Atlantic islands and the Mediterranean, respectively, whereas no distinct geographic clusters are evident in *L. purpurea* (Fig. 3C). However, I emphasize that sample size is very small and these phylogeographic patterns should thus be interpreted with caution.

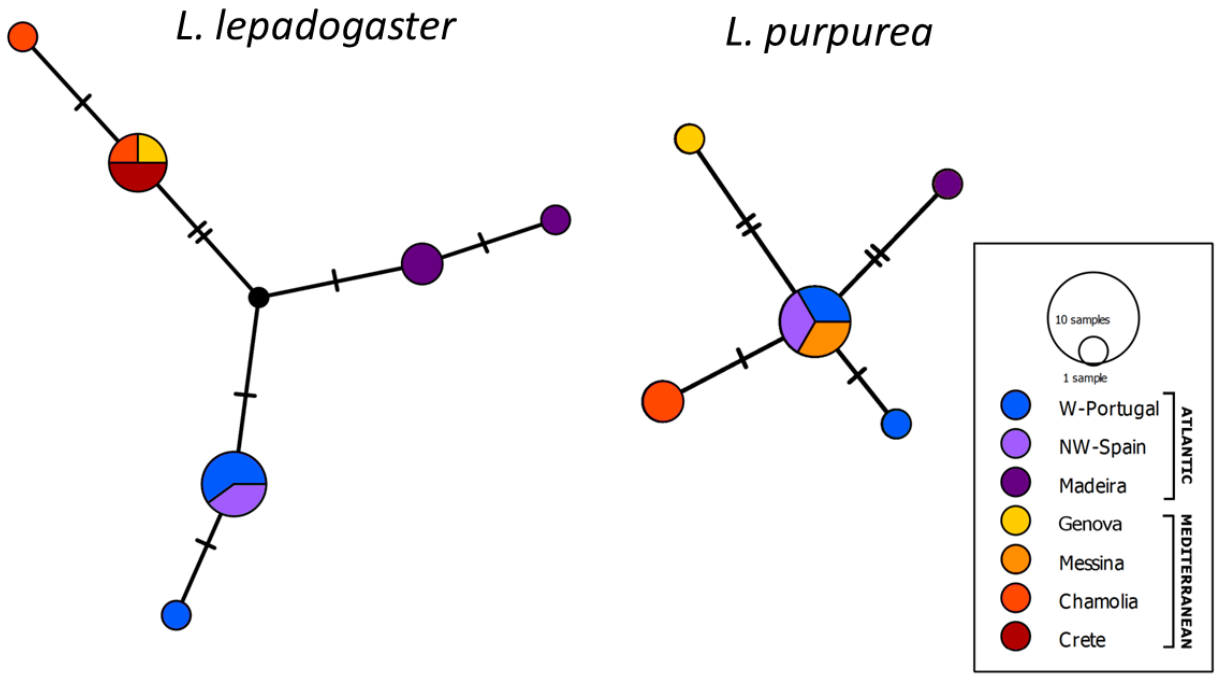

Fig. 11 12s rDNA statistical parsimony networks are shown for *Lepadogaster lepadogaster* and *L. purpurea*.

2.4 Discussion

Despite a very small sample size, the analysis of 12s rDNA data revealed first insights into the phylogeographic structure of *L. lepadogaster* and *L. purpurea*. Whereas the *L. lepadogaster* samples were grouped into three distinct clusters, European Atlantic coast, Atlantic island and Mediterranean, respectively, no distinct clustering became evident in *L. purpurea* (Fig. 11). The two Greek *L. purpurea* specimens shared a unique haplotype, but overall sample size is too small to confidently suggest a phylogeographic break between western and eastern Mediterranean basin. A recent study by Klein et al. (2016) gives very important insights into the population dynamics of *L. lepadogaster* by showing that there is high genetic connectivity among *L. lepadogaster* populations along the European Atlantic coast. Furthermore, that study also found that temporal genetic differences, caused by interannual changes in water currents or storms, play an important role for larval recruitment and dispersal and can even outweigh spatial genetic differences. The Mediterranean is characterized by seasonal and interannual shifts in current strengths and directions (Pinardi and Mosetti 2000; Fernández et al. 2005) and the impact of circulation systems on the genetic structure of Mediterranean fishes with low active dispersal activity has been already demonstrated previously (e.g. Galarza et al. 2009; Schunter et al. 2011; Koblmüller et al. 2015). As the two *Lepadogaster* species have different reproductive seasons (Henriques et al. 2002), it seems plausible that seasonal changes in water currents may be responsible for the observed differences in phylogeographic patterns.

It needs to be noted, however, that these are just preliminary interpretations, based on very few samples. A larger sample size in terms of individuals, geographic coverage and genetic markers is needed for future in depth investigations on patterns of intra-specific genetic variation (and the determinants thereof) in these two species, that are very similar in all their life history aspects apart from their breeding seasons.

III Conclusion and prospects

To sum up, the radiation of *Gouania* represents a promising model system for studying rapid speciation and the role of water currents in diversification processes in marine environments. Nevertheless, the use of high throughput sequencing methods (phylogenomics or transcriptomics) could illuminate early stages of this diversification and gain insights about underlying ontogenetic changes. However, a major taxonomic revision of the genus *Gouania*, including ecological and behavioural data, will be the prerequisite for interpreting such data. Furthermore, the investigation of additional regions in the Mediterranean Sea (i.e. northern African, western Spain, Turkey) might reveal further cryptic diversity and would be indispensable in order to get a full picture of the actual diversity in the genus *Gouania*.

Although quite a few studies have been conducted on the genus *Lepadogaster*, the results of this thesis underpin the urgent need of proper taxonomic expertise and new effective sample methods for studying cryptobenthic fishes. Apart from that, the preliminary phylogeographic results show high potential for future population-genetic (or genomic) studies. Hence, a pan-Mediterranean sample of the genus *Lepadogaster* could reveal major biogeographic insight that might be relevant also for other taxa.

In a nutshell, the results of this thesis show that cryptobenthic fishes, such as clingfishes, have high potential for future research. However, two major reasons hamper their exploration and are also responsible for their historical underrepresentation in the scientific literature. Firstly, the cryptic behaviour leads to a marginalized assessment of certain taxa and exclude in vivo ecological or behavioural observations. Secondly, the sole use of classical morphometrics and the hasty description of new species and genera led to extreme numbers of synonyms and incomplete and/ or messed up phylogenies. Even though modern molecular and morphometric methods cannot increase the chance of finding hidden taxa in the field, they can help in the detection of cryptic species and resolve, step by step, the cryptobenthic tree of life.

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V References

Ajmal AM, Gyulai G, Hidvégi N, et al (2014) The changing epitome of species identification - DNA barcoding. Saudi J Biol Sci 21:204–231. doi: 10.1016/j.sjbs.2014.03.003

Allen LG (1984) Gobiesociformes: development and relationships. Am Soc Ichthyol Herpetol 629–636

Almada F, Henriques M, Levy A, et al (2008) Reclassification of *Lepadogaster candollei* based on molecular and meristic evidence with a redefinition of the genus *Lepadogaster*. Mol Phylogenet Evol 46:1151–1156. doi: 10.1016/j.ympev.2007.05.021

Arendt J, Reznick D (2008) Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? Trends Ecol Evol 23:26–32. doi: 10.1016/j.tree.2007.09.011

Bilecenoğlu M (2015) First observation of the amphibious behaviour of *Gouania willdenowi* (Gobiesocidae) in the Eastern Mediterranean Sea. In: New Mediterranean Biodiversity Records (April 2015). Mediterranean Marine Science, p 270

Bilecenoğlu M, Yokeş MB, Kovačić M (2017) A new species of *Diplecogaster* (Actinopterygii: Gobiesocidae) from the Mediterranean Sea. Zoology in the Middle East:2326-2680. doi: 10.1080/09397140.2017.1349119

Bookstein FL (1991) Morphometric tools for landmark data: geometry and biology

Bouckaert R, Heled J (2014) DensiTree 2: Seeing trees through the forest. bioRxiv - Prepr Serv 1–11. doi: 10.1101/012401

Bouckaert R, Heled J, Kühnert D, et al (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Comput Biol 10:1–6. doi: 10.1371/journal.pcbi.1003537

Bowen BW, Bass AL, Muss A, et al (2006) Phylogeography of two Atlantic squirrelfishes (family Holocentridae): Exploring links between pelagic larval duration and population connectivity. Mar Biol 149:899–913. doi: 10.1007/s00227-006-0252-1

Brandl SJ, Wagner M, Hofrichter R, Patzner RA (2011) First record of the clingfish *Apletodon dentatus* (Gobiesocidae) in the Adriatic Sea and a description of a simple method to collect clingfishes. Bull Fish Biol 13:65–69

Briggs JC (1955) A monograph of the clingfishes (Order Xenopterygii)

Briggs JC (1986) Gobiesocidae. In: Whitehead PJP, Bauchot ML, Hureau JC, et al. (eds) Fishes of the north-eastern Atlantic and the Mediterranean, volume 3. United Nations Educational Scientific and Cultural Organization, pp 1351–1359

Briggs JC (1969) A New Genus and Species of Clingfish (Family Gobiesocidae) from the Bahama Islands. Copeia 2:332–334. doi: 10.2307/1443956

Clabaut C, Bunje PME, Salzburger W, Meyer A (2007) Geometric morphometric analyses provide evidence for the adaptive character of the Tanganyikan cichlid fish radiations. Evolution (N Y) 61:560–578. doi: 10.1111/j.1558-5646.2007.00045.x

Conway KW, Baldwin C, White MD (2014) Cryptic Diversity and Venom Glands in Western Atlantic Clingfishes of the Genus *Acyrtus* (Teleostei: Gobiesocidae). PLoS One 9:e97664. doi: 10.1371/journal.pone.0097664

Conway KW, Kim D, Rüber L, et al (2017a) Molecular systematics of the New World clingfish genus *Gobiesox* (Teleostei: Gobiesocidae) and the origin of a freshwater clade. Mol Phylogenet Evol 112:138–147. doi: 10.1016/j.ympev.2017.04.024

Conway KW, Stewart AL, King C (2017b) A new species of the clingfish genus *Trachelochismus* from bay and estuarine areas of New Zealand (Teleostei: Gobiesocidae). 4319:531–549

Depczynski M, Bellwood DR (2003) The role of cryptobenthic reef fishes in coral reef trophodynamics. Mar Ecol Prog Ser 256:183–191. doi: 10.3354/meps256183

Ditsche P, Wainwright DK, Summers AP (2014) Attachment to challenging substrates - fouling, roughness and limits of adhesion in the northern clingfish (*Gobiesox maeandricus*). J Exp Biol 217:2548–2554. doi: 10.1242/jeb.100149

Donley JM, Sepulveda C a, Konstantinidis P, et al (2004) Convergent evolution in mechanical design of lamnid sharks and tunas. Nature 429:61–65. doi: 10.1038/nature02435

Doyle JJ (1992) Gene Trees and Species Trees: Molecular Systematics as One-Character Taxonomy. Syst Bot 17:144–163

Duftner N, Koblmüller S, Sturmbauer C (2005) Evolutionary relationships of the Limnochromini, a tribe of benthic deepwater cichlid fish endemic to Lake Tanganyika, East Africa. J Mol Evol 60:277–289. doi: 10.1007/s00239-004-0017-8

Ebeling AW, Bernal P, Zuleta A (1970) Emersion of the Amphibious Chilean Clingfish, *Sicyases sanguineus*. Biol Bull 139:115–137

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. doi: 10.1093/chemse/bjs128

Endler JA (1982) Convergent and Divergent Effects of Natural Selection on Color Patterns in Two Fish Faunas. Evolution (NY) 36:178–188

Eschmeyer, Fricke R, van der Laan R (2006) Catalog of Fishes. Calif Acad Sci.

Facciola (1887) Intorno a due Lepadogastrini ed un nouvo Nettastoma del mare di Sicilia. Nat Sicil 6:163–167

Faria AM, Gonçalves EJ (2010) Ontogeny of swimming behaviour of two temperate clingfishes, *Lepadogaster lepadogaster* and *L. purpurea* (Gobiesocidae). Mar Ecol Prog Ser 414:237–248. doi: 10.3354/meps08692

Fernández V, Dietrich DE, Haney RL, Tintoré J (2005) Mesoscale, seasonal and interannual variability in the Mediterranean Sea using a numerical ocean model. Prog Oceanogr 66:321–340. doi: 10.1016/j.pocean.2004.07.010

Forest F (2009) Calibrating the tree of life: Fossils, molecules and evolutionary timescales. Ann Bot 104:789–794. doi: 10.1093/aob/mcp192

Fricke R, Wirtz P (2017) *Lecanogaster gorgoniphila,* a new species of clingfish (Teleostei: Gobiesocidae) from São Tomé and Principe, eastern Atlantic Ocean. Arquipelago 35:1–10.

Fricke R, Wirtz P, Brito A (2015) *Diplecogaster tonstricula*, a new species of cleaning clingfish (Teleostei: Gobiesocidae) from the Canary Islands and Senegal, eastern Atlantic Ocean, with a review of the *Diplecogaster-ctenocrypta* species-group. J Nat Hist 1–18. doi: 10.1080/00222933.2015.1079659

Fricke R, Wirtz P, Brito A (2010) A new species of the clingfish genus *Apletodon* (Teleostei: Gobiesocidae) from the Cape Verde Islands, Eastern Central Atlantic. Ichthyol Res 57:91–97. doi: 10.1007/s10228-009-0139-5

Galarza JA, Carreras-Carbonell J, Macpherson E, et al (2009) The influence of oceanic fronts and early life-life-history traits on connectivity among littoral fish species. Proc Natl Acad Sci USA 106:1473–1478. doi: 10.1073/pnas.0806804106

Gause GF (1934) The Struggle for Existence.

Gilg MR, Hilbish TJ (2008) The Geography of Marine Larval Dispersal: Coupling Genetics with Fine-Scale Physical Oceanography. Ecology 84:2989–2998

Gleiss AC, Jorgensen SJ, Liebsch N, et al (2011) Convergent evolution in locomotory patterns of flying and swimming animals. Nat Commun 2:1–7. doi: 10.1038/ncomms1350

Gonçalves DM, Gonçalves EJ, Almada VC, Almeida SP (1998) Comparative behaviour of two species of *Lepadogaster* (Pisces: Gobiesocidae) living at different depths. J Fish Biol 53:447–450. doi: 10.1006/jfbi.1998.0702

Gonçalves EJ, Almada VC, Almeida SP, et al (1996) Observations on the agonistic behaviour of *Lepadogaster lepadogaster purpurea* (Pisces: Gobiesocidae). J Fish Biol 49:367–369. doi: 10.1006/jfbi.1996.0163

Goncçalves EJ, Barbosa M, Cabral HN, Henriques M (2002) Ontogenetic shifts in patterns of microhabitat utilization in the small-headed clingfish, *Apletodon dentatus* (Gobiesocidae). Environ Biol Fishes 63:333–339. doi: 10.1023/A:1014302319622

Guzmán AF, Peréz HE, Miller RR (2001) Occurrence of the clingfish, *Gobiesox fluviatilis* (Gobiesociformes: Gobiesocidae), in the Rio Chapalagana, Mexico: Confirmation of a historical record. Southwest Nat 46:96–98

Haas TC (2011) Guide to the „Unbend specimens" module in tpsUtil. Tulane, University of New Orleans.

Hebert PDN, Gregory TR (2005) The promise of DNA barcoding for taxonomy. Syst Biol 54:852–859. doi: 10.1080/10635150500354886

Hebert PDN, Penton EH, Burns JM, et al (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci 101:14812–14817. doi: 10.1073/pnas.0406166101

Hebert PDN, Ratnasingham S, de Waard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc B Biol Sci 270:S96–S99. doi: 10.1098/rsbl.2003.0025

Heled J, Drummond AJ (2010) Bayesian Inference of Species Trees from Multilocus Data. Mol Biol Evol 27:570–580. doi: 10.1093/molbev/msp274

Hellberg ME (2009) Gene Flow and Isolation among Populations of Marine Animals. Annu Rev Ecol Evol Syst 40:291–310. doi: 10.1146/annurev.ecolsys.110308.120223

Henriques M, Lourenço R, Almada F, et al (2002) A revision of the status of *Lepadogaster lepadogaster* (Teleostei: Gobiesocidae): Sympatric subspecies or a long misunderstood blend of species? Biol J Linn Soc 76:327–338. doi: 10.1046/j.1095-8312.2002.00067.x

Herler J, Kerschbaumer M, Mitteroecker P, et al (2010) Sexual dimorphism and population divergence in the Lake Tanganyika cichlid fish genus *Tropheus*. Front Zool 7:e4. doi: 10.1186/1742-9994-7-4

Hickerson MJ, Ross JRP (2001) Post-glacial population history and genetic structure of the northern clingfish (*Gobbiesox maeandricus*), revealed from mtDNA analysis. Mar Biol 138:407–419. doi: 10.1007/s00227-002-0901-y

Hofrichter R (1995) Taxonomie, Verbreitung und Ökologie von Schildfischen der Unterfamilie Lepadogastrinae (Gobiesocidae, Teleostei). Phd Thesis.

Hofrichter R, Patzner RA (2000) Habitat and microhabitat of Mediterranean clingfishes (Teleostei: Gobiesociformes: Gobiesocidae). Mar Ecol 21:41–53. doi: 10.1046/j.1439-0485.2000.00689.x

Hutchings BJ (1991) Description of a new deepwater clingfish (Gobiesocidae) from New South Wales. Rec West Aust Museum 15:463–468

Ingram T, Kai Y (2014) The Geography of Morphological Convergence in the Radiations of Pacific *Sebastes* Rockfishes. Am Nat 184:E115–E131 . doi: 10.1086/678053

Jackson JBC, Sheldon PR (1994) Constancy and Change of Life in the Sea [and Discussion]. Philos Trans R Soc B Biol Sci 344:55–60. doi: 10.1098/rstb.1994.0051

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. doi: 10.1007/BF01731581

Klein M (2016) Recruitment patterns and processes of coastal fish species in a temperate rocky reef. Phd Thesis

Klein M, Teixeira S, Assis J, et al (2016) High Interannual Variability in Connectivity and Genetic Pool of a Temperate Clingfish Matches Oceanographic Transport Predictions. PLoS One 11:e0165881 . doi: 10.1371/journal.pone.0165881

Klingenberg CP (2011) MorphoJ: An integrated software package for geometric morphometrics. Mol Ecol Resour 11:353–357. doi: 10.1111/j.1755-0998.2010.02924.x

Klingenberg CP (1998) Heterochrony and allometry: the analy- sis of evolutionary change in ontogeny. Biol Rev 73:79–123

Koblmüller S, Steinwender B, Weiß S, Sefc KM (2015) Gene flow, population growth and a novel substitution rate estimate in a subtidal rock specialist, the black-faced blenny *Tripterygion delaisi* (Perciformes, Blennioidei, Tripterygiidae) from the Adriatic Sea. J Zool Syst Evol Res 53:291–299. doi: 10.1111/jzs.12110

Kovačić M (1998) Cryptobenthic gobies (Pisces, Perficormes, Gobiidae) and clingfishes (Pisces, Gobiesociformes, Gobiesocidae) in the Kvarner area, Adriatic Sea (1998): 1403. Oceanogr Lit Rev 8:1403

Kovačić M, Patzner RA, Schliewen U (2012) A first quantitative assessment of the ecology of cryptobenthic fishes in the Mediterranean Sea. Mar Biol 159:2731–2742. doi: 10.1007/s00227-012-2030-6

Kuhner MK (2009) Coalescent genealogy samplers: windows into population history. Trends Ecol Evol 24:86–93. doi: 10.1016/j.tree.2008.09.007

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874. doi: 10.1093/molbev/msw054

Lambert SM, Reeder TW, Wiens JJ (2015) When do species-tree and concatenated estimates disagree? An empirical analysis with higher-level scincid lizard phylogeny. Mol Phylogenet Evol 82:146–155. doi: 10.1016/j.ympev.2014.10.004

Leigh JW, Bryant D (2015) POPART: Full-feature software for haplotype network construction. Methods Ecol Evol 6:1110–1116. doi: 10.1111/2041-210X.12410

Li C, Ortí G, Zhang G, Lu G (2007) A practical approach to phylogenomics: the phylogeny of ray-finned fish (Actinopterygii) as a case study. BMC Evol Biol 7:44. doi: 10.1186/1471-2148-7-44

Limaye A (2012) Drishti: a volume exploration and presentation tool. doi: 10.1117/12.935640

Losos JB (2011) Convergence, adaptation, and constraint. Evolution (N Y) 65:1827–1840. doi: 10.1111/j.1558-5646.2011.01289.x

Ludt WB, Rocha LA (2015) Shifting seas: The impacts of Pleistocene sea-level fluctuations on the evolution of tropical marine taxa. J Biogeogr 42:25–38. doi: 10.1111/jbi.12416

Macpherson E, Raventos N (2005) Settlement patterns and post-settlement survival in two Mediterranean littoral fishes: Influences of early-life traits and environmental variables. Mar Biol 148:167–177. doi: 10.1007/s00227-005-0059-5

Maddison WP (1997) Gene trees in species trees. Syst Biol 46:523–536. doi: 10.1093/sysbio/46.3.523

Moore GI, Hutchins JB, Okamoto M (2012) A new species of the deepwater clingfish genus *Kopua* (Gobiesociformes: Gobiesocidae) from the East China Sea—an example of antitropicality? Zootaxa 3380:34–38

Moore WS (1995) Inferring Phylogenies from mtDNA Variation: Mitochondrial-Gene Trees Versus Nuclear-Gene Trees. Evolution (NY) 49:718–726

Moritz C, Cicero C (2004) DNA barcoding: Promise and pitfalls. PLoS Biol 2:10. doi: 10.1371/journal.pbio.0020354

Muschick M, Indermaur A, Salzburger W (2012) Convergent Evolution within an Adaptive Radiation of Cichlid Fishes. Curr Biol 22:2362–2368. doi: 10.1016/j.cub.2012.10.048

Near TJ, Dornburg A, Eytan RI, et al (2013) Phylogeny and tempo of diversification in the superradiation of spiny-rayed fishes. Proc Natl Acad Sci U S A 110:12738–12743. doi: 10.5061/dryad.d3mb4

Pamilo P, Nei M (1988) Relationships between gene trees and species trees. Mol Biol Evol 5:568–583. doi: 10.1093/oxfordjournals.molbev.a040517

Patzner RA (1999a) Sea Urchins as a Hiding-Place for juvenile benthic Teleosts (Gobiidae and Gobisocidae) in the Mediterranean Sea. Cybium 23:93–97

Patzner R a. (1999b) Habitat utilization and depth distribution of small cryptobenthic fishes (Blenniidae, Gobiidae, Trypyterigiidae) in Ibiza (western Mediterranean Sea). Environ Biol Fishes 55:207–214

Pennisi E (2012) Clingfish stick like geckos. Science (80) 335:277. doi: 10.1126/science.335.6066.277

Pimiento C, Griffin JN, Clements CF, et al (2017) The Pliocene marine megafauna extinction and its impact on functional diversity. Nat Ecol Evol 1:1100–1106. doi: 10.1038/s41559-017-0223-6

Pinardi N, Mosetti E (2000) Variability of the large-scale general circulation of the Mediterranean Sea from observations and modelling: a review. Palaeogeogr, Palaeoclim Palaeoecol 158:153–174

Rambaut, A., Drummond, A.J., 2009. Tracer v1.6, Available from <http://beast.bio.ed. ac.uk/Tracer>

Ratnasingham S, Hebert PDN (2007) BARCODING, BOLD: The Barcode of Life Data System (www.barcodinglife.org). Mol Ecol Notes 7:355–364. doi: 10.1111/j.1471-8286.2006.01678.x

Richlen ML, Barber PH (2005) A technique for the rapid extraction of microalgal DNA from single live and preserved cells. Mol Ecol Notes 5:688–691. doi: 10.1111/j.1471-8286.2005.01032.x

Rohlf FJ (2015) tpsUtil, version 1.65. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf FJ (2016a) tpsDig, version 2.26. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf FJ (2016b) tpsRelw, version 1.65. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf FJ, Slice D (1990) Extensions of the Procrustes Method for the Optimal Superimposition of Landmarks. Syst Zool 39:40–59. doi: 10.2307/2992207

Ronquist F, Teslenko M, Van Der Mark P, et al (2012) Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542. doi: 10.1093/sysbio/sys029

Rueffler C, Van Dooren TJM, Leimar O, Abrams PA (2006) Disruptive selection and then what? Trends Ecol Evol 21:238–245. doi: 10.1016/j.tree.2006.03.003

Rundle HDD, Nagel L, Boughman JW, et al (2000) Natural Selection and Parallel Speciation in Sympatric Sticklebacks. Science (80) 287:306–308. doi: 10.1126/science.287.5451.306

Schunter C, Carreras-Carbonell J, MacPherson E, et al (2011) Matching genetics with oceanography: Directional gene flow in a Mediterranean fish species. Mol Ecol 20:5167–5181. doi: 10.1111/j.1365-294X.2011.05355.x

Schwarzhans W, Carnevale G, Japundžić S, Bradić-Milinović K (2017) Otoliths in situ from Sarmatian (Middle Miocene) fishes of the Paratethys. Part V: Bothidae and Soleidae. Swiss J Palaeontol 136:109–127. doi: 10.1007/s13358-017-0128-7

Selkoe K a, Gaines SD, Caselle JE, et al (2014) Current Shifts and Kin Aggregation Explain Genetic Patchiness in Fish Recruits. Ecol Soc Am 87:3082–3094

Shorthouse DP (2010) SimpleMappr, an online tool to produce publication-quality point maps. Available from <http://www.simplemappr.net>

Sparks JS, Gruber DF (2012) A new mesophotic clingfish (Teleostei: Gobiesocidae) from the Bahamas. Copeia 2012:251–256. doi: 10.1643/CI-11-124

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. doi: 10.1093/bioinformatics/btu033

Stayton CT (2008) Is convergence surprising? An examination of the frequency of convergence in simulated datasets. J Theor Biol 252:1–14. doi: 10.1016/j.jtbi.2008.01.008

Tojeira I, Faria AM, Henriques S, et al (2012) Early development and larval behaviour of two clingfishes, *Lepadogaster purpurea* and *Lepadogaster lepadogaster* (Pisces: Gobiesocidae). Environ Biol Fishes 93:449–459. doi: 10.1007/s10641-011-9935-7

Untersteggaber L, Mitteroecker P, Herler J (2014) Coral architecture affects the habitat choice and form of associated gobiid fishes. Mar Biol 161:521–530. doi: 10.1007/s00227-013-2354-x

Wagner M, Bračun S, Kovačić M, et al (2017) *Lepadogaster purpurea* (Actinopterygii: Gobiesociformes: Gobiesocidae) from the eastern Mediterranean Sea: Significantly extended distribution range. Acta Ichthyol Piscat 47:417–421. doi: 10.3750/AIEP/02244

Wahlberg N, Braby MF, Brower AV., et al (2005) Synergistic effects of combining morphological and molecular data in resolving the phylogeny of butterflies and skippers. Proc R Soc B Biol Sci 272:1577–1586. doi: 10.1098/rspb.2005.3124

Wainwright DK, Kleinteich T, Kleinteich A, et al (2013) Stick tight: suction adhesion on irregular surfaces in the northern clingfish. Biol Lett 9:20130234. doi: 10.1098/rsbl.2013.0234

Wainwright PC, Smith WL, Price SA, et al (2012) The Evolution of Pharyngognathy: A Phylogenetic and Functional Appraisal of the Pharyngeal Jaw Key Innovation in Labroid Fishes and Beyond. Syst Biol 61:1001–1027. doi: 10.1093/sysbio/sys060

Ward RD, Zemlak TS, Innes BH, et al (2005) DNA barcoding Australia's fish species. Philos Trans R Soc Lond B Biol Sci 360:1847–1857. doi: 10.1098/rstb.2005.1716

Weersing K, Toonen RJ (2009) Population genetics, larval dispersal, and connectivity in marine systems. Mar Ecol Prog Ser 393:1–12. doi: 10.3354/meps08287

Wehrberger F, Herler J (2014) Microhabitat characteristics influence shape and size of coral-associated fishes. Mar Ecol Prog Ser 500:203–214. doi: 10.3354/meps10689

Weitzmann B, Mercader L (2012) First report of cleaning activity of *Lepadogaster candolii* (Gobiesocidae) in the Mediterranean Sea. Cybium 36:487–488

Wiemers M, Fiedler K (2007) Does the DNA barcoding gap exist? - A case study in blue butterflies (Lepidoptera: Lycaenidae). Front Zool 4:1–16. doi: 10.1186/1742-9994-4-8

Williams JT, Tyler JC (2003) Revision of the western Atlantic clingfishes of the genus *Tomicodon* (Gobiesocidae), with descriptions of five new species. Smithson Contrib to Zool 621:1–26. doi: 10.5479/si.00810282.621

Willis T (2001) Visual census methods underestimate density and diversity of cryptic reef fishes. J Fish Biol 59:1408–1411. doi: 10.1006/jfbi.2001.1721

Zachos FE (2016) Species Concepts in Biology - Historical Development, Theoretical Foundations and Practical Relevance. Springer International Publishing AG Switzerland

VI Appendix Part I

Supplementary Table 1 Sampling locations and dates

Locality	Country	Coordinates	Date
Krk (Glavotok)	Croatia	45°05'44.9"N 14°26'32.4"E	Jun. - Sep. 2014 and May 2015
Krk (St. Baska)	Croatia	44°56'45.3"N 14°42'22.2"E	May 2015
Pula (Stoja)	Croatia	44°51'38.4"N 13°49'05.0"E	Jul. 2016
Pula (Valsaline)	Croatia	44°51'01.1"N 13°50'11.0"E	Sep. 2016
Pula (Uvala Mužilj)	Croatia	44°51'40.5"N 13°48'24.4"E	Apr. 2016
Sv. Marina (Istria)	Croatia	45°01'42.1"N 14°09'17.4"E	Jul. 2016
Messina	Italy	38°14'47.1"N 15°34'59.5"E	Aug. 2014
Saronida	Greece	37°42'60.0"N 23°55'23.2"E	Aug. 2016
Chamolia	Greece	37°54'58.5"N 24°02'08.7"E	Aug. 2016
Thimari	Greece	37°41'06.4"N 23°56'16.6"E	Aug. 2016
Petres (Crete)	Greece	35°21'28.2"N 24°22'08.3"E	Aug. 2016
Vatos (Crete)	Greece	34°59'41.0"N 25°33'17.3"E	Aug. 2016
Mades (Crete)	Greece	35°24'01.1"N 25°02'01.6"E	Aug. 2016
Souda Beach (Crete)	Greece	35°11'32.1"N 24°22'04.9"E	Aug. 2016
Plakias (Crete)	Greece	35°11'40.8"N 24°22'50.9"E	Aug. 2016
Cagnes-sur-mer (Nice)	France	43°39'22.1"N 7°10'25.3"E	Oct. 2016
Antibes (Nice)	France	43°34'12.2"N 7°08'11.5"E	Oct. 2016
Banyuls-sur-mer	France	42°29'18.6"N 3°07'43.9"E	Oct. 2016
Le Port d'Alon (Toulon)	France	43°08'47.8"N 5°42'27.2"E	Oct. 2016

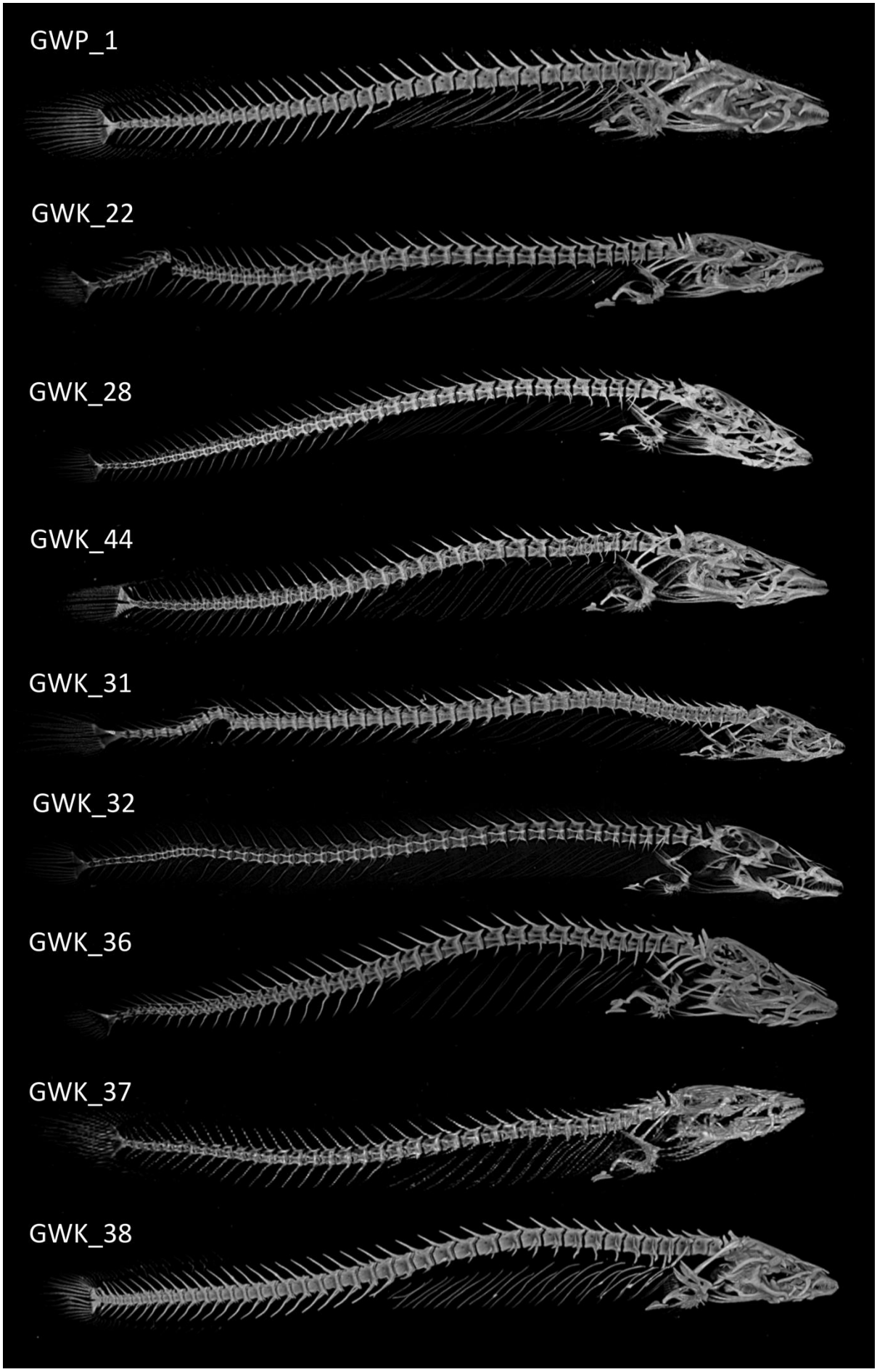
Supplementary Table 2 Horizontal eye diameter (eye) and Standard length measurements in [mm]

ID	Lineage	eye	SL	eye/SL	ID	Lineage	eye	SL	eye/SL	ID	Lineage	eye	SL	eye/SL	ID	Lineage	eye	SL	eye/SL	ID	Lineage	eye	SL	eye/SL
G1_10	G.will	0.76	31.71	0.024	G2_10	Croatia~	0.67	35.44	0.019	G3_10	Croatia*	0.78	24.03	0.032	G4_10	Greece/Crete*	1.00	35.71	0.028	G5_10	Greece~	0.56	24.96	0.022
G1_11	G.will	0.67	35.36	0.019	G2_11	Croatia~	0.68	36.29	0.018	G3_11	Croatia*	0.56	14.80	0.038	G4_11	Greece/Crete*	0.86	26.29	0.033	G5_11	Greece~	0.61	25.72	0.024
G1_12	G.will	1.05	42.59	0.025	G2_12	Croatia~	0.59	32.84	0.018	G3_12	Croatia*	0.62	18.04	0.034	G4_12	Greece/Crete*	0.87	27.61	0.031	G5_12	Greece~	0.59	26.87	0.022
G1_13	G.will	0.98	39.15	0.025	G2_13	Croatia~	0.63	34.48	0.019	G3_13	Croatia*	0.98	36.57	0.027	G4_13	Greece/Crete*	0.69	17.87	0.039	G5_13	Greece~	0.64	31.06	0.021
G1_14	G.will	0.95	35.56	0.027	G2_14	Croatia~	0.64	34.15	0.021	G3_14	Croatia*	0.76	23.58	0.032	G4_14	Greece/Crete*	0.62	17.96	0.035	G5_14	Greece~	0.54	30.81	0.018
G1_15	G.will	0.94	38.04	0.025	G2_15	Croatia~	0.57	26.90	0.018	G3_15	Croatia*	1.10	37.87	0.029	G4_15	Greece/Crete*	0.62	19.05	0.033	G5_15	Greece~	0.60	29.26	0.020
G1_16	G.will	0.70	25.46	0.028	G2_16	Croatia~	0.81	45.29	0.019	G3_16	Croatia*	1.09	42.10	0.026	G4_16	Greece/Crete*	0.67	18.81	0.035	G5_16	Greece~	0.58	32.33	0.018
G1_17	G.will	0.93	34.02	0.027	G2_17	Croatia~	0.80	42.68	0.017	G3_17	Croatia*	0.62	17.34	0.036	G4_17	Greece/Crete*	0.59	14.20	0.042	G5_17	Greece~	0.65	30.37	0.021
G1_18	G.will	0.85	33.59	0.025	G2_18	Croatia~	0.68	40.05	0.018	G3_21	Croatia*	0.88	31.54	0.028	G4_18	Greece/Crete*	0.60	15.77	0.038	G5_18	Greece~	0.57	27.90	0.021
G1_19	G.will	0.94	36.11	0.026	G2_19	Croatia~	0.66	37.05	0.016	G3_22	Croatia*	0.96	31.67	0.030	G4_19	Greece/Crete*	0.76	20.11	0.038	G5_19	Greece~	0.61	30.56	0.020
G1_1	G.will	1.21	44.80	0.027	G2_1	Croatia~	0.67	40.89	0.017	G3_23	Croatia*	0.92	36.81	0.025	G4_1	Greece/Crete*	0.85	29.77	0.028	G5_1	Greece~	0.57	31.02	0.018
G1_20	G.will	0.84	31.28	0.027	G2_20	Croatia~	0.53	31.75	0.018	G3_2	Croatia*	0.72	24.37	0.030	G4_20	Greece/Crete*	0.72	20.85	0.034	G5_20	Greece~	0.50	24.08	0.021
G1_21	G.will	0.63	23.10	0.027	G2_21	Croatia~	0.67	38.02	0.017	G3_3	Croatia*	0.97	29.96	0.032	G4_21	Greece/Crete*	0.44	13.86	0.032	G5_21	Greece~	0.53	26.17	0.020
G1_22	G.will	0.59	23.28	0.025	G2_22	Croatia~	0.60	35.67	0.017	G3_4	Croatia*	0.82	29.50	0.028	G4_22	Greece/Crete*	0.58	16.47	0.035	G5_22	Greece~	0.42	14.74	0.028
G1_23	G.will	0.66	22.87	0.029	G2_23	Croatia~	0.63	36.13	0.017	G3_5	Croatia*	0.86	30.11	0.029	G4_23	Greece/Crete*	0.65	19.74	0.033	G5_23	Greece~	0.51	21.24	0.024
G1_24	G.will	0.60	20.70	0.029	G2_24	Croatia~	0.62	36.22	0.018	G3_6	Croatia*	1.03	30.63	0.034	G4_24	Greece/Crete*	0.68	22.03	0.031	G5_24	Greece~	0.67	29.77	0.022
G1_25	G.will	0.86	30.56	0.028	G2_25	Croatia~	0.57	31.04	0.021	G3_7	Croatia*	0.97	29.29	0.033	G4_25	Greece/Crete*	0.72	21.18	0.034	G5_25	Greece~	0.53	26.26	0.020
G1_26	G.will	0.85	31.92	0.027	G2_26	Croatia~	0.62	29.22	0.018	G3_8	Croatia*	1.31	48.84	0.027	G4_26	Greece/Crete*	0.63	19.31	0.033	G5_26	Greece~	0.48	21.74	0.022
G1_27	G.will	0.62	18.71	0.033	G2_27	Croatia~	0.51	28.57	0.020	G3_9	Croatia*	1.00	38.80	0.026	G4_27	Greece/Crete*	0.61	14.63	0.042	G5_27	Greece~	0.40	15.40	0.026
G1_28	G.will	0.76	26.47	0.029	G2_28	Croatia~	0.61	30.16	0.019						G4_28	Greece/Crete*	0.64	19.00	0.034	G5_28	Greece~	0.38	16.39	0.023
G1_29	G.will	0.64	24.52	0.026	G2_29	Croatia~	0.59	31.12	0.018						G4_29	Greece/Crete*	0.49	13.82	0.036	G5_29	Greece~	0.53	20.46	0.026
G1_2	G.will	1.11	45.87	0.024	G2_2	Croatia~	0.60	33.21	0.019						G4_2	Greece/Crete*	0.70	21.67	0.032	G5_2	Greece~	0.62	30.55	0.020
G1_30	G.will	0.63	21.95	0.029	G2_30	Croatia~	0.52	27.28	0.017						G4_30	Greece/Crete*	0.56	15.90	0.035	G5_30	Greece~	0.51	18.65	0.027
G1_3	G.will	1.10	42.17	0.026	G2_3	Croatia~	0.68	39.74	0.019						G4_3	Greece/Crete*	0.63	20.06	0.031	G5_3	Greece~	0.64	28.72	0.022
G1_4	G.will	0.95	37.70	0.025	G2_4	Croatia~	0.53	28.71	0.019						G4_4	Greece/Crete*	0.61	16.81	0.036	G5_4	Greece~	0.63	30.34	0.021
G1_5	G.will	1.05	37.12	0.028	G2_5	Croatia~	0.66	34.25	0.018						G4_5	Greece/Crete*	0.63	17.01	0.037	G5_5	Greece~	0.70	31.15	0.022
G1_6	G.will	0.88	33.80	0.026	G2_6	Croatia~	0.65	35.22	0.018						G4_6	Greece/Crete*	0.66	16.88	0.039	G5_6	Greece~	0.61	26.08	0.023
G1_7	G.will	0.88	34.10	0.026	G2_7	Croatia~	0.65	35.76	0.018						G4_7	Greece/Crete*	0.77	19.17	0.040	G5_7	Greece~	0.63	29.58	0.021
G1_8	G.will	0.94	34.09	0.028	G2_8	Croatia~	0.59	33.40	0.018						G4_8	Greece/Crete*	0.66	17.05	0.038	G5_9	Greece~	0.55	25.81	0.021
G1_9	G.will	0.85	33.00	0.026	G2_9	Croatia~	0.59	33.12	0.019						G4_9	Greece/Crete*	0.88	28.54	0.031					

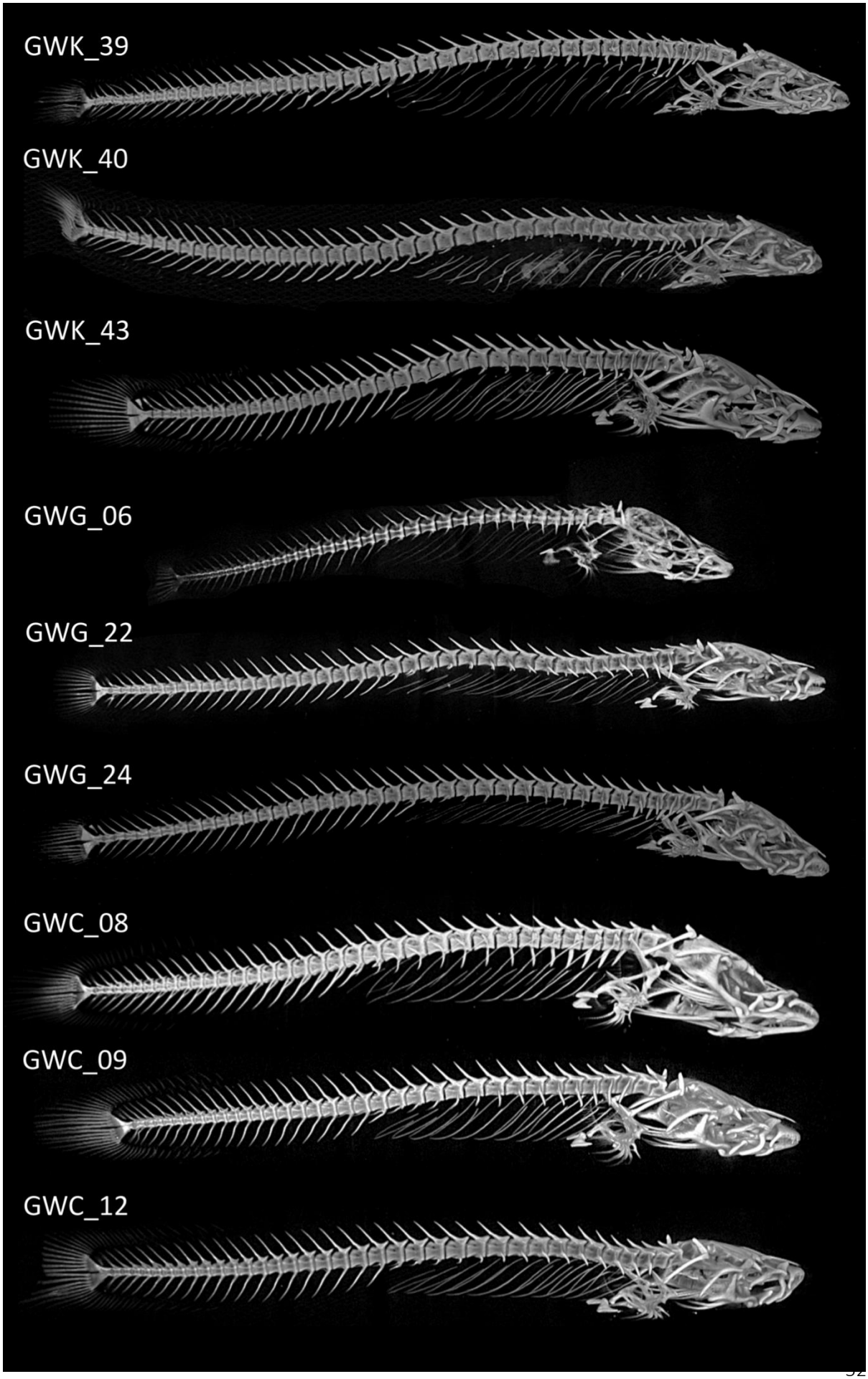
Supplementary Table 3 Vertebrae counts

ID	Vertebrae	Complex
GWK_28	40	Croatia~
GWK_31	40	Croatia~
GWK_32	39	Croatia~
GWK_38	40	Croatia~
GWK_39	40	Croatia~
GWK_40	40	Croatia~
GWP_1	35	Croatia*
GWK_22	35	Croatia*
GWK_36	35	Croatia*
GWK_37	35	Croatia*
GWK_43	35	Croatia*
GWK_44	35	Croatia*
GWG_22	38	Greece~
GWG_24	39	Greece~
GWC_85	40	Greece~
GWG_46	39	Greece~
GWG_35	39	Greece~
GWG_6	35	Greece/Crete*
GWC_8	35	Greece/Crete*
GWC_9	35	Greece/Crete*
GWC_12	36	Greece/Crete*
GWC_54	36	Greece/Crete*
GWC_86	35	Greece/Crete*
GWF_B1	37	West*
GWF_B5	37	West*
GWF_N1	37	West*
GWF_N6	37	West*
GWF_N7	38	West*

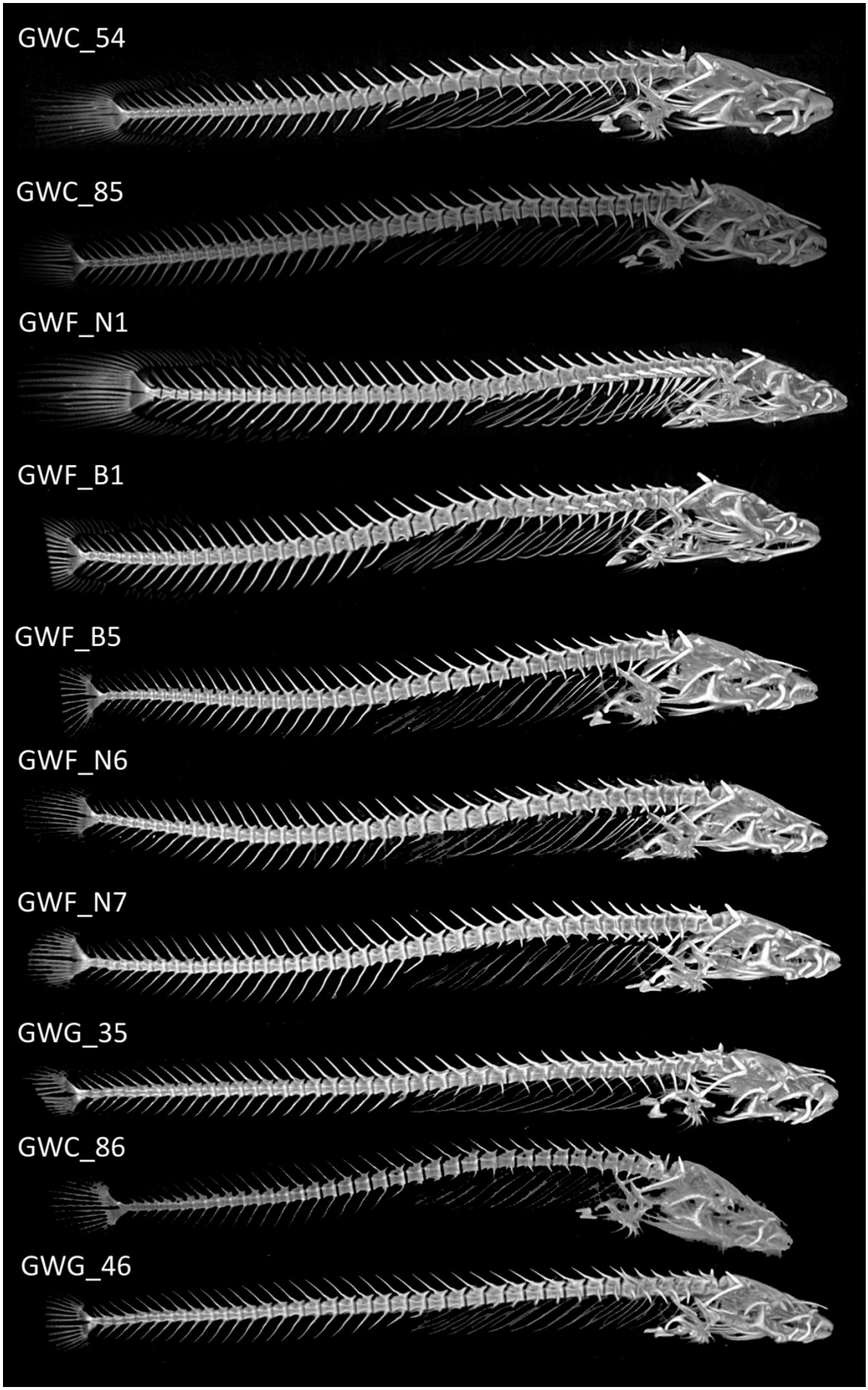
Supplementary Fig. 1 On the next pages, lateral screenshots of all 3D models are shown.



… Supplementary Fig. 1 …



… Supplementary Fig. 1 …



Landmark (roman numerals) and semilandmark (arab numerals) configurations

Dorsal configurations:

I… right center of orbit

II… left center of orbit

III… anterior tip of snout

IV… Right Upper Lip Invagination

V… Right invagination of head (~eyes)

VI… right Right Intersection between Pectoralis and Head

VII… Tip of Caudalis

VIII – X… conform to IV – VI (but left side)

1.. between III and IV

2… betw. IV and V

3,4… betw. V and VI

5 – 9… betw. VI and VII

10 – 18 correspond to the numbers 1 – 9

19… betw. I and II

29… betw. VI and VIII

21… betw. 6 and 13;

Lateral configurations:

I… center of orbit

II… Anterior tip of snout

III… Most posterior point of lips

IV… Upper end of caudal peduncle

V… Midpoint of origin of caudal fin

VI… Lower edge of caudal peduncle

VII… intersection betw. pectoralis and body

VIII… intersection betw. sucking disc and head

3… point of largest head width

1,2… betw. II and 3

7… above IV

4 – 6… betw. 3 and 7

11… posterior tip of caudalis

8 – 10… betw. 7 and 11

15… below VI

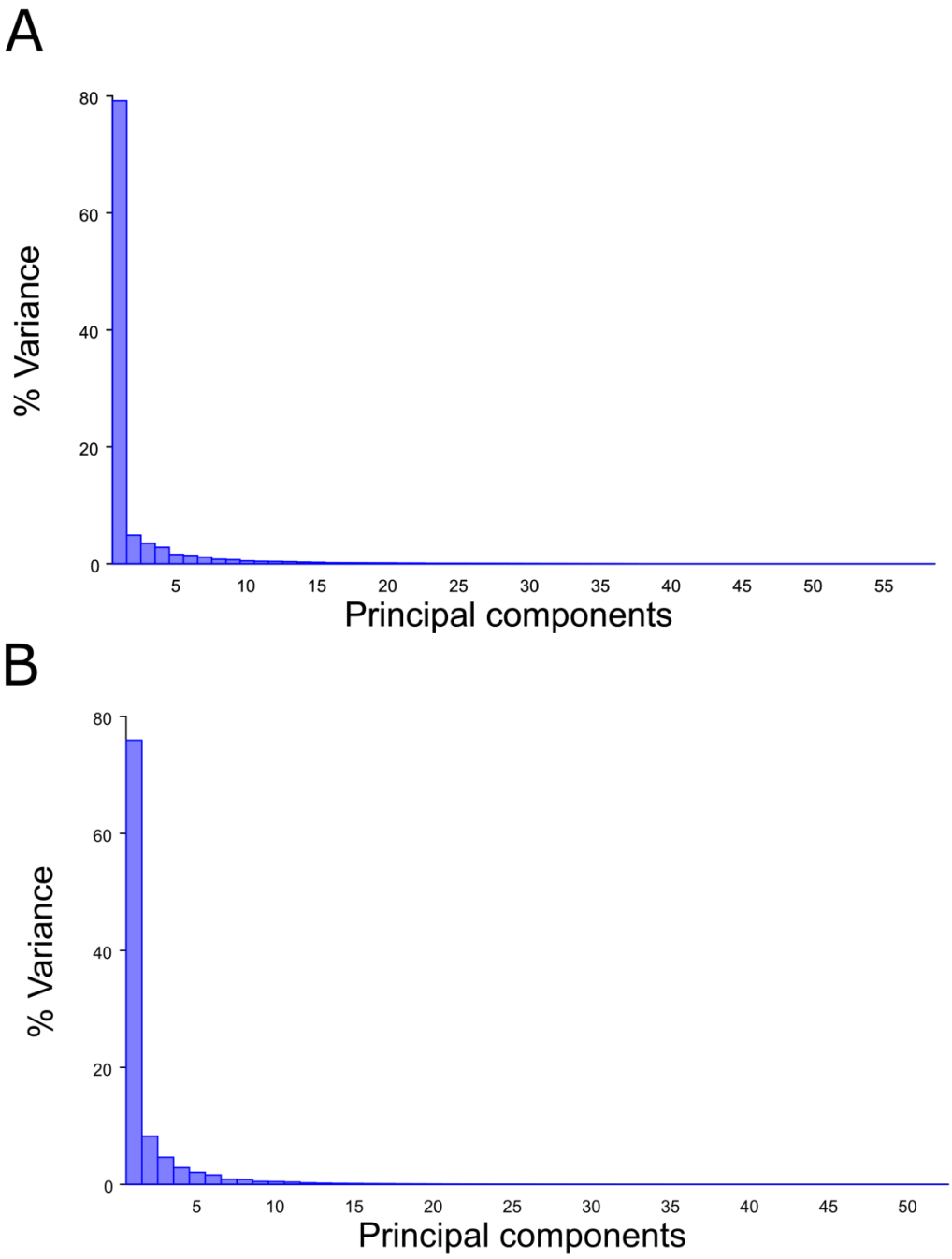
12 – 14 betw. 11 and 15

16, 17… betw. 15 and VII

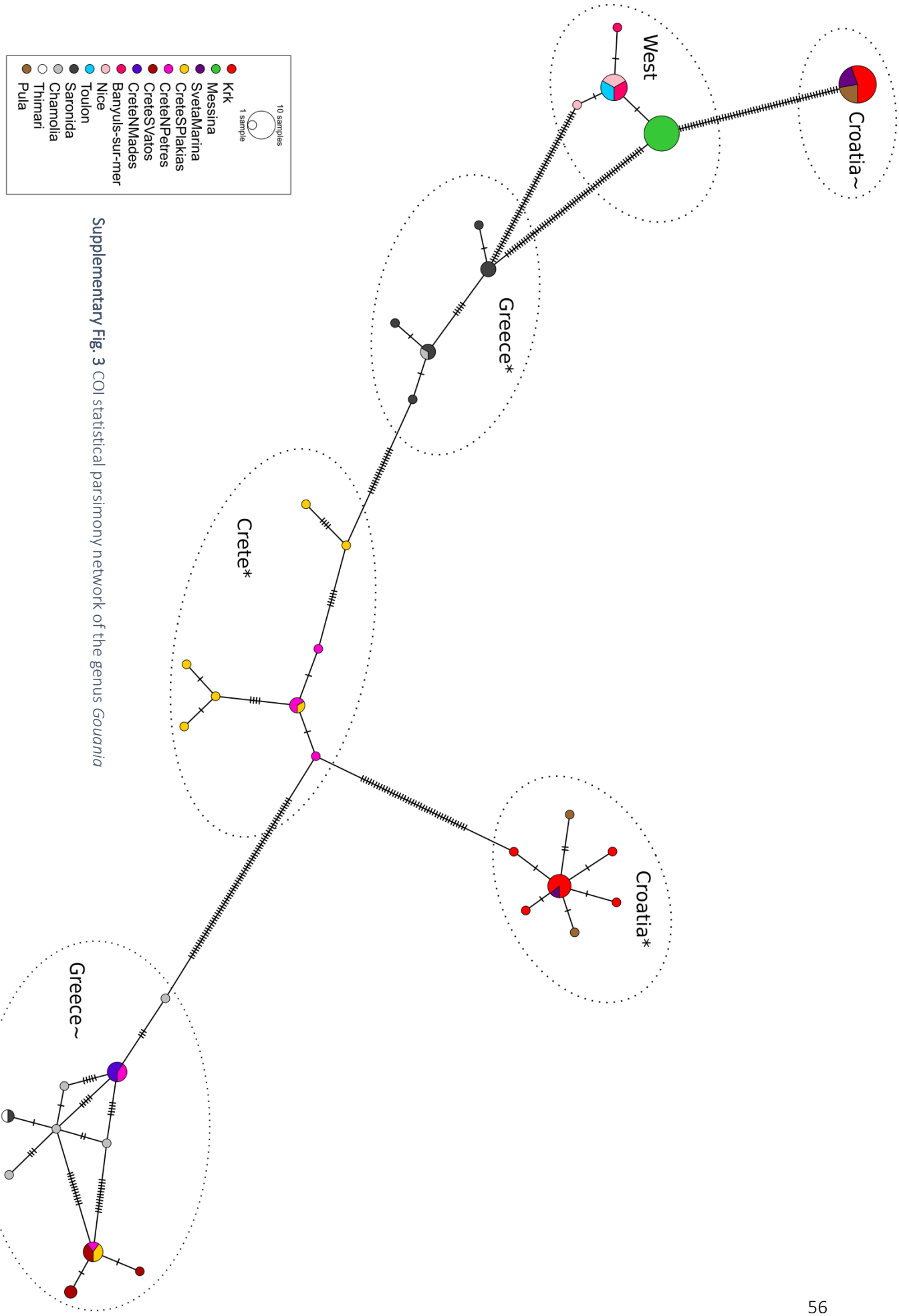
19… lower jaw tip

18… betw. VIII and 19

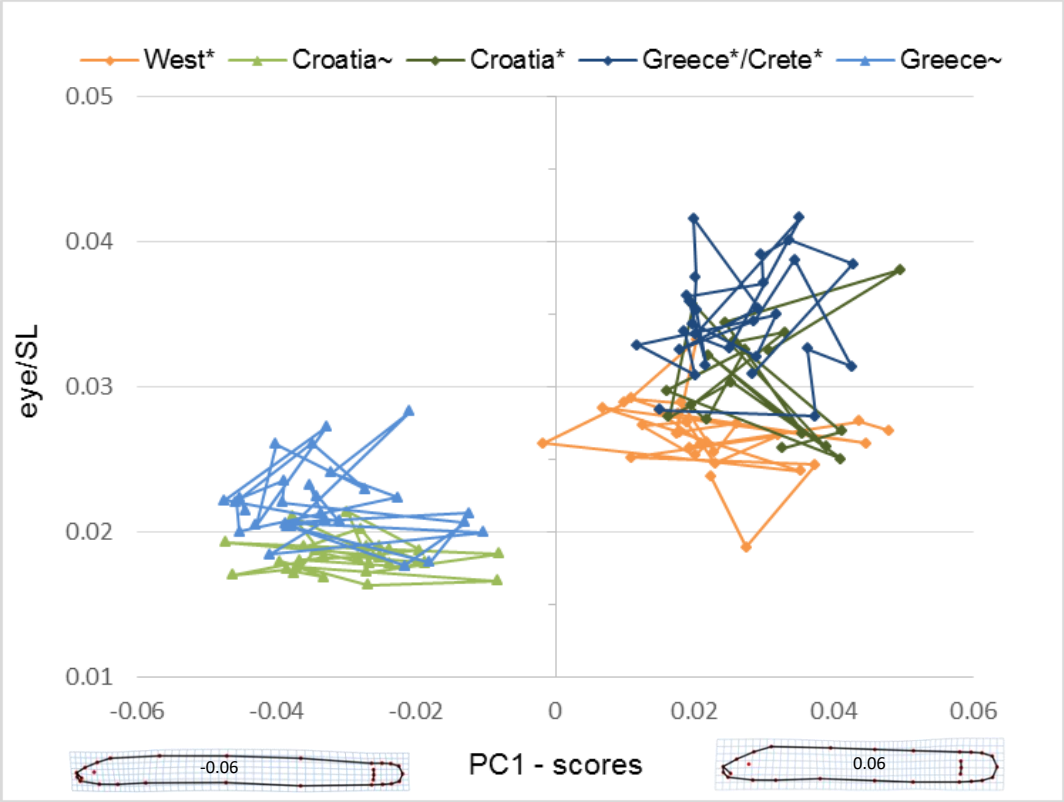
20… betw. 19



Supplementary Fig. 2 Scree plots of the Principal Component analysis of the geometric Morphometric approach (see 1.3.4)



Supplementary Fig. 3 COI statistical parsimony network of the genus *Gouania*



Supplementary Fig. 4 Eye size matches body shape. The PC1 scores of the Principal component analysis described 75.93 % of the variance, hence is a good representation about overall body shape. There is a correlation between body shape and size of eyes. Furthermore, deformation grids are given.

VII Appendix Part II

ACTA ICHTHYOLOGICA ET PISCATORIA (2017) 47 (4): 417–421 DOI: 10.3750/AIEP/02244

LEPADOGASTER PURPUREA (ACTINOPTERYGII: GOBIESOCIFORMES: GOBIESOCIDAE) FROM THE EASTERN MEDITERRANEAN SEA: SIGNIFICANTLY EXTENDED DISTRIBUTION RANGE

Maximilian WAGNER[1*], Sandra BRAČUN[1, 2], Marcelo KOVAČIĆ[3], Samuel P. IGLÉSIAS[4, 5], Daniel Y. SELLOS[4], Stamatis ZOGARIS[6], and Stephan KOBLMÜLLER[1*]

[1] *Institute of Zoology, University of Graz, Graz, Austria*

[2] *Morska Škola Pula, Pula, Croatia*

[3] *Natural History Museum Rijeka, Rijeka, Croatia*

[4] *Muséum National d'Histoire Naturelle, Station de Biologie Marine de Concarneau, Concarneau, France*

[5] *Département Systématique et Evolution, Institut de Systématique, Evolution, Biodiversité, Sorbonne Universités, Paris, France*

[6] *Institute of Marine Biological Resources and Inland Waters, Hellenic Centre for Marine Research (HCMR), Anavissos, Greece*

Wagner M., Bračun M., Kovačič M., Iglésias S.P., Sellos D.Y., Zogaris S., Koblmüller S. 2017. *Lepadogaster purpurea* (Actinopterygii: Gobiesociformes: Gobiesocidae) from the eastern Mediterranean Sea: Significantly extended distribution range. Acta Ichthyol. Piscat. 47 (4): 417–421.

Abstract. The Cornish sucker, *Lepadogaster purpurea* (Bonnaterre, 1788), a clingfish species thus far known from the north-eastern Atlantic south to western Africa, the Canary Islands and Madeira, and the western Mediterranean basin, was recently collected in Sicily (Italy), Croatia and Greece. Species identification was based on morphological and/or molecular data. These new Mediterranean records of *L. purpurea* are the first evidence of the species' occurrence in the eastern Mediterranean basin and significantly extend its known distribution range, which likely mirrors that of its sister species *Lepadogaster lepadogaster* (Bonnaterre, 1788).

Keywords: clingfish, biogeography, overlooked diversity, sister species

Clingfishes (Gobiesocidae) are small cryptobenthic fishes that inhabit crevices in rocks or sea grass rhizomes or are found under large boulders and in pebble interstices. Among the eight species reported from the Mediterranean Sea, the species of the genus *Lepadogaster* are certainly the best known. Restricted to temperate waters, members of this genus inhabit mainly shore reefs of the intertidal zone (Hofrichter unpublished[**]). Although being quite abundant in suitable habitat (e.g., 23 individuals of *L. lepadogaster* per m^2; Hofrichter and Patzner 2000) the taxonomy of the genus *Lepadogaster* has long been unclear. Briggs (1955) recognized three species of this genus: *Lepadogaster candolii* (Risso 1810), *Lepadogaster zebrina* Lowe 1839, as well as two subspecies of *Lepadogaster lepadogaster* (Bonnaterre 1788)—*L. lepadogaster lepadogaster* and *L. lepadogaster purpurea*. However, Henriques et al. (2002) invalidated the species-status of *L. zebrina*, which is currently regarded as a *L. lepadogaster* population from Madeira. Moreover, Henriques et al. (2002) showed that *L. lepadogaster* and *Lepadogaster purpurea* (Bonnaterre, 1788) are two closely related, but clearly distinct species living sympatrically in the interstices of boulder beaches. Subsequent molecular phylogenetic analyses showed that the genus *Lepadogaster* is polyphyletic, with the genus *Gouania* being the sister taxon of the species pair *L. lepadogaster* and *L. purpurea* (see Almada et al. 2008).

Even though *L. purpurea* and *L. lepadogaster* are very similar in overall appearance, the species can be distinguished by a number of morphological traits, such as size and number of papillae on the sucking disc, size of head-marks and eyespots, different body coloration, snout length and interorbital distance (Henriques et al.

* Correspondence: Priv.-Doz. Dr. Stephan Koblmüller, Institut für Zoologie, Universität Graz, Universitätsplatz 2, 8010 Graz, Austria, phone: 00433163803978, fax: 00433163809875, e-mail: (SK) *stephan.koblmueller@uni-graz.at*, (MW) *maximilian.wagner@edu.uni-graz.at*, (SB) *marebracun@gmail.com*, (MK) *marcelo@prirodoslovni.com*, (SPI) *samuel.iglesias@mnhn.fr*, (DYS) *sellos@mnhn.fr*, (SZ) *zogaris@gmail.com*.

** Hofrichter R. 1995. Taxonomie, Verbreitung und Ökologie von Schildfischen der Unterfamilie Lepadogastrinae (Gobiesocidae, Teleostei). PhD Thesis. University of Salzburg, Austria.

2002). Furthermore, they show clear differences in larval development and behaviour (Faria and Gonçalves 2010, Tojeira et al. 2012). The two species further differ in their microhabitat preferences and breeding seasons (Patzner 1999, Henriques et al. 2002). Both species are broadly sympatric along the European and north-western African Atlantic coasts and islands, whereas only *L. lepadogaster* is thought to be present and common throughout the Mediterranean and the Black Sea. Only a few positive records of *L. purpurea* are available for the Mediterranean Sea, with the easternmost reported occurrence from Genova, Italy (Henriques et al. 2002).

Here, based on morphological and molecular data, we provide evidence for the occurrence of *L. purpurea* also in the eastern Mediterranean basin.

In August 2016, specimens of the genus *Lepadogaster* were caught in Greek waters south of Athens at Chamolia (37.916250°N, 24.035750°E), Thimari (37.685111°N, 23.937944°E), and at three locations on Crete (Plakias: 35.194667°N, 24.380806°E; Petres: 35.357833°N,24.368972°E;Myrtos:34.994722°N,25.554806°E)in around one meter water depth, underneath boulders and pebbles with a diameter of about 5–20 cm. Following euthanization with MS-222, standardized photographs were taken and fishes were preserved in ethanol (>95%, small individuals) or formalin (7%, large individuals; prior to fixation in formalin a finclip was taken from the right pectoral fin and preserved in ethanol) for subsequent genetic and morphological analysis. In addition, two ethanol-preserved tissue samples of putative *L. purpurea* from Messina, Italy (38.219137°N, 15.567788°E), collected in 2014 were included in the genetic analysis. For one putative individual of *L. purpurea* collected on the island of Ilovik, Croatia (44.445389°N, 14.57625°E) in October 2014 only morphological data were taken, as DNA quality proved to be insufficient for PCR and sequencing.

Morphometric and meristic measurements followed Hofrichter (unpublished*). Total length (TL), standard length (SL), head length (Hl), body depth (Bd), body width (Bw), sucking disc length (SDl), sucking disc width (SDw), interorbital distance (iO) and number of papillae in sucking disc regions A, B, C (papA, papB, papC) was measured/counted in two individuals of putative *L. purpurea* and *L. lepadogaster* from Greece, and one putative *L. purpurea* individual from Croatia. Voucher specimens of all individuals used for morphological analysis were deposited at the Natural History Museum Rijeka, Croatia (*L. purpurea* voucher IDs: PMR VP3580, Prisliga, island of Ilovik, 10 October 2014; PMR VP4054 LG2, Chamolia, south to Athens, Greece, 6 August 2016; PMR VP4055 LG3, Chamolia, south to Athens, Greece, 6 August 2016; *L. lepadogaster* voucher IDs: PMR VP4053 LG1, Chamolia, south to Athens, Greece, 6 August 2016; PMR VP4056 LG7, Petres, Crete, Greece, 10 August 2016).

DNA was extracted from fin tissue using a rapid Chelex protocol (Richlen and Barber 2005). A 390 bp long fragment of the third domain of the mitochondrial 12S rDNA and a 577 bp long part of the mitochondrial COI gene were amplified and sequenced according to the protocols described in Henriques et al. (2002) and Duftner et al. (2005), respectively. The primer pairs used for PCR and chain termination sequencing were 12sFor/12sRev (Henriques et al. 2002) and FishF1/FishR1 (Ward et al. 2005) for 12S and COI respectively. DNA fragments were purified with Sephadex™ G-50 (GE Healthcare) and visualized on an ABI 3130xl capillary sequencer (Applied Biosystems). In addition, following sequences were downloaded from GenBank and added to the dataset: for 12S rDNA AY036587 and AY036589–AY036605 (Henriques et al. 2002), and for COI KF369136, KJ616457 and KJ768244–KJ768246 (Lobo et al. 2013, Conway et al. 2014, Landi et al. 2014). Sequences were aligned in MEGA 6.0 (Tamura et al. 2013) using MUSCLE (Edgar 2004). All newly generated sequences are deposited in GenBank under the accession numbers MF425769–MF425781 and MF544114–MF544120. Some sequences from Greece (of the individuals also used for morphological analysis) are also available from BOLD (project MEDLP, Mediterranean *Lepadogaster purpurea*). For phylogenetic tree inference, sequences were collapsed into haplotypes. Unrooted maximum likelihood (ML) trees were inferred in PhyML 3.0 (Guindon et al. 2010), employing the best fitting substitution models selected based on the Bayesian Information Criterion (BIC) in MEGA. Statistical support was assessed from 1000 bootstrap replicates.

Based on morphology, two specimens from Chamolia (Greece) and one individual from Ilovik (Croatia) were identified as *L. purpurea*, whereas all the other eight Greek specimens were identified as *L. lepadogaster*. The morphological characteristics of the collected specimens usually fell within the ranges provided earlier by other researchers for the two focal species (Henriques et al. 2002). Interestingly, one of the *L. purpurea* specimens (PMR VP4054 LG2) had fewer rows of papillae in sucking disc region B than is typical for this species (see Henriques et al. 2002). The morphological species identification was further supported by DNA sequence data, which clustered the individuals into two distinct groups corresponding to *L. lepadogaster* and *L. purpurea* (Fig. 1; only the 12S rDNA tree is shown as COI shows the same pattern). 12S and COI net divergence (uncorrected p-distance) between *L. purpurea* and *L. lepadogaster* was 2.7% and 8.5%, respectively.

Our new *Lepadogaster purpurea* records from Messina (Italy, molecular), the island of Ilovik (Croatia, morphological) and Chamolia (Greece, molecular and morphological) are the easternmost records of this species so far and include the first definitive records from the eastern Mediterranean basin. A possible occurrence of *L. purpurea* in the Black Sea was documented by Briggs (1986), who refers to a record by Murgoci (1964). Taking our findings into account, it seems very likely that Murgoci's (1964) individuals of putative *L. purpurea* from the Black Sea are indeed *L. purpurea*. Consequently, the actual distribution

* See footnote on page 409

range of *L. purpurea* in the Mediterranean (and possibly also Black) Sea is much larger than previously assumed and mirrors that of its sister species *L. lepadogaster*.

Considering its widespread distribution in the Mediterranean Sea and its occurrence in shallow water, it is quite astonishing that *L. purpurea* has been overlooked for such a long time. A likely reason therefore might be its cryptobenthic life style (Henriques et al. 2002). *Lepadogaster purpurea* preferentially occurs under large boulders (Henriques et al. 2002) and thus could be very easily missed in ichthyofaunal surveys. However, all individuals of *L. purpurea* collected in the present study were found in pebble or cobble sized substrates (~5–20 cm diameter). As only juveniles (<4 cm SL) were caught in the presently reported study, this indicates that juvenile *L. purpurea* are not restricted to large boulders and do also occur in regular pebble fields. Whether this is a general pattern or only true for *L. purpurea* from the eastern Mediterranean basin remains to be confirmed through further studies.

It is very likely that *Lepadogaster purpurea* has been erroneously recorded as *L. lepadogaster* in the past. This is not at all surprising considering that the two species were regarded as two sub-species of *L. lepadogaster*. On the other hand, the two species are easy to tell apart based on a number of characters (Henriques et al. 2002). Size (smaller in *L. purpurea*) and number of papillae on the sucking disc region A (5–6 rows in *L. purpurea*, 3–4 rows in *L. lepadogaster*) and B (5–6 rows in *L. purpurea*, 3–4 rows in *L. lepadogaster*), as well as the eyespots on the back (see Fig. 2) are reliable characters for species identification. Whereas the papillae of the sucking disc stay intact even after preservation of fishes, the size of the eyespots (head markings) becomes useless in lab identification.

Our study shows that even though the importance of small cryptobenthic fishes for coastal marine ecosystems has been assessed in a variety of studies (e.g., Allen et al. 1992, Ackermann and Bellwood 2000, Depczynski and Bellwood 2003, Smith-Vaniz et al. 2006), the level of knowledge about these fishes is still very poor. The general presumption that *L. lepadogaster* has a much larger distribution range in the Mediterranean Sea than its sister species *L. purpurea* appears to be due to a lack of taxonomic expertise combined with the fact that *L. lepadogaster* and *L. purpurea* were elevated to species rank just a few years ago. We assume that the distribution of *L. purpurea* has been underestimated and expect this species to have a pan-Mediterranean distribution. Probably, species assignment of many specimens deposited in museums is incorrect and needs to be updated, which should be straightforward employing the characters listed by Henriques et al. (2002).

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REFERENCES

Ackerman J.L., Bellwood D.R. 2000. Reef fish assemblages: A re-evaluation using enclosed rotenone stations. Marine Ecology Progress Series **206**: 227–237.
DOI: 10.3354/meps206227

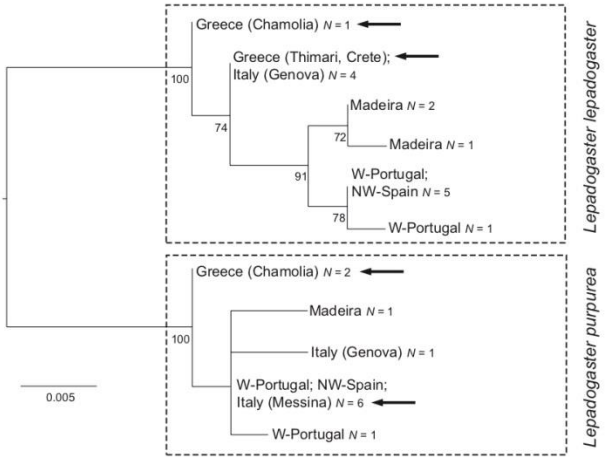

Fig. 1. Maximum likelihood tree based on 12s rDNA data (only bootstrap values > 50 are shown) showing the phylogenetic relations between *Lepadogaster lepadogaster* and *L. purpurea*; the tree was rooted using the midpoint rooting criterion; arrows indicate the haplotypes found in newly sequenced *Lepadogaster* specimens from the Mediterranean Sea

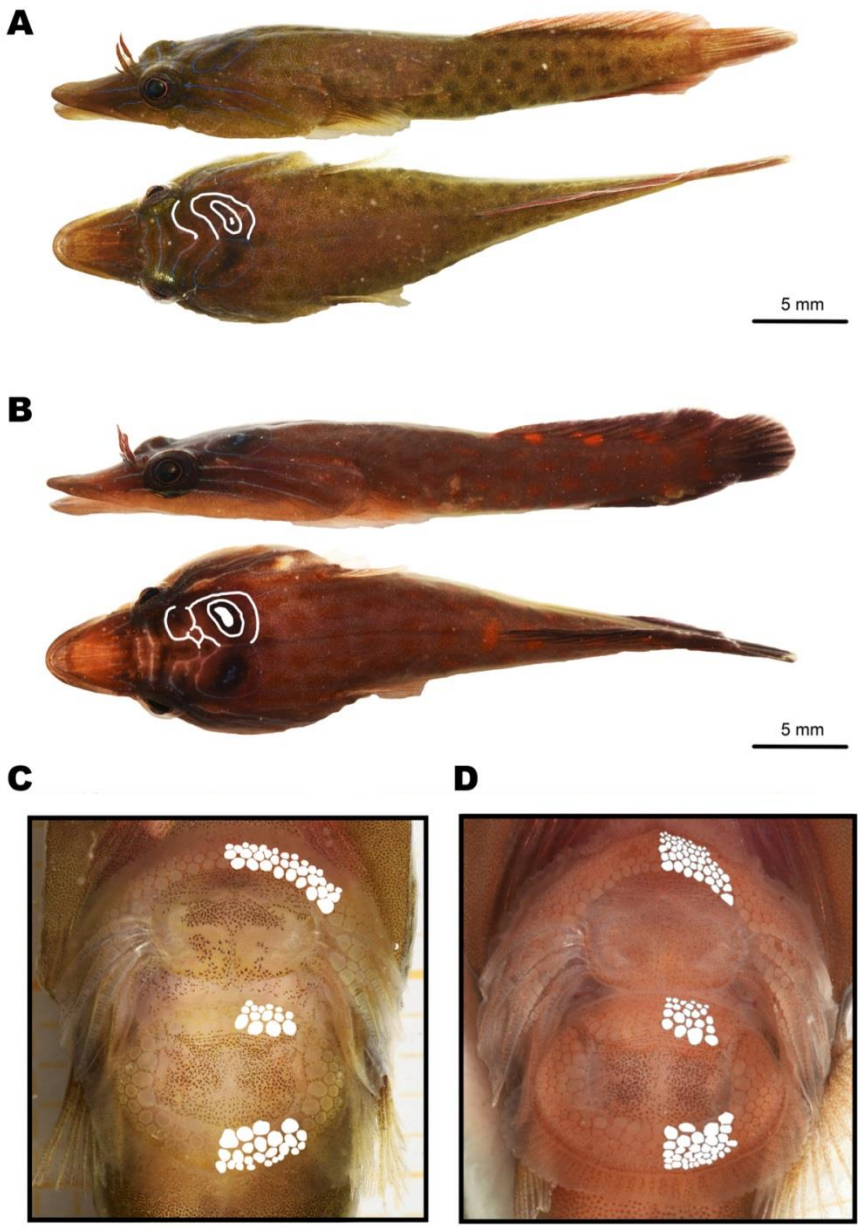

Fig. 2. Photographs and overlaid drawings highlighting the distinctive phenotypic characters that distinguish the two *Lepadogaster* species: (**A**) *L. lepadogaster* has smaller eyespots on the head than (**B**) *L. purpurea*; sucking-disc papillae differ in size and number of rows between (**C**) *L. lepadogaster* and (**D**) *L. purpurea*; the specimens shown are: *L. lepadogaster*, PMR VP4053 LG1, and *L. purpurea*, PMR VP4055 LG3, both from Chamolia, Greece

Allen L.G., Bouvier L.S., Jensen R.E. 1992. Abundance, diversity, and seasonality of cryptic fishes and their contribution to a temperate reef fish assemblage off Santa Catalina Island, California. Bulletin of the Southern California Academy of Sciences **91** (2): 55–69.

Almada F., Henriques M., Levy A., Pereira A., Robalo J., Almada V.C. 2008. Reclassification of *Lepadogaster candollei* based on molecular and meristic evidence with a redefinition of the genus *Lepadogaster*. Molecular Phylogenetics and Evolution **46** (3): 1151–1156.
DOI: 10.1016/j.ympev.2007.05.021

Briggs J.C. 1955. A monograph of the clingfishes (Order Xenopterygii). Stanford Ichthyological Bulletin **6**: 33–39.

Briggs J.C. 1986. Gobiesocidae. Pp. 1351–1359. *In:* Whitehead P.J.P., Bauchot M.-L., Hureau J.-C., Nielsen J., Tortonese E. (eds.) Fishes of the north-eastern Atlantic and the Mediterranean. Volume 3. UNESCO, Paris.

Conway K.W., Baldwin C., White M.D. 2014. Cryptic diversity and venom glands in western Atlantic clingfishes of the genus *Acyrtus* (Teleostei: Gobiesocidae). PLoS ONE **9** (5): e97664.
DOI: 10.1371/journal.pone.0097664

Depczynski M., Bellwood D.R. 2003. The role of cryptobenthic reef fishes in coral reef trophodynamics. Marine Ecology Progress Series **256**: 183–191.
DOI: 10.3354/meps256183

Duftner N., Koblmüller S., Sturmbauer C. 2005. Evolutionary relationships of the limnochromini, a tribe of benthic deepwater cichlid fish endemic to Lake Tanganyika, East Africa. Journal of Molecular Evolution **60** (3): 277-289.
DOI: 10.1007/s00239-004-0017-8

Edgar R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research **32** (5): 1792–1797.
DOI: 10.1093/nar/gkh340

Faria A.M., Gonçalves E.J. 2010. Ontogeny of swimming behaviour of two temperate clingfishes, *Lepadogaster lepadogaster* and *L. purpurea* (Gobiesocidae). Marine Ecology Progress Series **414**: 237–248.
DOI: 10.3354/meps08692

Guindon S., Dufayard J.-F., Lefort V., Anisimova M., Hordijk W., Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Systematic Biology **59** (3): 307–321.
DOI: 10.1093/sysbio/syq010

Henriques M., Lourenço R., Almada F., Calado G., Gonçalves D., Guillemaud T., Cancela M.L., Almada V.C. 2002. A revision of the status of *Lepadogaster lepadogaster* (Teleostei: Gobiesocidae): Sympatric subspecies or a long misunderstood blend of species? Biological Journal of the Linnean Society **76** (3): 327–338.
DOI: 10.1046/j.1095-8312.2002.00067.x

Hofrichter R., Patzner R.A. 2000. Habitat and microhabitat of Mediterranean clingfishes (Teleostei: Gobiesociformes: Gobiesocidae). Marine Ecology **21** (1): 41–53.
DOI: 10.1046/j.1439-0485.2000.00689.x

Landi M., Dimech M., Arculeo M., Biondo G., Martins R., Carneiro M., Carvalho G.R., Brutto S.L., Costa F.O. 2014. DNA barcoding for species assignment: The case of Mediterranean marine fishes. PLoS ONE **9** (9): e106135.
DOI: 10.1371/journal.pone.0106135

Lobo J., Costa P.M., Teixeira M.A.L., Ferreira M.S.G., Costa M.H., Costa F.O. 2013. Enhanced primers for amplification of DNA barcodes from a broad range of marine metazoans. BMC Ecology **13** (1): 34.
DOI: 10.1186/1472-6785-13-34

Murgoci A.A. 1964. Contribution à la connaissance des gobiesocides (ordre des Xenopterygii) de la mer Noire. Revue Roumaine de Biologie, Série de Zoologie **9**: 297–306.

Patzner R.A. 1999. Habitat utilization and depth distribution of small cryptobenthic fishes (Blenniidae, Gobiesocidae, Gobiidae, Tripterygiidae) in Ibiza (western Mediterranean Sea). Environmental Biology of Fishes **55** (3): 207–214.
DOI: 10.1023/A:1007535808710

Richlen M.L., Barber P.H. 2005. A technique for the rapid extraction of microalgal DNA from single live and preserved cells. Molecular Ecology Notes **5** (3): 688–691.
DOI: 10.1111/j.1471-8286.2005.01032.x

Smith-Vaniz W.F., Jelks H.L., Rocha L.A. 2006. Relevance of cryptic fishes in biodiversity assessments: a case study at Buck Island Reef National Monument, St. Croix. Bulletin of Marine Science **79** (1): 17–48.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution **30** (12): 2725–2729.
DOI: 10.1093/molbev/mst197

Tojeira I., Faria A.M., Henriques S., Faria C., Gonçalves E.J. 2012. Early development and larval behaviour of two clingfishes, *Lepadogaster purpurea* and *Lepadogaster lepadogaster* (Pisces: Gobiesocidae). Environmental Biology of Fishes **93** (4): 449–459.
DOI: 10.1007/s10641-011-9935-7

Ward R.D., Zemlak T.S., Innes B.H., Last P.R., Hebert P.D.N. 2005. DNA barcoding of Australia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences **360** (1462): 1847–1857.
DOI: 10.1098/rstb.2005.1716

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