REGULAR PAPER

DNA reconciles morphology and colouration in the drunk blenny genus *Scartichthys* (Teleostei: Blenniidae) and provides insights into their evolutionary history

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Abstract

The blenniids of the genus *Scartichthys* represent key herbivore species of central and south American Pacific coastal reefs. Yet *Scartichthys* spp. remain difficult to identify in the field, especially across the c.a 6000 km where three of the four currently accepted species are known to occur in sympatry. The main diagnostic characters from traditional taxonomy that have been used to revise this genus are indeed elusive. At the same time, species can display multiple colour patterns in the field, depending on the ontogenetic stage, habitat association, and/or reproductive behaviour. Overall, molecular characterization is warranted to help address these issues. Here, we used a combination of colouration, morphological and molecular data for the first time, including specimens representative of the four currently valid species and seven described colour patterns. Our integrative approach revealed that only three of the four species should be considered as valid; *Scartichthys gigas* (Steindachner, 1876), *S. variolatus* (Valenciennes, 1836) and *S. viridis* (Valenciennes, 1836); while S. *crapulatus* Williams 1990 should be synonymized with *S. viridis*. In the same way, our analyses show that one of the colour patterns attributed so far to *S. gigas* is characteristic of the juvenile stages of *S. viridis*. Our time-calibrated phylogeny shows that this genus is relatively young, with an estimated time of divergence between *Scartichthys gigas* and *S. viridis* of around 1.71 Ma. In comparison, the Desventuradas and Juan Fernandez Islands endemic *S. variolatus* diverged about 1.95 Ma. Our results help to clarify the taxonomy of the *Scartichthys* genus.

Keywords biogeography, Chile, integrative taxonomy, kelp forests, molecular phylogeny, species delimitation.

1 | INTRODUCTION

Species are the core units of any analysis in ecological, biogeographical, conservation, or evolutionary studies. Taxonomists used to describe and name species using solely morphological characters, being the only tool available until the development of modern molecular biology tools (Teletchea, 2010). The recent development of molecular biology with the use of DNA sequences data, combined with global initiative such as the Barcode of Life initiative (Hebert & Gregory, 2005) have offered new tools and framework to not only complete taxonomic description but also challenge how species are described. If molecular approaches were at the beginning the subject of debate in the taxonomists community (Will et al., 2005), it is now recognized that the combination of molecules and morphology can improve and accelerate the process of describing new species (Kekkonen & Hebert, 2014a; Pante et al., 2015). In this context, the integrative taxonomy framework has been proposed to examine the congruence of the diversity of data available such as morphology, colouration, behaviour, and molecules to help delimiting species (Padial et al., 2010).

The blenniids of the genus *Scartichthys* are one of the most abundant herbivorous fishes of intertidal and shallow subtidal rocky environments along the Pacific coast of South America from western Panama to the latitude 33ºS in Chile. They also occur at the Juan Fernández Archipelago and the Desventuradas Islands (Stepien, 1990; Pérez-Matus et al., 2017a, 2017b). They are famous among fishermen for the unfounded side effects associated with its consumption leading to its common name “borrachillas” [the drunken ones] (Williams, 1990; Méndez-Abarca & Mundaca, 2016). Yet, these giants among blenniids, adults reaching up to 300 mm total length, remain difficult (Pérez-Matus et al., 2007; Riquelme-Pérez et al., 2019) or even impossible to identify at the species level in the field (Villegas et al., 2019), especially across the 6000 km where three of them are known to occur in sympatry. Elusive characters such as the number of dental incisors (DI) and colour patterns described mainly from preserved specimens, the only material available at that time (Williams 1990), are indeed the main diagnostic characters for the four currently valid species: (1) *Scartichthys variolatus* (Valenciennes, 1836) is endemic to the islands of the Juan Fernández Archipelago (33°SL) and the San Ambrosio and San Félix Islands

(26°SL); (2) *S. gigas* (Steindachner, 1876) is distributed from Panama (9 ° NL) to Northern Chile (Antofagasta, 23°SL) and in contrast to the three other species, display less than 73 DI and typically 17 dorsal rays; (3) *S. viridis* (Valenciennes, 1836) with a geographic distribution from Peru (Independence Bay, 14°SL) to Central Chile (Valparaíso, 33 ° SL), display more than 73 DI while the distinct colour patterns of preserved specimen (*"Tiny, dark-brown (rarely pale) spots on posterior half of body*") have been commonly used to described it; (4) *S. crapulatus* Williams 1990, as a species endemic to Central Chile, reported only from Central Chile (Barquito (26°S) and Valparaiso (33°S)) where it occurs in sympatry with *S. gigas* and *S. viridis*.

Using colouration patterns solely to identify species can be problematic and can lead to confusion in the field. Five different colour patterns based on live specimens were reported (Méndez-Abarca & Mundaca, 2016) for two species, *S. viridis* and *S. gigas*. A clear link has been made between the "reticulated colour pattern" reported by Williams (1990) from preserved specimens of *S. gigas* and three live colour patterns reported by Méndez-Abarca & Mundaca (2016), respectively the "two-bar front head covered", the "two-bar front head uncovered", and the "reticulated bar-stained". In addition to these three colour patterns, a new colour pattern has been attributed to juveniles of *S. gigas*; the "uniform orange-brown". This last colour pattern is problematic because it is very similar to one of the colours in life described for *S. crapulatus* ("reddish-brown to golden") by Williams (1990). However, *S. crapulatus* are also presenting "orange-brown dots on posterior half of the body" (Williams, 1990) difficult to see on the field. A "dark-light bluish green" pattern has been attributed to juveniles and adults of *S. viridis* (Méndez-Abarca & Mundaca, 2016). Together with the "circular red spots in head and body" pattern of *S. variolatus*, a total of seven different colour patterns have been described for the four species of this genus. The diversity of live colour patterns has been attributed to ontogeny, habitat association, and/or reproductive behavior. Unfortunately, no explicit references have been made to classic morphological characters such as the number of DI for the new colour patterns described. It is worth noting that assessing these characters are of particular complexity for juveniles.

Finally, the recent phylogeny of blenniids (Hundt & Simons, 2018) represents the first attempts to reconstruct the evolutionary history of *Scartichthys* including one representative of the four currently valid species based on five nuclear markers (refer to Supplementary Figure S1 in Hundt & Simons (2018)). This molecular phylogeny confirmed the monophyly of the genus *Scartichthys*. Molecular evidence (Hundt et al., 2014; Hundt & Simons, 2018) confirmed that *Scartichthys* was sister to *Ophioblennius* as formerly hypothesized by Williams (1990) using morphological data. Interestingly, a minimal divergence between *S. crapulatus* and *S. viridis* was retrieved, questioning whether this divergence was of the order of inter or intraspecific divergence and paralleling the concern expressed by Stepien (1990) regarding the validity of *S. crapulatus.*

The elusive diagnostic morphological characters, the controversy regarding the validity of *S. crapulatus*, the lack of diagnostic molecular data, and the multitude of colour patterns described so far for the genus *Scartichthys* called for a reappraisal of the different diagnostic characters available so far. This study aims at clarifying the taxonomy of *Scartichthys* using for the first-time colouration, morphological and molecular data in combination. We reconstructed a phylogeny, including the different live colour patterns described so far for the four currently valid species of this genus to investigate the validity of the species described so far and reconstruct the evolutionary history of this genus.

2 | MATERIALS AND METHODS

2.1 | Ethical statement sampling

Fishes were collected according to Chilean environmental laws through R.EX 2231, R.EX 556 and R.EX 1489 permits, and procedures for collection, maintenance, and analyses of fishes followed the international guidelines for animal experiments through ethical permits of Universidad de Valparaiso and Pontificia Universidad Católica de Chile.

2.2 | Taxon sampling

We analysed 66 specimens from 8 locations, covering most of the four species' geographic range composing the *Scartichthys* complex (Figure 1a, Table S1). Fifty-five specimens were sampled using hand nets and fishing lines between 0 and 15 m depth from December 2018 to February 2019 for this study. Also, eleven specimens (early juveniles) were captured between Montemar, 33°SL, and El Quisco, 33°SL, between September 2015 and February 2017 using Ecocean light traps (CARE, Ecocean, Montpellier, France) and preserved in 96% EtOH (specimens from Díaz-Astudillo *et al*. (2019). All specimens were euthanized with an overdose of benzocaine under bioethical standards before preservation. A small piece of pectoral fin tissue from each specimen was preserved in 96% EtOH at -20°C.

2.3 | Morphological analyses

Counts of incisor teeth and dorsal fin rays followed Williams (1990) and were taken using a Leica model EZ4 binocular. Specimens were photographed using a Nikon D90 and a Canon EOS T5 digital cameras, upon collection when possible, and in the laboratory to identify their fresh colouration patterns, following (Williams, 1990) and (Méndez-Abarca & Mundaca, 2016). The total counts of dentary incisors (DI) are diagnostic for only two of the four species of this genus (*S. gigas* and *S. variolatus*), while the seven colour patterns in life currently reported so far allow distinguishing in the field the four currently described *Scartichthys;* one for *Scartichthys variolatus*, one for *S. viridis*, one for *S. crapulatus* and four for *S. gigas*. Only part of morphological data (colouration) was collected for specimens collected in San Juan de Marcona (SJM), Perú. No morphological data could be obtained from specimens kept in alcohol upon capture (Table S1).

2.4 | DNA Extraction, amplification and sequencing

Whole genomic DNA was extracted from fin tissue preserved in 96% EtOH. DNA extraction was performed following the HotSHOT method (Truett *et al*., 2000), using 50mM NaOH and 1M Tris-HCL. For each specimen, we amplified a fragment of 652 bp of the mitochondrial gene coding for cytochrome C oxidase subunit I (COI) with the primers

F2 and R2 designed by (Ward et al., 2005). Fragments were amplified using PCR protocols as described by (Williams et al., 2012), with modifications in the final reactions (10 µl), containing 5 µl of KAPAG Fast Multiplex Mix multiple mixing solution (KAPA2G Fast HotStart DNA Polymerase (1 U per 25 µL reaction), KAPA2G Buffer A (1.5X at 1X), dNTPs (0.2 mM each dNTP at 1X), $MgCl_2$ (3 mM at 1X) and stabilizers), 1 µl of a mixture of F2 and R2 primers (2mM each primer), 3.0 µl H_2O, and 1 µl of genomic DNA. After PCRs, 1 µl of each PCR product (mixed with 1 µl of Red Gel dye) was separated by electrophoresis on a 1% agarose gel at 100 V for 30 minutes and visualized with a UV transilluminator. When the PCR products showed a clear and unique band of the correct expected length, all PCR products were purified by adding 1.3 µl of alkaline exonuclease phosphatase and then placed in the thermal cycler at 37°C for 60 min and then at 85°C for 15 min. The sequencing was performed bi-directionally with the same PCR primers using a BigDye ™ Terminator v3.1 cycle sequencing kit and an ABI 3500 XL Applied Biosystems sequencer. Sequences were aligned with Clustal W (Thompson et al., 1994) and edited using GENEIOUS 9.0.5 (http://www.geneious.com, (Kearse et al., 2012)). All generated sequences were deposited in GenBank (Accession numbers: *forthcoming*).

2.5 | Phylogenetic analyses

We first constructed a haplotype network using the haplonet function of the package "pegas" (Paradis, 2010) in the R statistical environment (R Core Team, 2020) to visualize the relationships between haplotypes of *Scartichthys* spp. among the different sampling localities. We then implemented Neighbour-Joining (NJ), Maximum Likelihood (ML), and Bayesian inference (BI) methods to reconstruct phylogenetic relationships of *Scartichthys* spp. The NJ analysis based on the Kimura 2-parameter (K2P) model of sequence evolution (Kimura, 1980) was conducted using the software package MEGA 6 (Tamura et al., 2013). Confidence in topology was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985). The Maximum Likelihood (ML) analysis was performed using the online version of IQ□TREE (Minh et al., 2013; Nguyen et al., 2015) available at http://iqtree.cibiv.univie.ac.at (Trifinopoulos et al., 2016). ModelFinder implemented in IQ□ TREE was used to assess the best model of evolution using the Bayesian Information

Criterion (BIC) prior to the construction of the ML tree (Kalyaanamoorthy et al., 2017). The ultrafast bootstrap approximation (UFboot) (Minh et al., 2013) and the SH□like approximate likelihood ratio test (SH□aLRT), both with 1,000 bootstrap replicates (Guindon et al., 2010) was conducted to evaluate the reliability of the nodes. Sequences of *Ophioblennius macclurei* (KF930203), *Cirripectes variolosus* (MH707881), *Cirripectes polyzona* (HQ168554) and *Exallias brevis* (MF409572) were downloaded from GenBank and used to root the trees in all analyses.

Finally, a time-calibrated phylogeny (BI) of *Scartichthys* spp. using one specimen per species based on our mitochondrial marker (COI) and five nuclear markers (ENC1, myh6, ptr, sreb2 and tbr1) was constructed with the software BEAST2 2.5.2 (Bouckaert et al., 2019). We used COI sequences representatives of *S. viridis*, *S. gigas* and *S. variolatus* together with nuclear sequences corresponding to these three species produced by Hundt & Simons (2018) (Table S2; *Scartichthys crapulatus* discarded given results from all previous analyses (see Results)). Blenniids are relatively rare and fragmented in the fossil record (Bannikov 1998), but see Methods and Supplementary Information section of Liu *et al.* (2018) for a recent review), preventing their use as reliable calibration point. Deep secondary calibrations have thus been generally used for Blenniids (e.g. Lin & Hastings, 2013; Liu *et al.*, 2018), which can lead to overestimation of divergence times among taxa that have recently diverged (Ho et al., 2008) or in such small scale survey. We thus chose to use an informative prior for the evolutionary rate of COI based on the substitution rate of 1.2 % per million years commonly used for fishes for this marker (e.g Bermingham, McCafferty, & Martin, 1997; Lessios, 2008; Tea *et al.*, 2019). We assumed a strict clock for each of the six markers, with the relative rates of ENC1, myh6, ptr, sreb2 and tbr1 being inferred in our analyses, a Birth□Death model as tree prior, with a chain length of 30 million generations. ModelFinder implemented in IQ□ TREE was used to assess the best model of evolution for each marker using the Bayesian Information Criterion (BIC). Trees and parameters were sampled every 3000 generations, and the first 10% of the samples were discarded as burn-in. We assessed the convergence and appropriate burn□in of each analysis using TRACER 1.5 (Drummond & Rambaut, 2007). Three independent analyses were run to ensure convergence. A maximum clade credibility tree was constructed using

TreeAnnotator 2.5.2 (Bouckaert et al., 2019) to get median ages and 95% highest posterior density (HPD) intervals for each node. The 95% HPD represents the smallest interval that contains 95% of the posterior probability and can be loosely thought of as a Bayesian analog to a confidence interval (Gelman et al., 2013).

2.6 | Sequence-based species delimitation analysis

As each sequence-based species delimitation method is susceptible to pitfalls, we used a 50% consensus to produce a robust delimitation scheme among five different methods (Kekkonen & Hebert, 2014b; Hubert & Hanner, 2015; Kekkonen et al., 2015): (1) Automatic Barcode Gap Discovery (ABGD; (Puillandre et al., 2012)) available at https://bioinfo.mnhn.fr/abi/public/abgd/, Poisson Tree Process (PTP, Zhang *et al.*, 2013) in its (2) single (sPTP) and (3) multiple rate version (mPTP) available at https://mptp.h-its.org/#/tree and General Mixed Yule-Coalescent (GMYC) in its (5) single rate version (sGMYC) and (6) multiple rate version (mGMYC) as implemented in the R package Splits 1.0-19 (Fujisawa & Barraclough, 2013). ABGD need a DNA alignment as input while we used the ML tree as input for PTP. Finally, the ultrametric and fully resolved tree needed to conduct GMYC analyses was reconstructed using the Bayesian approach implemented in BEAST 2.5.2. Two Markov chains of 10 million were run independently using a Yule pure birth model tree prior, a strict-clock model of 1.2% of genetic distance per million years. Trees were sampled every 1,000 states after an initial burn-in period of 1 million, both runs were combined using LogCombiner 2.5.2, and the maximum credibility tree was constructed using TreeAnnotator 2.5.2 (Bouckaert et al., 2019). Duplicated sequences were pruned prior to the Bayesian analysis.

3 | RESULTS

The total length (TL) of specimens collected ranged from 50 to 246 mm (Figure 2, Table S1). Specimen displaying the “uniform orange-brown” and the “orange-brown dots on posterior half of the body” colour patterns were the smallest, with mean size 80 mm (± 24 mm) and 106 mm (± 20 mm), respectively. In contrast, specimens displaying the reticulated

patterns were the largest with a mean size 156 mm (± 44 mm). The number of dorsal rays retrieved for the "reticulated bar stained" and the "two bar front head covered" colour patterns were typically 17 while the five other colour patterns displayed typically 18 (Figure 2). The number of dentary incisors (DI) was counted for 66 of the specimens and ranged from 52 to 123 (Figure 2). Among the three-colour pattern attributed to *S. gigas*, only the "reticulated" and the "two-bar front head covered" displayed a number of DI in accordance with the diagnostic description. Specimens with the "uniform orange-brown" colour pattern presented up to 118 DI. Interestingly, red/orange spots (dots) that are usually a diagnostic character of *S. crapulatus* were found on specimens with the "uniform orange-brown" colour pattern and all specimens of dark-light bluish-green colouration when observed under the binocular (see Figure S1). *S. variolatus* displayed a unique colour pattern (circular red spots in head and body) and DI number (80-93). Both characters can be used to distinguish this island species from the remaining continental species. We retrieved DI numbers in accordance with the diagnostic report. Finally, the colour pattern is the only character distinguishing *S. crapulatus* from *S. viridis*; we retrieved similar DI numbers for both colour patterns described so far for these two species (Figure 2). It is worth noting that the range of values retrieved for specimen displaying the "uniform orange-brown" colour pattern attributed so far to *S. gigas* correspond with those of *S. crapulatus* and *S. viridis.*

Specimen displaying the "uniform orange-brown" and the "orange-brown dots on posterior half of the body" colour patterns were the smallest, with mean size 80 mm (± 24 mm) and 106 mm (± 20 mm), respectively. In contrast, specimens displaying the reticulated patterns were the largest with a mean size 156 mm (± 44 mm). The number of dorsal rays retrieved for the "reticulated bar stained" and the "two bar front head covered" colour patterns were typically 17 while the five other colour patterns displayed typically 18 (Figure 2). The number of dentary incisors (DI) was counted for 66 of the specimens and ranged from 52 to 123 (Figure 2). Among the three-colour pattern attributed to *S. gigas*, only the "reticulated" and the "two-bar front head covered" displayed a number of DI in accordance with the diagnostic description. Specimens with the "uniform orange-brown" colour pattern presented up to 118 DI. Interestingly, red/orange spots (dots) that are usually a diagnostic character of *S. crapulatus* were found on specimens with the "uniform orange-

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325
326 For the molecular analyses, we worked from an alignment of 652 base pairs from
327 the mitochondrial COI region. While specimens of all currently four valid *Scartichthys*
328 species were sampled, our molecular analysis only shows the existence of three well
329 supported and highly divergent clades. The haplotype network analysis shows 3 distinct
330 groups, separated by 36 and 26 mutations, respectively: a first one composed of all
331 Robinson Crusoe specimens, a second one composed of specimens collected in the two
332 northernmost sampling localities (San Juan de Marcona (Peru) and Antofagasta (Chile)),
333 and finally a third group composed of specimens caught in continental Chile (Figure 1b).
334 The best nucleotide substitution model using the Bayesian information criterion (BIC) was
335 HKY+I+G. The NJ and ML produce the same tree topology with strong bootstrap support
336 (Figure 3). Similarly, the Bayesian analysis produced the same tree topology (Figure 3)
337 across all three runs with high posterior probabilities (PP) and parameters that reached
338 effective sample sizes higher than 200.
339
340 All three methods revealed that *Scartichthys* is a monophyletic group composed of
341 three well supported and highly divergent clades: (1) the first clade is composed of all
342 specimen from Robinson Crusoe Island identified as *S. variolatus;* (2) the second clade is
343 composed of 11 out of the 20 specimens identified as *S. gigas*; representing all specimens
344 with the "reticulated" and the "two-bar front head covered" colour pattern and (3) the third
345 clade is composed of not only all specimens identified as *S. viridis*, but also of all
346 specimens identified as *S. crapulatus*, and all specimens displaying the "uniform orange-

brown" colour pattern, described so far as a juvenile colour pattern for *S. gigas* (Figure 3). Species delimitation analyses provided concordant number of Molecular Operational Taxonomic Units (MOTUs) among the different methods: 3 for ABGD, PTP, mPTP, GMYC and 4 for mGMYC, leading to a consensus delimitation scheme of 3 MOTUs. It is worth noting that the fourth MOTU delimitated by mGMYC correspond to a single specimen among the *S. gigas* clade presenting a "reticulated bar-stained" colour pattern.

The same topology has been retrieved in our time calibrated phylogeny based on six markers, on mitochondrial (COI) and five nuclear (ENC1, myh6, ptr, sreb2 and tbr1) (Figure 4). All but one node (S. gigas – S. viridis ; 0.50) were well supported (above 0.9). We found that *Scartichthys* and *Ophioblennius* diverged 7.84 Ma (6.20 – 9.41, 95% HPD). The estimated time of divergence between the *S. variolatus* (Clade 1) and the other *Scartichthys* spp. is approximately 1.95 Ma (1.41- 2.49, 95% HPD; Figure 4) while *S. gigas* (Clade 2) and *S. viridis* (Clade 3) diverged 1.71 Ma (1.00 - 1.97, 95% HPD, Figure 4).

4 | DISCUSSION

The present study represents an updated phylogeny of the *Scartichthys* genus. Using morphological, colouration, and molecular evidence in combination, we show that this genus is composed of three species. Our integrative approach revealed indeed that the colour pattern used to diagnose *S. crapulatus* and the "uniform orange-brown" colour patterns recently attributed to juveniles of *S. gigas* are both juvenile colour patterns of *S. viridis*. Finally, we show that the diversification of this genus is relatively recent, beginning around 1.95 Ma.

A diversity of information has been produced so far to describe and characterize species of the genus *Scartichthys*. Our study proposed to use for the first time this broad array of evidence in an integrative approach by congruence to clarify the taxonomy of *Scartichthys.* Five lines of evidence taken together indicate that the "small dark-brown spots on the posterior half of the body" colour pattern, used to characterize *Scartichthys crapulatus*, is also a colour pattern of juveniles of *S. viridis* and that the "uniform orange-

brown" colour pattern is also a colouration of *S. viridis* juveniles, not of *S. gigas* as previously proposed. These conclusions are based on:

(1) *Size*: the "small dark-brown spots on the posterior half of the body" and the "uniform orange-brown" specimens observed in the field and collected measured respectively 106 mm (± 20 mm) and 80 mm (± 24 mm), substantially smaller than what *Scartichthys* adults usually are. These two colour patterns are thus likely characteristic of juveniles. Stepien (1990) first suggested that the colour pattern attributed to *S. crapulatus* ("small dark-brown spots on the posterior half of the body") was actually one of the juvenile forms of an already existing species (*S. viridis*). Indeed, both juveniles and adults of *S. viridis* can be found displaying a "Dark-light bluish green" pattern ;

(2) *Colouration:* we found small dark-brown spots on the posterior half of the body not only on adult specimens presenting the "Dark-light bluish green" pattern characteristic of *S. viridis*, similarly to Méndez-Abarca & Mundaca (2016), but also on specimen displaying the "uniform orange-brown" pattern (so far attributed to juveniles of *S. gigas*). The main diagnostic of *S. crapulatus* is thus not anymore diagnostic, three colour patterns sharing this character. Interestingly, Williams (1990) mentioned that colour in life of *S. crapulatus* were highly variable, with "Body colours [ranging] from green or reddish brown to golden with small brownish orange or brown spots on posterior half of body and on segmented-ray portion of dorsal fin.", and it is possible that specimen presenting an "uniform orange-brown" pattern were actually included in *S. crapulatus* at that time. It should be emphasized that the "two bar front head uncovered" colour pattern described by Méndez-Abarca and Mundaca (2016) and attributed specifically to *S. gigas* corresponds to the "Orange-brown dots on posterior half of body" colour pattern described by Williams (1990) and is used in the present study as a character of *S. viridis* (Figure 5). If Méndez-Abarca & Mundaca (2016) did not mention orange-brown dots, we found that specimens with these two colour pattern have the two dark bars and the front head uncovered, and it is likely that dots were not easily noticeable on live specimens (see live specimens in Figure 5).

(3) *Dental incisors and dorsal rays:* The three colour patterns sharing the "small dark-brown spots on the posterior half of the body" ("dark-light bluish green", "uniform orange-brown" and "orange-brown dots on posterior half of the body" patterns) also share a similar number of DI, clearly higher than the number of DI observed in *S. gigas* (Figure 2) and generally higher than the number of DI observed in *S. variolatus*. They also share similar a higher number of dorsal rays, typically 18, compared to the number of dorsal rays observed typically in *S. gigas*. The more variable number of DI for the "uniform orange-brown" retrieved here (109 ± 8), but as low as 58 in small specimens and the variable number of dorsal rays (typically 18, but as low as 17) might have led (Méndez-Abarca & Mundaca, 2016) to attribute this colour pattern to *S. gigas*, the species displaying the lowest number of DI and dorsal rays in this complex.

(4) *Molecular data*: Molecular analyses showed a lack of reciprocal mtDNA monophyly for the "small dark-brown spots on the posterior half of the body", the "uniform orange-brown" and "Dark-light bluish green" patterns. Indeed, phylogenetic analyses (distance-based, Maximum Likelihood and Bayesian) and sequence-based delimitation methods all showed that these three-colour patterns were part of a single clade.

(5) *Distribution:* Our extensive sampling allowed us to find the juvenile "uniform orange-brown" patterns in a geographic region where *S. gigas* has not yet been recorded in central Chile (Valparaiso region). No *S. gigas* adults have been observed, excluding the possibility of a range extension of *S. gigas* and reinforcing the hypothesis that the "uniform orange-brown" is one of a juvenile colour patterns in *S. viridis*. The same reasoning applies for the "two bar front head uncovered" colour pattern. Specimens displaying the "uniform orange-brown" colour pattern are frequently observed in the subtidal kelp, *Lessonia trabeculata*, down to 20 m depth, and we thus hypothesize that this colouration is involved in camouflage (Gaither et al., 2020). In line with Gaither et al. (2020), different colour morphs of *S. viridis* have been observed together inhabiting the same environments. This colour polymorphism could thus be related to either juvenile stages or microhabitat preferences as both colour

morphs are more algae associated (Pérez-Matus *et al.*, 2017; Riquelme-Pérez *et al.*, 2019).

Our study also allowed us to depict the evolutionary history of the *Scartichthys* genus. The phylogenetic analyses all retrieved the monophyly of the genus *Scartichthys*, as previously proposed using a single representative per species (Hundt & Simons, 2018). Based on multiple specimens per colour patterns and currently valid species, we show here that the extremely shallow divergence Hundt & Simons (2018) observed between *S. viridis* and *S. crapulatus* can be attributed to intraspecific divergence. Indeed, all our analysis converged in the existence of three and not four well supported clades within *Scartichthys*, and resulted in trees presenting the same topology whether analysis were performed on COI only or on the mitochondrial marker and the five nuclear markers.

The topology retrieved here differs from the one previously proposed based on nuclear markers only in that *S. gigas* was early diverging from the other *Scartichthys* (Hundt & Simons, 2018). Mitochondrial markers such as COI indeed often possess numerous informative sites to untangle the relationship within species complex but are more prone to saturation and homoplasy than conserved nuclear markers. These conserved markers are often preferred to untangle relationships between genera (Clabaut et al., 2005; Dornburg et al., 2014). *Scartichthys* is sister to *Ophioblennius*, which are found in the Atlantic (*O.atlanticus, O. macclurei*, and *O. trinitatis*) and in Eastern Pacific (*O. clippertonensis* and *O. steindachneri*) (Froese & Pauly, 2019). Our estimates for the divergence between these two genera (7.84 Ma (6.20 – 9.41, 95% HPD)) are in agreement with the first estimations of Liu *et al.* (2018) (around 12 Ma (5 – 19 Ma) a secondary calibration (age of the crown Blenniidae: 66 Mya). *Scartichthys and Ophioblennius* genera diverging from Indo-Pacific *Cirripectes* and *Exallia*, an Eastward dispersal, from the Indo-West Pacific to Eastern Pacific to finally reach the Atlantic is a likely dispersal hypothesis at the origin of the emergence of *Scartichthys* in the Eastern Pacific (Duncan et al., 2006; Hou & Li, 2018).

Three latitudinal biogeographic regions have been described for the continental Chilean species based on species geographic range distributions that inhabit intertidal habitats which coincide with the ecoregions delineated within the Warm Temperate Southeastern Pacific Province (Spalding et al., 2007); the central Chile that ranges from 20° to 36° SL, the Araucanian ecoregion from 39° to 43° SL, and the Chiloense region from 45° to 55° SL (Spalding et al., 2007; Navarrete et al., 2014). Here, the two closely related continental species *Scartichthys viridis* and *S. gigas* are both distributed within this first biogeographic region. However, with *S. gigas* occurring from 9 ° NL to 23 ° SL and *S. viridis* from 14 ° SL to 33 ° SL, the two species occur in sympatry only from 14°SL to 23°SL. They differ indeed in their success to colonize warmer waters, *S. viridis* being restricted to the Humboldt Current system and found only up to 14 ° SL while *S. gigas* can be found not as south as S. viridis but as north as 9 ° NL, outside of the Humboldt Current system, within the Tropical Eastern Pacific Province.

The Desventuradas and Juan Fernández Islands represent a distinct biogeographic unit (Dyer & Westneat, 2010), a hotspot of endemism with more than 42 % of the species observed in its water occurring nowhere else in the world (Dyer & Westneat, 2010). Their origin and the processes that led to their emergence remain somehow a mystery. Biogeographic analyses based on species distribution showed that the Desventuradas Islands were grouped with Easter Island, and Sala y Gomez (Kulbicki et al., 2013) and related to the “Hawaiian Archipelago” and the “South Western Pacific Ocean” province, extending from western Australia all the way to the Kermadec islands (Kulbicki et al., 2013). Phylogenetic analysis including Juan Fernandez endemics are scarce and showed that Juan Fernandez endemic species were related so far to (a) either southwest Pacific species; with Juan Fernandez endemics *Chironemus bicornis* (Steindachner, 1898), *C. delfini* (Porter, 1914), *Amphichaetodon melbae* Burgess & Caldwell, 1978 and *Girella albostriata* Steindachner, 1898 being all closely related to Australian / New Zealand / Lord How Island species (Burridge et al., 2006; Cowman & Bellwood, 2011; Gaboriau et al., 2018; Delrieu-Trottin et al., 2019; Beldade et al., 2020),(b) or southern Pacific species, occurring from Australia to Easter Island, with *Pseudolabrus gayi* (Valenciennes, 1839) being closely related to *P. fuentesi* (Regan, 1913) (Delrieu-Trottin et al., 2019). Our

phylogenetic analyses showed however that Juan Fernandez endemic species could also be related to continental Chile. The distinct origins so far retrieve call for a more extensive study including a larger number of Juan Fernandez endemic species.

The time tree produced here showed that the divergence time *Scartichthys variolatus* is around 1.95 Ma (1.41- 2.49, 95% HPD) and can be considered as a Neoendemic, being younger than the geological age of the Juan Fernandez and Desventuradas Islands (Santa Clara: 5.8 Ma, Robinson Crusoe: 3.7 Ma (Clouard & Bonneville, 2005; Lara et al., 2018)). Since Desventudas Islands are younger, with San Ambrosio being 2.9 million years old (Clouard & Bonneville, 2005), it is likely that speciation occurred in Juan Fernandez Island followed by a colonization of the Desventudas Islands. Similar divergence time have been retrieved for *Pseudolabrus gayi*, dated around 2.35 Ma (1.34-3.63, 95% HPD) (Delrieu-Trottin et al., 2019) while divergence estimates are older than the geological age of Juan Fernandez islands for *Amphichaetodon melbae* (7.26 Ma (4.23-10.97, 95% HPD)) (Delrieu-Trottin et al., 2019). A comprehensive survey of the divergence estimates of Juan Fernandez endemics would provide a better insight into the origin of this unique fauna.

5 | CONCLUSION

This study presents evidence that *S. crapulatus* should be synonymized with *S. viridis*, resolving a 30-year-old discord among taxonomists. We also showed that specimens displaying the colouration newly pattern described in 2016 (Méndez-Abarca & Mundaca, 2016) are juvenile of *S. viridis*, using different habitats (mainly occupying subtidal kelp sporophytes) than adult, greenish individuals. This study is a novel example of how molecular genetics can help to address the problem of species delimitation. We contribute to the clarification of the systematics of the *Scartichthys* genus, reconciling morphological, distributional, colouration and molecular evidence. We also showed that the Juan Fernandez endemic species is relatively young and likely to be a neoendemic species.

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SUPPORTING INFORMATION

Supplementary material is available at https://doi.org/xxx.

AUTHOR CONTRIBUTIONS

EDT, HHS, PSA, MFL, and APM conceived the study; PSA, MFL, and APM acquired the funding; HHS, MFL, and APM collected the field data; EDT, HHS, PSA, and APM produced the data; EDT, HHS, PSA, and APM analysed the data; and all authors contributed to the writing and approved the final version of the manuscript.

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SIGNIFICANCE STATEMENT

The blenniids of the genus Scartichthys represent key herbivore species of central and south American Pacific coastal reefs. Yet, they remain difficult to identify in the field. Here we provide an updated phylogeny of this genus, comparing for the first time morphological, coloration, and molecular data in combination, resolving a 30-year-old discord among ecologists and taxonomists.

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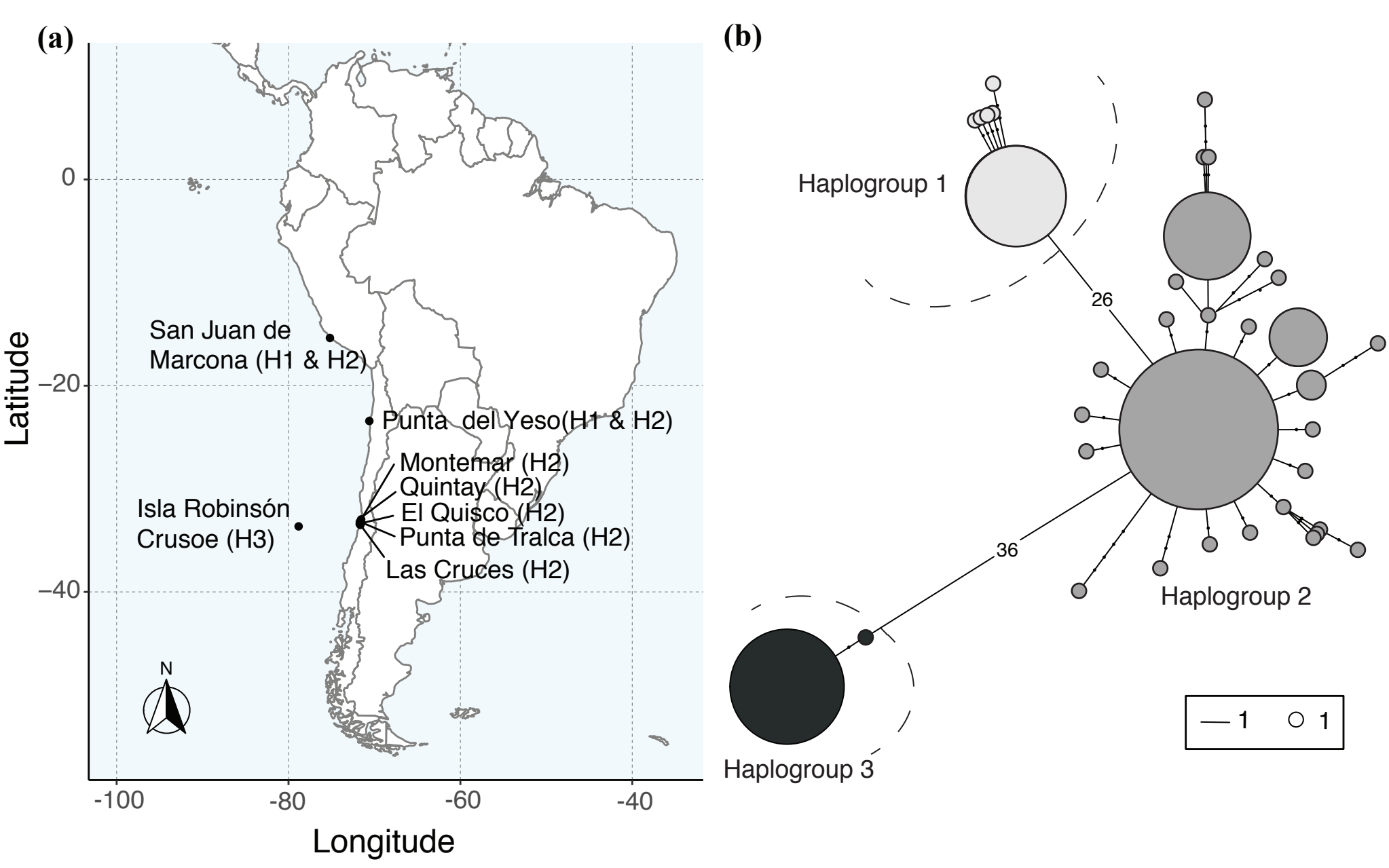
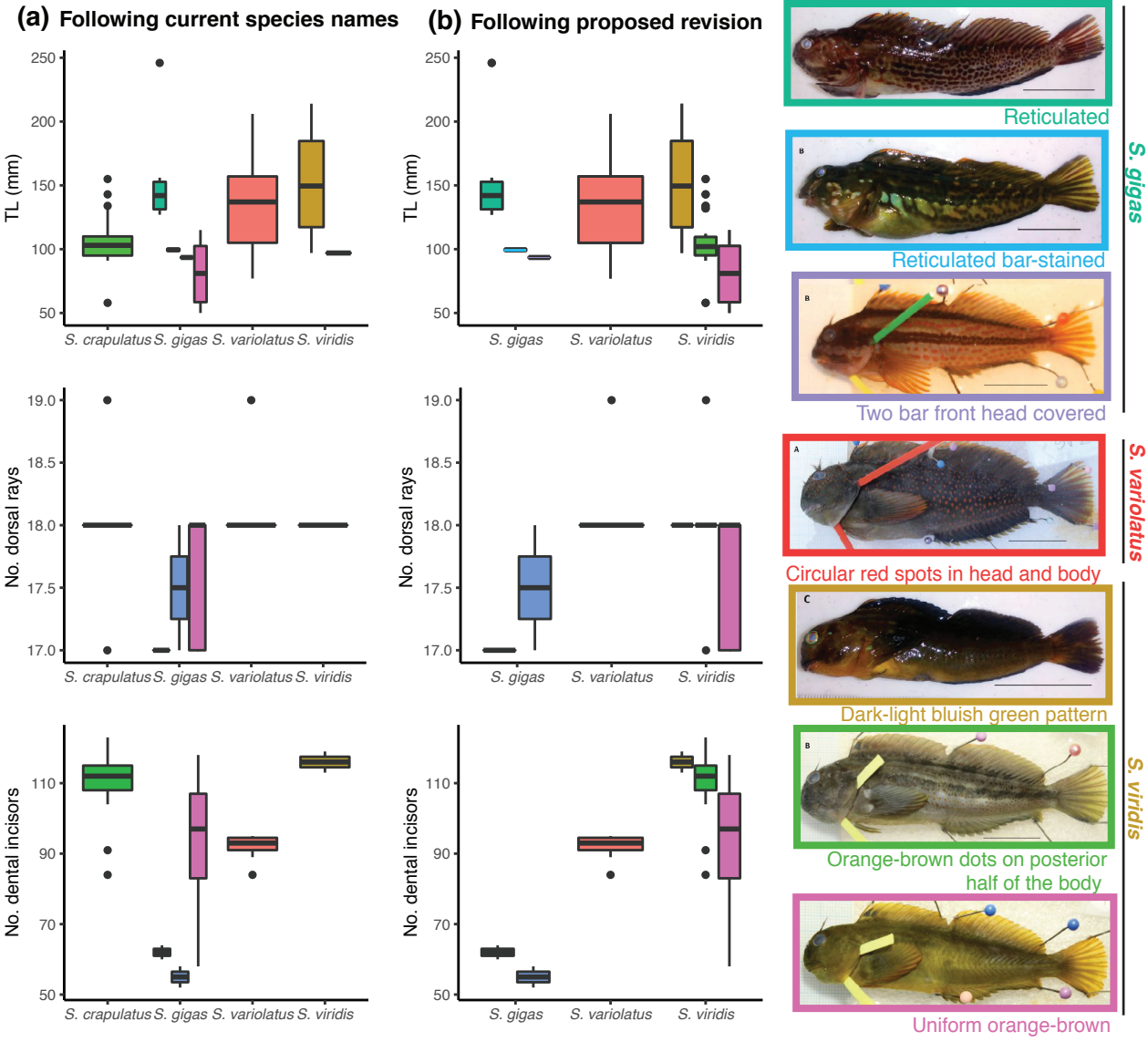

(a)
0
−20
−40
Latitude
San Juan de
Marcona (H1 & H2)
Punta del Yeso(H1 & H2)
Montemar (H2)
Quintay (H2)
El Quisco (H2)
Punta de Tralca (H2)
Las Cruces (H2)
Isla Robinsón
Crusoe (H3)
N
-100
-80
-60
-40
Longitude
(b)
Haplogroup 1
26
36
Haplogroup 2
Haplogroup 3
1
1


(a) Following current species names
(b) Following proposed revision
TL (mm)
No. dorsal rays
No. dental incisors
S. crapulatus
S. gigas
S. variolatus
S. viridis
Reticulated
Reticulated bar-stained
Two bar front head covered
Circular red spots in head and body
Dark-light bluish green pattern
Orange-brown dots on posterior half of the body
Uniform orange-brown
S. gigas
S. variolatus
S. viridis

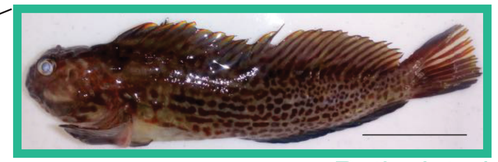
Coloration
ABGD
PTP
mPTT
GMYC
mGMYC
Consensus

A1
P1
A4
A3
A6
P8
P2
P9
P3
P6
A2
PT6
A5
P5
M7
PT4
Q3
LC5
LC4
EQ2
LC10
PT3
LC15
LC11
P10
M2
LC6
LC9
M3
A7
A8
EQ4
LC7
PT1
P7
M4
A9
PT5
Q2
EQ3
LC17
LC1
LC3
LC18
M1
EQ1
M5
LC13
M6
PT2
LC14
LC12
LC8
P4
LC2
Q1
LC16
IRC1
IRC2
IRC3
IRC4
IRC5
IRC6
IRC7
IRC9
IRC8

Outgroup

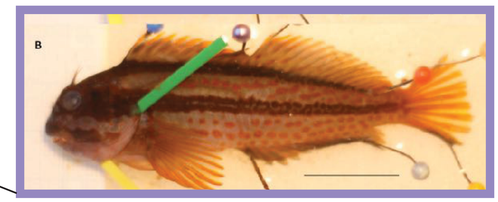
2.0

S. gigas



Reticulated

Reticulated bar-stained



Two bar front head covered

Dark-light bluish green pattern

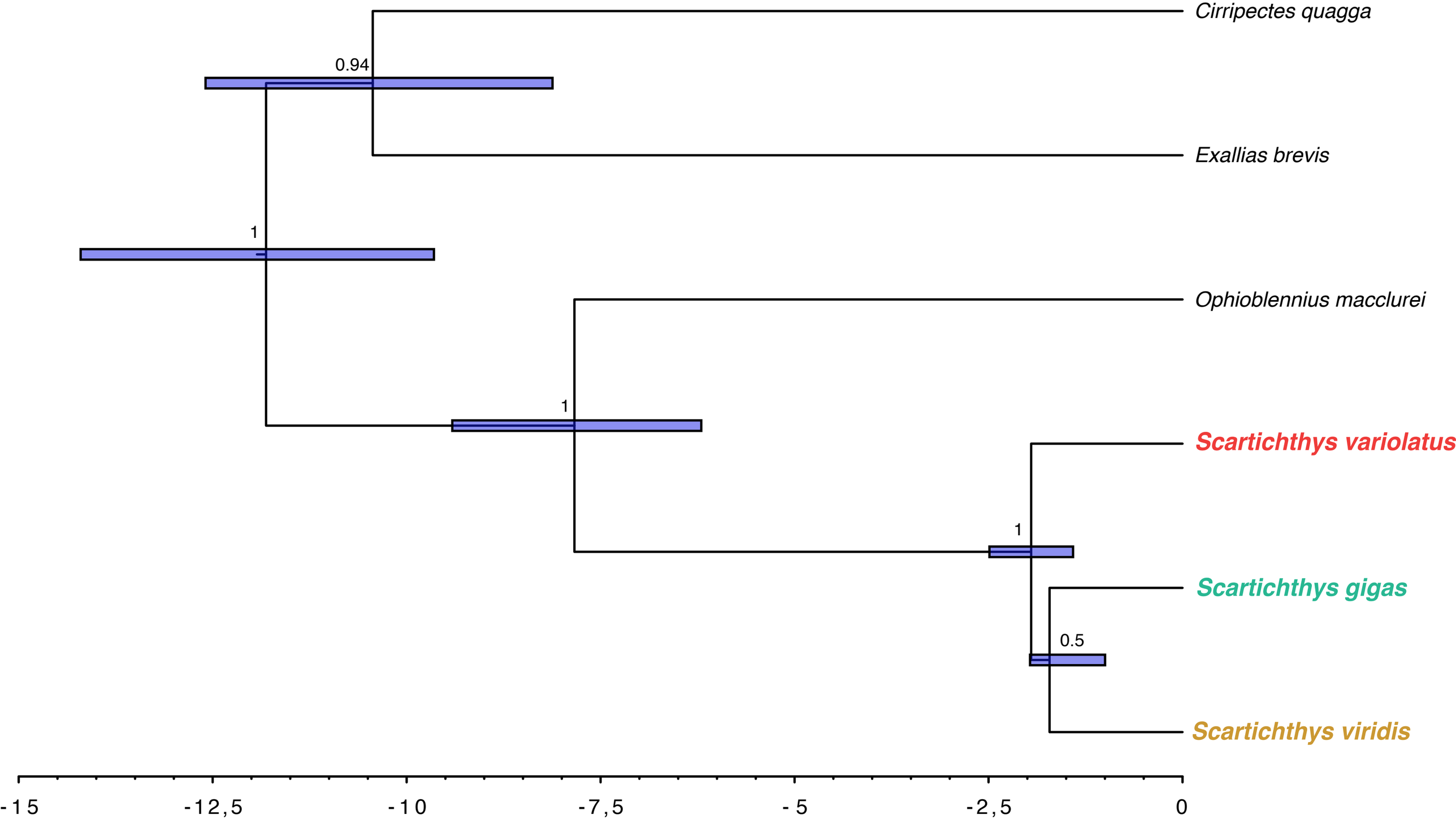
S. viridis

Small dark-brown spots on posterior half of the body

Uniform orange-brown

S. variolatus

Circular red spots in head and body


Cirripectes quagga
0.94
Exallias brevis
1
Ophioblennius macclurei
1
Scartichthys variolatus
1
Scartichthys gigas
0.5
Scartichthys viridis
-15
-12,5
-10
-7,5
-5
-2,5
0