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Evolutionary origin of the Atlantic Cabo Verde nibbler (*Girella stuebeli*), a member of a primarily Pacific Ocean family of antitropical herbivorous reef fishes

Ricardo Beldade [a], Gary C. Longo [b], Kendall D. Clements [c], D. Ross Robertson [d], Alejandro Perez-Matus [a], Shiro Itoi [e], Haruo Sugita [e], Giacomo Bernardi [f,*]

[a] Estación Costera de Investigaciones Marinas, Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica Chile, Santiago, Chile
[b] NRC Research Associateship Program, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Blvd E, Seattle, WA 98112, USA
[c] School of Biological Sciences, University of Auckland, Private Bag 92019 Auckland, New Zealand
[d] Smithsonian Tropical Research Institute, Balboa, Unit 0948, APO AA 34002, Panama
[e] Department of Marine Science and Resources, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan
[f] Department of Ecology and Evolutionary Biology, University of California Santa Cruz, CA, USA

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ABSTRACT

Nibblers (family Girellidae) are reef fishes that are mostly distributed in the Indo-Pacific, with one exception: *Girella stuebeli*, which is found in the Cabo Verde Archipelago, in the Atlantic Ocean. We capitalized on this unusual distribution to study the evolutionary history of the girellids, and determine the relationship between *G. stuebeli* and the remaining nibbler taxa. Based on thousands of genomic markers (RAD sequences), we identified the closest relatives of *G. stuebeli* as being a clade of three species endemic to the northwestern Pacific, restricted to the Sea of Japan and vicinity. This clade diverged from *G. stuebeli* approximately 2.2 Mya. Two alternative potential routes of migration may explain this affinity: a western route, from the Tropical Eastern Pacific and the Tropical Western Atlantic, and an eastern route via the Indian Ocean and Southern Africa. The geological history and oceanography of the regions combined with molecular data presented here, suggest that the eastern route of invasion (via the Indian Ocean and Southern Africa) is a more likely scenario.

1. Introduction

The fish fauna of the Tropical Eastern Atlantic, a biogeographic region that includes northwest Africa, tropical western Africa, and the Archipelagos of Cabo Verde, and São Tomé and Principe, has a general affinity with the north eastern Atlantic fauna (Floeter et al., 2008). The Cabo Verde archipelago is better known for its explosive radiation of cone snails (genus *Conus*) (Cunha et al., 2005; Duda and Rolán, 2005), and keyhole limpets (Cunha et al., 2017). Yet, with approximately 10% of its marine fish species being endemic, the Cabo Verde ichthyofauna is also of particular interest for its unique evolutionary history in a broad variety of taxa (Avila et al., 2020; Domingues et al., 2008; Floeter et al., 2008; Freitas, 2014; Wirtz et al., 2013). As an example, the reef fish genera *Similiparma, Viridentex,* and possibly *Diplodus* and *Scartella,* have originated there (Araujo et al., 2020; Floeter et al., 2008), suggesting that this location may have factors that enhance speciation. Unlike the general pattern described above (which calls for an affinity with the Tropical Eastern Atlantic), a number of species found at Cabo Verde are of western origin, either directly from the Western Atlantic (Freitas, 2014; Freitas et al., 2014), or from the Tropical Eastern Pacific with a presumably extinct Western Atlantic lineage (Choat et al., 2012; Ludt et al., 2015; Piñeros et al., 2019). In addition, a few species are of eastern origin, with an Indian Ocean affinity. For example, the angelfish genus *Holacanthus*, which includes species from the Tropical Eastern Pacific and the Tropical Western Atlantic, has one early diverging species, *H. africanus,* that is found in the Tropical Eastern Atlantic (including Cabo Verde), and displays an evolutionary origin in the Indian Ocean (Tariel et al., 2016). For the genera that include both Atlantic and Pacific members, but with a much wider overall distribution, understanding the origin of Atlantic species is more challenging (Cowman et al., 2017). For

* Corresponding author at: Department of Ecology and Evolutionary Biology, University of California Santa Cruz, 115 McAllister Way, Santa Cruz, CA 95060, USA.
E-mail address: bernardi@ucsc.edu (G. Bernardi).

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example, the Indian Ocean appears to be the most likely evolutionary origin for Atlantic pygmy angelfishes (genus *Centropyge*, Bowen et al., 2006). A similar pattern was also found for the goby genus *Gnatholepis* (Rocha et al., 2005), and potentially the emperor species pair *Lethrinus atlanticus/L. genivittatum* (but with low statistical support, Lo Galbo et al., 2002), suggesting that an Indian Ocean origin for Tropical Eastern Atlantic taxa might be more common than previously thought.

In that respect the genus *Girella* offers a useful model system to study the underlying complexity of evolutionary history of reef fishes. The nibblers (family Girellidae, Eupercaria: Centrarchiformes: Terapontoidei) (Davis and Betancur-R, 2017), which appeared approximately 30 Mya (25–38 Mya) (Knudsen et al., 2019), comprise 17 species of omnivorous/herbivorous fishes. These include a single species, *G. stuebeli,* endemic to Cabo Verde, and the only species in the Atlantic Ocean, and 16 Indo-Pacific Ocean species ranging from Northwest America to Japan, and from Southwest America to Southwestern Australia, (Fig. 1). While 14 of those species are restricted to the Pacific Ocean, two are found in the southeastern Indian Ocean: *G. zebra* which ranges from Tasmania to Western Australia, and *G. tephraeops,* which is endemic to Southwestern Australia. There are two invalid species, *G. feliciana* and *G. zonata,* that were misidentified due to ontogenetic differences between juvenile and adult forms of *G. albostriata* and *G. stuebeli*, respectively (Brito et al., 2007; Dyer and Westneat, 2010; Wirtz et al., 2013). One other species, *G. melanichthys,* has been synonymized with *G. punctata* (Froese and Pauly, 2000), although some molecular data separate those two species (Davis and Betancur-R, 2017; Knudsen et al., 2019), or even potentially synonymize this species with *G. leonina* instead (Kim et al., 2020). Several species of *Girella* are endemic to small remote islands, including the Juan Fernandez Islands off Chile (*G. albostriata*), the Kermadec Islands off northern New Zealand (*G. fimbriata)*, the Galápagos Islands off Ecuador (*G. freminvillei*), Easter Island (*G. nebulosa*), and Cabo Verde (*G. stuebeli*) (Fig. 1). Finally, the family Girellidae includes a single species outside of the genus *Girella.* This species, *Graus nigra*, is found along the west coast of South America (Johnson and Fritzsche, 1989; Perez-Matus et al., 2012).

Graus nigra is a large (up to 90 cm) carnivorous species that shows irregular spots that tend to occasionally form vertical bars, and has two white spots in the posterior region of the body. In contrast, *Girella* spp. are medium-sized (varying in size between 30 and 50 cm) omnivorous reef-associated fishes that usually display a solid dark background color varying from bluish to greenish (Figs. 2 and 3). Over this solid background, some variations are found in different species. Two species have pale mottling (*Girella fimbriata, G. nebulosa*), which might be the ancestral state since *Graus nigra* is also mottled. One species, *G. punctata,* which is the type for the genus, has very small pale dots on the body (*Girella* means small dots). Two species (*G. nigricans, G. simplicidens*) are either uniformly colored, or display few distinct large white dots on their backs (typically one to six dots) (Davis, 2001; Terry et al., 2000). Five species have dark or light bodies with contrastingly colored vertical bars; two have pale bodies with ~ 10 dark bars (*G. zebra, G. tricuspidata*), one species has a dark body with several faint pale bars (*G. albostriata*), and two species have dark bodies with a single white bar (*G. mezina, G. stuebeli*). Girellids are broadcast spawners, with larvae that remain in the water column for approximately two months (Mansur et al., 2014; Stevens et al., 1989; Terry et al., 2000; Waples, 1987).

Studies pertaining to girellids have mostly focused on dentition and feeding (Clements and Choat, 1997; Johnson and Fritzsche, 1989; Norris, 1963; Norris and Prescott, 1959; Orton, 1989; Yagishita and Nakabo, 2003), or morphological analyses (Johnson and Fritzsche, 1989; Orton, 1989; Vial and Ojeda, 1990; Yagishita and Nakabo, 2000). The only comprehensive attempt at investigating the evolutionary relationships of Girellidae was based on morphological characters (Orton, 1989), and while it presented an interesting phylogenetic hypothesis, it resulted in several polytomies, and raised a number of yet unresolved questions (Fig. 2). Previous girellid molecular analyses have only included a small subset of (up to three) species (Cerda et al., 2019; Ito et al., 2018; Itoi et al., 2007b, 2007a; Ohara and Taniguchi, 2003; Orton and Buth, 1984; Terry et al., 2000; Umino et al., 2009), or were included within a much larger phylogenetic context (Davis and Betancur-R, 2017; Knudsen et al., 2019; Knudsen and Clements, 2016) (Fig. 3). Presently, a full molecular phylogeny of the family is lacking, and, importantly, the phylogenetic position of the enigmatic Atlantic species, *Girella stuebeli*, has yet to be examined.

The unique geographic position of *Girella stuebeli* in Cabo Verde points either to a western colonization involving a Tropical Eastern Pacific/Western Atlantic origin, combined with extinction in the neotropics, or to an eastern colonization from the Pacific Ocean via the Indian Ocean and South Africa, combined with extinctions throughout the Indian Ocean and the South Atlantic, unless the Cabo Verde species is related to the Western Australia representatives (Floeter et al., 2008; Freitas, 2014).

Here we discuss the evolutionary and taxonomic history of *Girella stuebeli,* the Cabo Verde nibbler, the only girellid found in the Atlantic Ocean. It has been considered a junior synonym of *G. zonata,* a species originally described by Günther (Günther, 1859), but this description has been discussed and criticized before (Wirtz et al., 2013). Günther

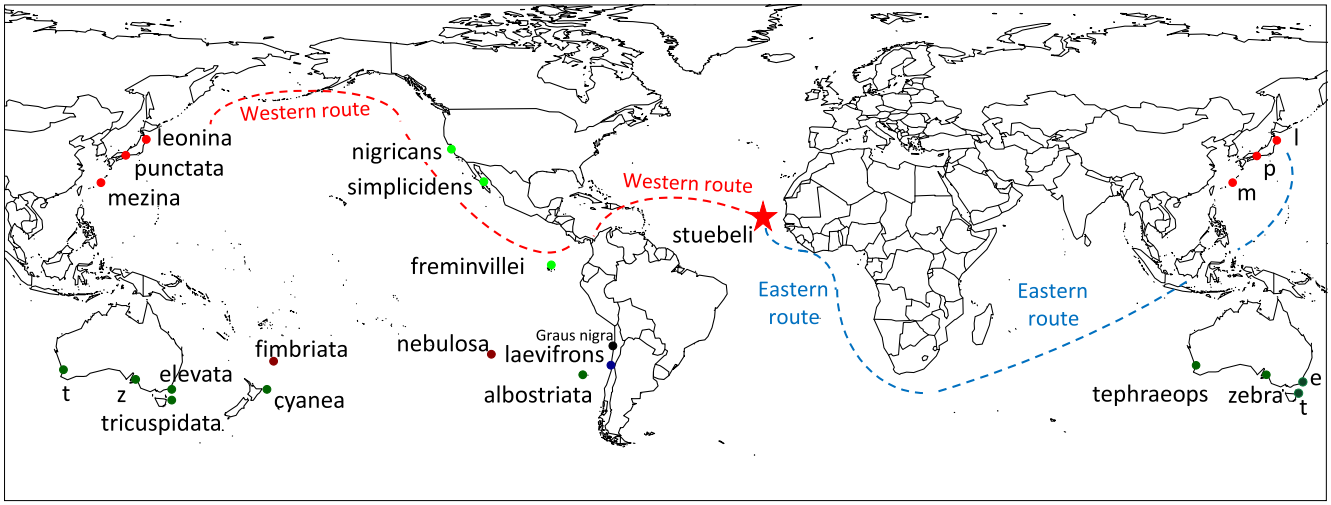

Fig. 1. Map of collection sites of girellid species. Species names are given for the genus *Girella, Graus nigra* is fully spelled out to avoid confusion. Locality (Cabo Verde) for *Girella stuebeli*, the focal species of this study, is marked by a star. Potential eastern and western routes of invasion are shown in dashes. Additional information is provided on Table 1.

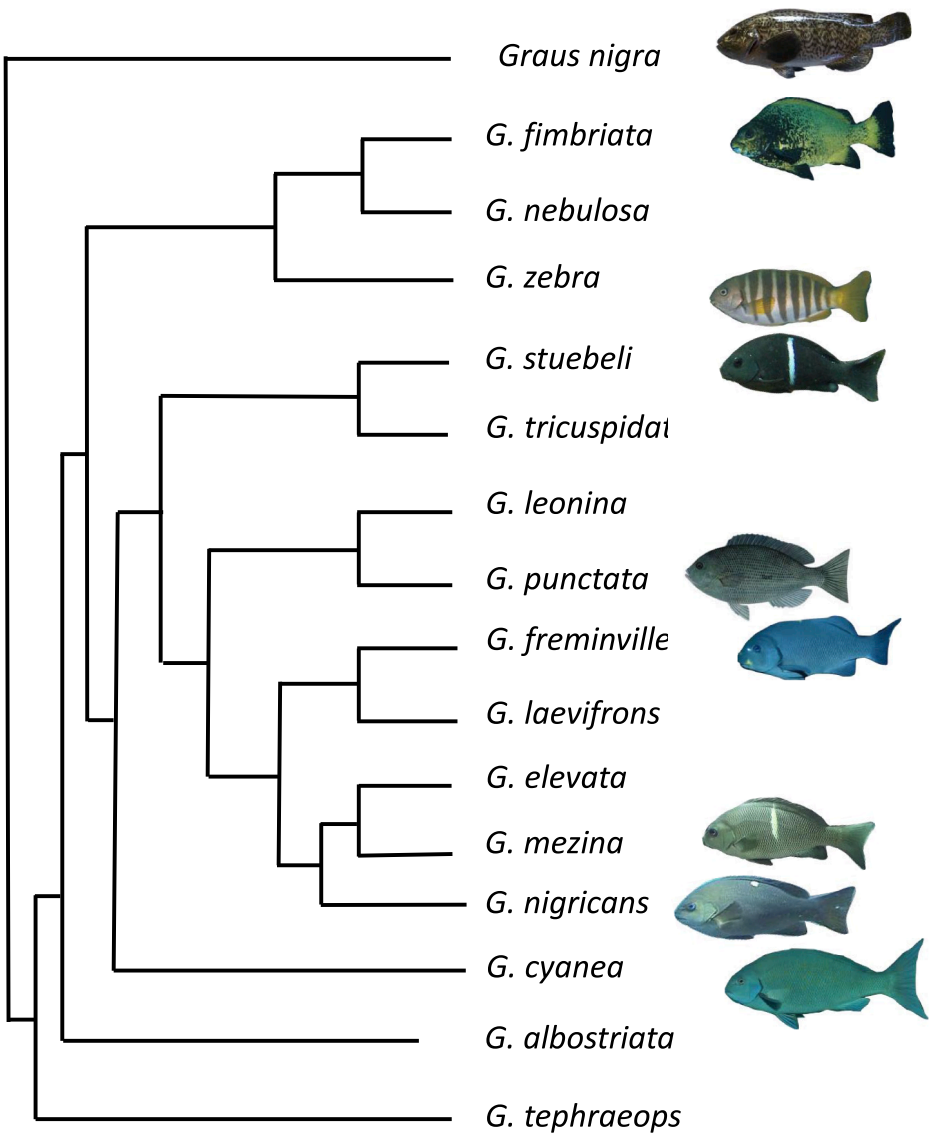

Fig. 2. Phylogenetic hypothesis based on morphological characters, redrawn from Orton (1989), placing *Graus nigra* as an outgroup. Species with characteristic color patterns are shown.

obtained this specimen from the Haslar Collection, and gives its location as "from Australian seas", but without additional information. The specimen, currently held at the British Museum (BMNH:1855.9.19.286), still has a visible vertical light bar (viewed by S. Knudsen, personal communication), a character that is found in only two species in the genus*: G. stuebeli*, from the Cabo Verde Islands, and *G. mezina,* from Japan (Figs. 2 and 3). Considering that no adult or juvenile *Girella* from Australia displays this color pattern, we place *G. zonata* as a *nomen dubium.* Importantly, in his original description of *G. stuebeli,* Troschell was aware of the description of *G. zonata*, which he considered to be a juvenile of *G. stuebeli* and dismissed its name for its incorrect provenance (Eschmeyer, 2013; Troschell, 1866). Incidentally, Jordan and Starks astutely noticed the resemblance between *G. stuebeli* and *G. mezina* in their original description of *G. mezina* from Japan, and asked C. Tate Regan to check the BMNH individual to ascertain that *G. mezina* was different than *G. stuebeli* (called then *G. zonata*), which he confirmed (Jordan and Starks, 1907).

The focus of the present study was to test two alternative evolutionary hypotheses for the presence of *Girella stuebeli* in the Atlantic in a molecular phylogenetic framework. To accomplish this goal, we used RADseq markers with nine girellid species, to reconstruct the evolutionary history of *G. stuebeli.* We also considered two published girellid phylogenies (which also were based on a subset of species, neither including *G. stuebeli*) and constructed a molecular phylogenetic hypothesis (a gene tree) based on a single mitochondrial molecular marker (16S rRNA) using all species of *Girella* and *Graus* (i.e. all species in the family Girellidae) to test whether species missing from the RAD phylogeny would change the evolutionary scenario drawn for *G. stuebeli* based on RAD markers alone. Our RAD data show that *G. stuebeli* is most closely related to a group of species currently found in the Sea of Japan. Two alternative pathways to the Atlantic are discussed (Fig. 1): a western route of invasion via the Tropical Eastern Pacific (where the ancestor of *G. stuebeli* subsequently was replaced by another congeneric lineage) and the Tropical Western Atlantic (where it disappeared), and an eastern route via the Indian Ocean and South Atlantic (where it disappeared from most of that area). Both routes have geological and oceanographic time constraints. For migration to have taken the western route, it would have to predate the closure of the Isthmus of Panama. The time of closure is much debated, but most agree that it occurred ~ 3.0 Mya, between 3.5 and 2.8 Mya (Bacon et al., 2015b, 2015a; Coates and Stallard, 2013; Jaramillo et al., 2017; Lessios, 2015, 2008; Marko et al., 2015; O'Dea et al., 2016). A western route of invasion would

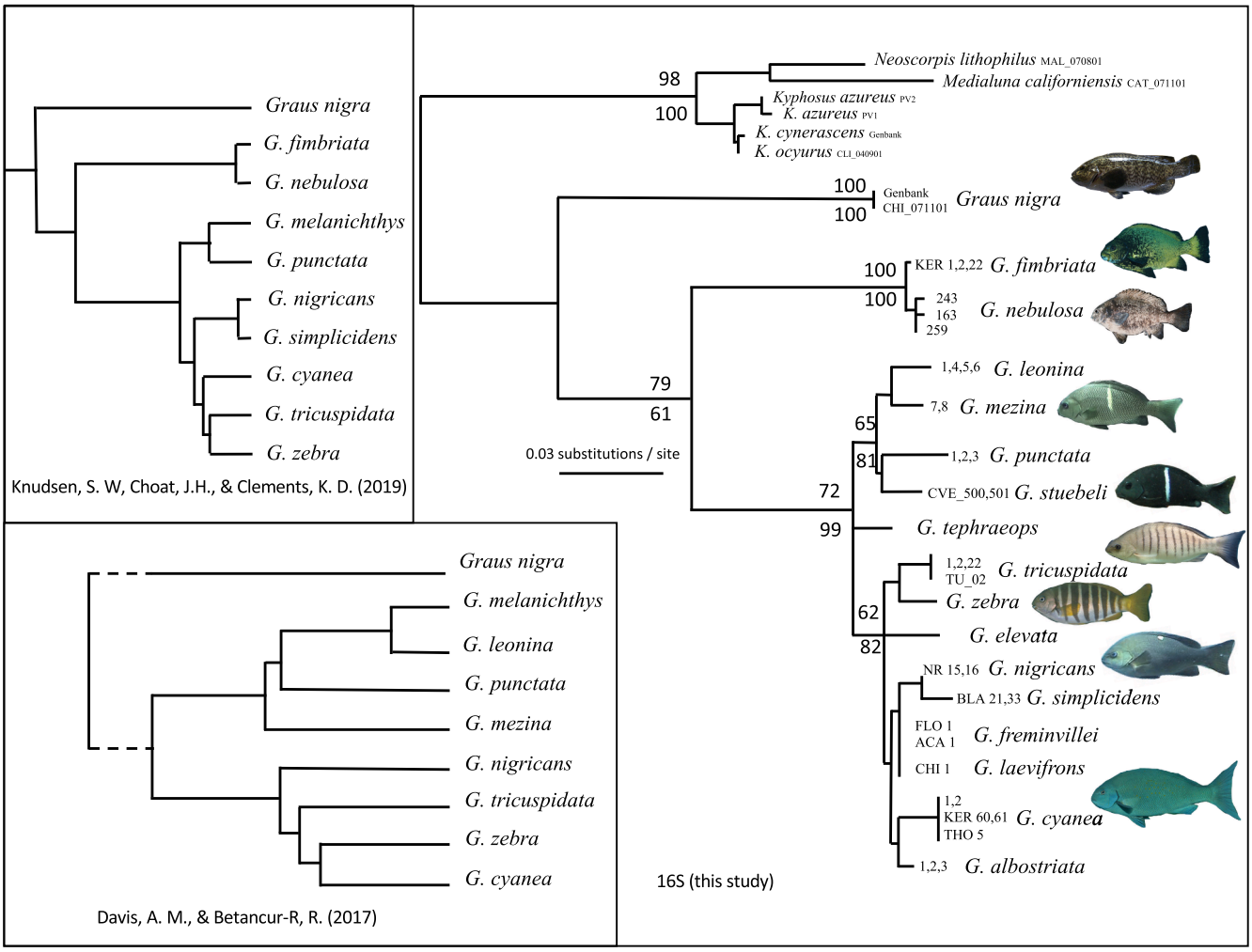

Fig. 3. Left Panels: published phylogenies of a subset of girellid species. Right panel: Molecular phylogeny of all girellid species and outgroups based on the mitochondrial 16S rRNA sequence marker. Pictures of typical girellids are shown on the right of the species labels. The main coloration patterns are: mottled (*Graus nigra, Girella fimbriata, G. nebulosa*), a single white band (*G. mezina, G. stuebeli*), several band (*G. zebra, G. tricuspidata*). All other species display a uniform dark color (e.g. *G. cyanea*). Two species occasionally display white dots on the back (*G. nigricans, G. simplicidens*). Bootstrap values for Maximum Likelihood and Neighbor-Joining methods are shown above and below the nodes, respectively.

therefore require a passage, at a minimum, before 2.8 Mya, probably before 3.1 Mya. The eastern route of invasion presents its own set of obstacles. Chiefly, the onset of cold-water upwelling off southwestern Africa occurred approximately 2 Mya, and would therefore have created a semi-permeable barrier to transport around that time (Byrne et al., 1995; Nencioli et al., 2018; Peeters et al., 2004). An eastern route of invasion would therefore most likely require a migration prior to 2 Mya, although some more recent occasional transport across that barrier might have been possible (through water eddies known as Agulhas rings, Beal et al., 2011; Villar et al., 2015). These two hypotheses have different constraints, forcing an older passage for the western route and allowing a more recent passage for the eastern route. Our goal here was to assess those two alternatives

2. Materials and methods

2.1. Sampling and DNA preparations

All samples were obtained from the field using spears while free- or scuba diving, or with hand nets from tidepools. In a few cases, we were able to obtain tissue samples from museum specimens. The full list of samples used in this study is described in Table 1. Tissue samples were stored in 95% ethanol and DNA was extracted from fin clips using DNeasy Blood & Tissue kits (Qiagen) according to the manufacturer's protocol.

2.2. RADseq analyses

Due to insufficient amounts of tissue material, we were not able to include all the samples used for the mitochondrial analyses for the construction of RAD libraries. Therefore, we were only able to use a subset of the original taxa, which comprised 26 individuals, belonging to 11 species (nine girellids and two outgroup species, Table 1). We constructed RAD libraries using the original protocol with the restriction enzyme SbfI (Baird et al., 2008; Miller et al., 2007; Omar et al., 2016) and minor variations reported below. Initial genomic DNA amounts for each individual were 400 ng. We sheared libraries on a Covaris S2 sonicator with an intensity of 5, duty cycle of 10%, cycles/burst of 200, and a cycle time of 30 s. The final PCR amplification step was carried out in 50 µl reaction volumes with 10 amplification cycles. For all size selection and purification steps, we used Ampure XP beads (Agencourt). Samples used in this study were sequenced in one of two libraries, each containing 96 individually barcoded samples. Libraries were sequenced in a single lane on an Illumina HiSeq 4000 at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley.

Table 1
Sampling locations for girellids and outgroups used in this study. Species names, sampling locations, and number of samples used to construct 16S rRNA and RADseq/SNP phylogenies are presented in this order from left to right.

Species	Sampling site	Number of samples
		16S/SNPs
Ingroup		
Girella albostriata	Juan Fernandez Islands	3/3
Girella cyanea	New Zealand (North shore)	5/0
Girella fimbriata	Kermadec Islands	4/4
Girella freminvillei	Galápagos Islands	2/1
Girella elevata	Eastern Australia	1/0
Girella laevifrons	Chile	1/0
Girella leonina	Japan	4/2
Girella mezina	Japan	2/0
Girella nebulosa	Easter Island	3/0
Girella nigricans	California, USA-Baja California, Mexico	2/4
Girella punctata	Japan	3/3
Girella simplicidens	Sea of Cortez, Mexico	2/2
Girella stuebeli	Santa Maria, Praia, Cabo Verde	2/4
Girella tephraeops	Perth, Australia	1/0
Girella tricuspidata	Tasmania, Australia	4/0
Girella zebra	Australia	1/0
Graus nigra	Chile	1/1
Graus nigra	GenBank	1/0
Outgroup		
Kyphosus azureus	California, USA	2/0
Kyphosus cynerescens	GenBank	1/0
Kyphosus ocyurus	Clipperton Island, France	1/1
Kyphosus vaigiensis	Ulithi, Micronesia	0/1
Medialuna californiensis	California, USA	1/0
Neoscorpis lithophilus	Ponta Malongane, Mozambique	1/0

2.3. Quality filtering

Raw reads were trimmed to 92 bp on the 3′ end, quality filtered, and then split according to the 6 bp unique barcode custom Perl scripts (Miller et al., 2012). Sequences were dropped if the product of quality scores for their 92 bases was below 80%. The barcode (6 bp) and restriction site residue (6 bp) were then removed from the 5′ end, resulting in a final sequence length of 80 bp.

We used the software program Stacks version 2.2 (Catchen et al., 2013, 2011) to identify orthologous sequences among taxa. We followed published recommendations to optimize the stacks parameters (Rochette and Catchen, 2017). These optimizations resulted in the use of a value of three for the parameters M and n in the denovo_map. The minimum stack depth (μm) was set at three, following recommendations (Rochette and Catchen, 2017). We then ran multiple iterations of the Stacks routine 'populations' to generate output files for input into downstream phylogenetic programs. Stacks allows for fine control of which markers will be exported by specifying the number of species (p) and the percentage of individuals in each species (r) that must possess that marker. We ran the population program both considering either each species as a population or each individual as a population. We only retained a single SNP per locus (write-single-snp option in stacks). The number of final loci varied very slightly but the results did not. We therefore retained the population map that considered each individual as a population so as to be most stringent. Full sequence RAD markers and fixed SNPs of each individual were exported for downstream analyses. The quality filtered sequences are deposited at the National Center for Biotechnology Information short-read archive (accession no. SRP056799).

2.4. Phylogenetic reconstructions

For each Stacks populations parameter set, we built supermatrices with complete RAD sequences (80 bp) and identified phylogenetically informative sites using FASconCAT-G (Kück and Longo, 2014). Supermatrices were generated both with individual sequence data and species consensus sequence data with IUPAC ambiguity codes for polymorphic data. We also used the stacks population output to generate phylogenetic trees (output code –phylip). This output retains the fixed differences between groups (in this case each individual). Results generated from this output did not differ from the full matrix, therefore we used this simplified output that was found to be as robust but more computationally manageable. Phylogenetic relationships were assessed using a Neighbor-Joining approach (NJ) implemented in PAUP v. 4.0a (Swofford, 2003), and Maximum Likelihood (ML) implemented in GARLI v. 0.951 (Zwickl, 2006). Statistical confidence in nodes was evaluated using 100 non-parametric bootstrap replicates (Felsenstein, 1985) (using the automated stopping criterion set at 10,000 generations).

2.5. Estimating times of divergence

In order to estimate times of divergence, we assessed molecular divergence by first determining the best substitution model to match our data, using JModeltest v. 2.1.4 (Darriba et al., 2012). We then used a previously published calibrated molecular phylogeny of Kyphosidae (Knudsen et al., 2019), where the divergence of *Graus nigra* and the genus *Girella* was estimated to have occurred 25–38 Mya to calibrate divergences within girellids. Importantly, the divergence of the genus *Girella* from *Graus* has also been estimated at approximately 50 Mya (Davis and Betancur-R, 2017). However, since this estimate included *Tilodon* as a kyphosid (rather than the Microcanthid, it actually is, Eschmeyer 2013), it might have over-estimated the time of divergence of that divergence (Davis and Betancur-R, 2017). Interestingly, the divergence of the crown group (the genus *Girella*) does not differ much between the two studies and therefore both are consistent with our results when considering evolutionary events occurring within the genus *Girella*.

Divergence times were estimated using standard models of evolution implemented in BEAST v. 2.6.3 (Drummond et al., 2012). We used a lognormal relaxed clock-model, in combination with a birth–death (BD) prior for rates of cladogenesis (Drummond and Rambaut, 2007) with a GTR + G model of substitution. One run was conducted with 10 million generations each, with sampling every 1000 generations. A time tree was obtained using TreeAnnotator v 2.6.3 (Drummond et al., 2012). We used one prior with normal distributions as an internal calibration point, with the minimum and maximum bounds implemented with the 95th percentile of the distribution. We used an internal calibration point based on the estimated age of Girellid origin of 25–38 Mya (mean at 30 Mya) as discussed above (Knudsen et al., 2019).

2.6. Mitochondrial analyses

Amplifications of the 16S rRNA segments were performed using the universal primers 16SAR-16SBR (Palumbi et al., 1991) with 35 cycles at a denaturation temperature of 94 °C for 30 s, an annealing temperature of 52 °C, and an extension of 30 s at 72 °C. After purification of the PCR products, following the manufacturer's protocol (Applied Biosystems, Foster City, CA), sequencing was performed in both directions with the primers used in the PCR amplification.

2.7. Phylogenetic inferences

We used the computer program MAFFT implemented in Geneious v. 2020.2.4 to align the DNA sequences. Overall, few insertions and deletions (indels) were observed (accounting for a total of 9 bp). These indels were removed from the subsequent phylogenetic analyses, yet their inclusion did not change the results. We used both Neighbor-Joining and Maximum Likelihood methods and bootstrapping as described above to infer phylogenetic relationships.

3. Results

3.1. RAD phylogeny

RAD libraries using 26 individuals from 11 species resulted in a mean coverage of 29.1x, ranging from 14.2x to 65.0x (StDev = 13.2x) with a total of 168,627 loci. The phylogeny based on RAD markers comprised two outgroup individuals (two species) and 24 ingroup individuals (9 species), resulting in an alignment of 46165 bp (which is also the number of loci, only one SNP was kept for each locus). However, for subsequent analyses, we only included those sites that were present in all individuals, bringing the number of usable loci to 82. When removing the two outgroups, the number of sites present in all ingroup species was 977. The ingroup topology of the tree based on the full species dataset (ingroup + outgroup, 82 bp), and the topology solely based on ingroup species (977 bp) were identical. Hence, we proceeded to use the larger dataset. Based on those 977 bp, we were able to obtain a fully resolved phylogeny (Fig. 4). For the most part, this phylogeny was identical to the one obtained with the mitochondrial marker, and other published phylogenies, where *Graus nigra* was found to be sister to the *Girella* clade, and *Girella fimbriata* being the sister to the remaining *Girella* species. The tree splits into two major clusters: one cluster included *G. stuebeli* and the species from Japan (*G. punctata* and *G. leonina*), and the other cluster included all remaining species.

3.2. Mitochondrial 16S phylogeny and published phylogenies

We sampled all species of Girellidae and include them in a phylogenetic hypothesis based on a portion of the 16SrRNA gene. The dataset comprised six outgroup individuals (five species) and 42 ingroup individuals (16 species). The aligned sequences resulted in a fragment of 496 bp. Phylogenetic analyses resulted in a mostly resolved phylogeny (Fig. 3). The ancestral girellid was found to be *Graus nigra*. Then, within the genus *Girella,* a lineage comprising *G. fimbriata* and *G. nebulosa* (from the south Pacific Kermadec Islands and Easter Island, respectively), was sister to all remaining *Girella* species. The remaining species separated in three distinct clades: a monophyletic clade of *G. tephraeops* (south-western Australia), a clade comprised of the Cabo Verde species, *G. stuebeli,* clustered with the three Japanese species (*G. leonina, G. mezina,* and *G. punctata*), and a third clade including all remaining species. In this case, the tree is fully resolved except for two southeastern Pacific species *G. freminvillei* (from the Galápagos Islands) and *G. laevifrons* (from Chile), which were not distinguishable based on the 16S rRNA marker. Those two species cluster with the northeastern Pacific California and Sea of Cortez species *G. nigricans* and *G. simplicidens.* We found that trees based on mitochondrial data, RAD data, and the two published trees were generally congruent (Figs. 3 and 4). Importantly, there was no evidence of inconsistency with the grouping of *G. stuebeli* with the clade of species from Japan.

3.3. Divergence times

Divergence times were estimated using RAD markers (Fig. 5). Based on a divergence time of 30 Mya for Girellids, we found that divergence between *G. stuebeli* and *Girella* spp. from Japan was estimated at 2.22 Mya (range of 1.6–3.0 Mya). The time of divergence of the Tropical

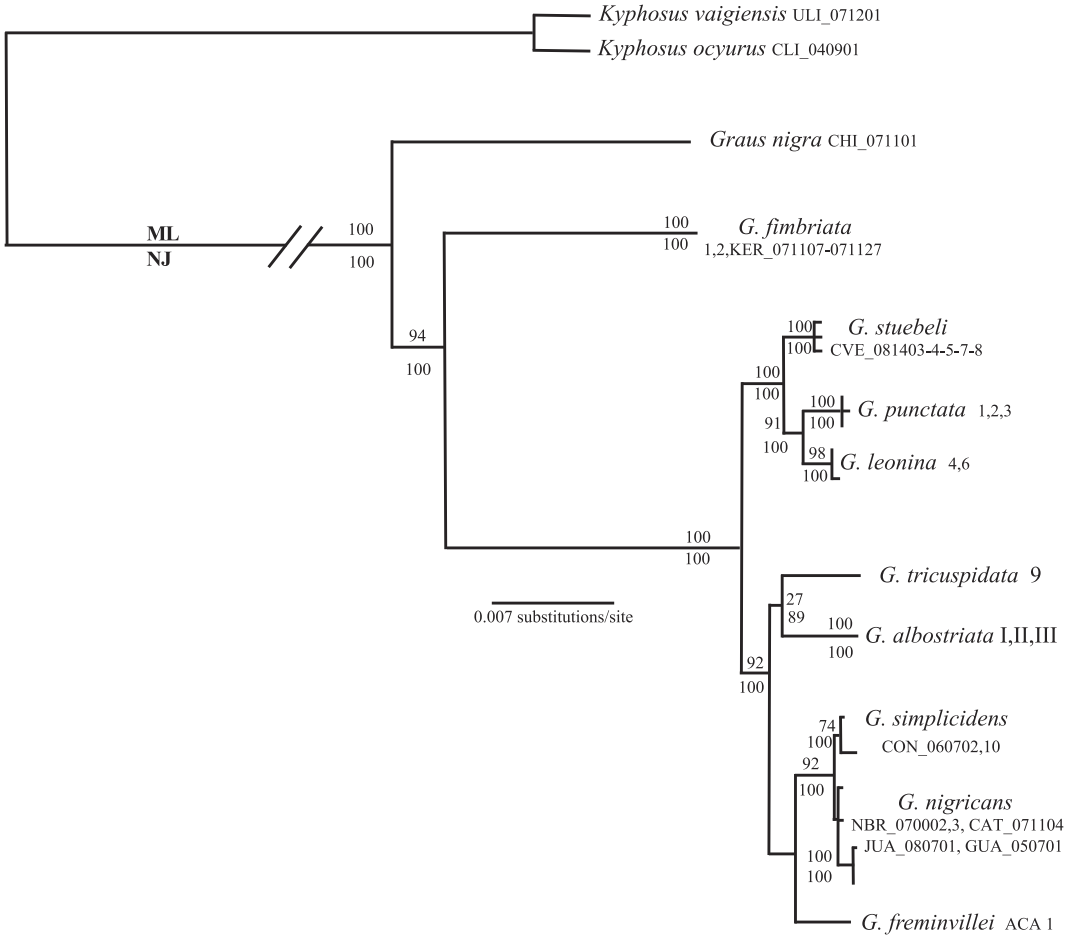

Fig. 4. Molecular phylogeny of a subset of girellid species and outgroups based on 977 RAD seq markers. Bootstrap values for Maximum Likelihood and Neighbor-Joining methods are shown above and below the nodes, respectively.

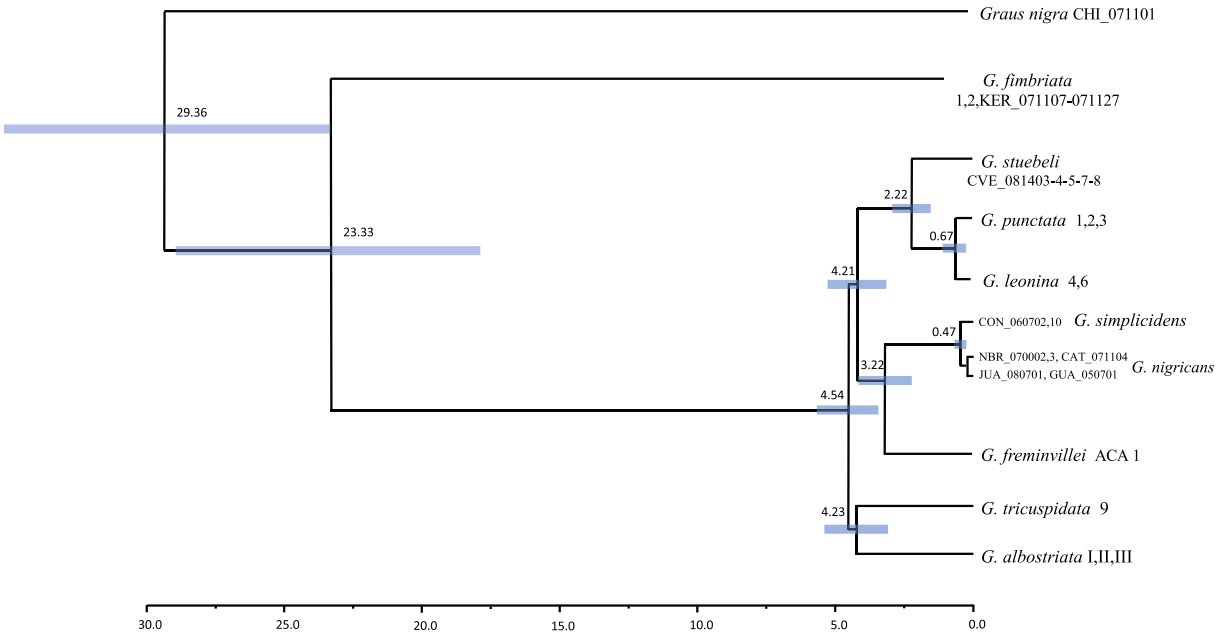

Fig. 5. Time calibrated phylogeny of the Girellidae based RAD markers implemented in BEAST using internal time calibration based on the divergence of Girellidae (average 30 Mya, range 25–38 Mya). Horizontal blue bars at the nodes represent the 95% confidence intervals for each date estimate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Eastern Pacific clade was estimated at approximately 3.22 Mya. Within that clade, the estimated time of divergence between two of the three members of that TEP clade, *G. nigricans* and *G. simplicidens* was approximately 0.47 Mya, which is consistent with a previously published divergence estimate of 0.3–2.2 Mya based on mitochondrial D-loops (Terry et al., 2000).

4. Discussion

4.1. Taxonomic and ecological considerations

Girellids are generally viewed as herbivorous species, although analyses of gut contents suggest that *Girella* species should be considered omnivorous that complement readily-available energy from algae with protein from invertebrates (Clements and Choat, 1997; Clements and Zemke-White, 2008). While at least six morphological synapomorphies unite *Graus* and *Girella*, the relative position of these two genera was still under discussion, in the context of primarily herbivorous *Girella,* and a more primarily omnivorous and secondarily carnivorous *Graus nigra* (Flores and Smith, 2010; Johnson and Fritzsche, 1989; Muñoz and Ojeda, 1997; Perez-Matus et al., 2012). Our data point to an early divergence of *Graus nigra* (Figs. 3–5), thus providing a window to understand the ancestral diet of *Girella*. Benthic juveniles of several *Girella* species are omnivorous and, after an unusual change in teeth morphology, become predominantly herbivorous as adults (Norris and Prescott, 1959; Yagishita and Nakabo, 2003). We see the phylogenetic hypothesis produced by the results of the present study as a starting point that needs further buttressing by obtaining a genomic phylogeny based on all girellid species. The concordance between previously published phylogenies and those presented here suggests that the relative position of *Graus* and *Girella* is unlikely to change in future analyses.

Graus nigra is a temperate species currently found on the southwest coast of South America. Based on a calibrated phylogenetic tree, the early divergence between *Graus* and *Girella* occurred approximately 30 Mya (Knudsen et al., 2019). Later in the evolutionary history of girellids, our data indicate that the earliest split of the genus *Girella* separates south Pacific species found in the Kermadec Islands and Easter Island from the remainder of the *Girella* species that are found at the edges of the distribution of the entire genus, a potentially interesting example of centrifugal speciation (Bernardi et al., 2004; Briggs, 2000). Those latter *Girella* species are found on the periphery of the Pacific Ocean: in Japan, California, Mexico, the Galápagos Islands, the southwest coast of South America, and Australia (Fig. 1).

4.2. The origin of Girella stuebeli

Girellidae offer a unique opportunity to study the evolutionary history of an endemic Cabo Verde fish; *Girella stuebeli* is the sole Atlantic member of an otherwise Pacific Ocean family. Here we tested the alternative hypotheses of a western (Eastern Pacific/Western Atlantic), or eastern (Indian Ocean/Western Pacific) origin of that taxon. Both phylogenetic reconstructions, based on a mitochondrial locus and RAD seq markers yielded the same result, where *Girella stuebeli,* the Cabo Verde Islands endemic, was found to cluster with *Girella* representatives from Japan (*G. leonina, G. punctata, G. mezina*). This clustering is also consistent with the fact that *G. mezina* and *G. stuebeli* are the only members of the genus showing a white band in the middle of the dark body, a unique feature that might have been peculiar to this clade. Interestingly, other color patterns such as multiple dark bands on a pale body (*G. zebra, G. tricuspidata*), pale dots on a dark body (*G. nigricans, G. simplicidens*), and mottled patterns (*G. fimbriata, G. nebulosa*) are also phylogenetically consistent (Fig. 3).

4.3. Western route hypothesis

The western route hypothesis presumes that the ancestor of the Atlantic species would have first migrated from Japan to the Tropical Eastern Pacific, cross the Panama region before the closure of the Isthmus, and later migrated across the Atlantic to reach the Cabo Verde Archipelago. The timing of the closure of the Isthmus of Panama has extensively been used to calibrate molecular clocks and explain biogeographic patterns (e.g. Banford et al., 2004; Bermingham et al., 1997; Bernardi and Lape, 2005; Garcia et al., 2020; Lessios, 2008; Lessios and Robertson, 2013; Tariel et al., 2016). As mentioned above, the timing of the closure is subject to much debate, but it can be narrowed to a window of time between 3.5 Mya to 2.8 Mya, with a reasonable

average estimate of ~ 3.0 Mya (Bacon et al., 2015a, 2015b; Coates and Stallard, 2013; Jaramillo et al., 2017; Lessios, 2015, 2008; Marko et al., 2015; O'Dea et al., 2016). There are several examples of fish groups that are common to Japan and the Eastern Pacific, such as greenling (genus *Hexagrammos*), rockfish (genus *Sebastes*), Sheephead (genus *Semicossyphus*), surfperches (family *Embiotocidae*) and several others (Crow et al., 2004; Longo and Bernardi, 2015; Longo et al., 2018; Poortvliet et al., 2013; Rocha-Olivares et al., 1999). In addition, the African snapper (*Lutjanus dentatus*) and the Guinean parrotfish (*Scarus hoefleri*) are closest to a TEP relative (*Lutjanus colorado,* and *Scarus perrico*, respectively), and several species have closest representatives between the Eastern and Western Atlantic (e.g. genera *Sparisoma, Thalassoma*), indicating that a western route of invasion is possible (Bernardi et al., 2004, 2000; Frédérich and Santini, 2017; Luiz et al., 2012; Robertson et al., 2006). The divergence of the Japanese species and *G. stuebeli* clade (2.2 Mya, range 1.6–3.0 Mya) are slightly more recent than the closure of the Isthmus of Panama. While the earliest genetic divergence estimate (3.0 Mya) is compatible with the latest closure of the Isthmus of Panama estimate (2.8 Mya), the passage into the Atlantic seems unlikely, but difficult to exclude categorically. The TEP clade (*G. nigricans*, *G. simplicidens*, and *G. freminvillei*) predates the divergence of the *G. stuebeli* – Sea of Japan species clade (3.22 v.s. 2.22 Mya, respectively), indicating that a *G. stuebeli* ancestor would have had to compete against congeneric species when in the region, which may explain its current absence from the region (but makes its passage there difficult). Its absence from the Caribbean might be explained by the major change in the ecological conditions of the region, which became a tropical, coral-reef dominated region following the closure of the Isthmus of Panama (although appropriate habitat and ecological conditions do exist along the coast of Brazil). *Girella* is a temperate genus that does not seem to be very competitive in coral reef environments. The only additional step in this sequence that is not seen in other taxa is the extinction of a population in the TEP as well as of one in the Caribbean.

4.4. Eastern route hypothesis

The alternative hypothesis posits that migration would first need to connect individuals from Japan to the Indian Ocean, and then from there to the Atlantic. The last major tropical coastal connection between Atlantic and Indo–Pacific habitats closed approximately 2 million years ago (Mya), with the onset of cold-water upwelling off southwestern Africa. Contemporary eddies of warm water from the Agulhas current (Agulhas rings) wrap around the Cape of Good Hope and then move away to the west into the Atlantic (Byrne et al., 1995; Nencioli et al., 2018; Peeters et al., 2004). This creates an existing warm water connection between the Indian Ocean and the Atlantic. Therefore, an eastern route of migration would either require an older invasion (before 2 Mya), or Agulhas rings that could have transported Indian Ocean *Girella* larvae into the South Eastern and central (St Helena is > 7 my old, Ascension is only ~ 1my) Atlantic. This tempo of this route seems well within the divergence time estimate that was found for *G. stuebeli* (1.6–3.0 Mya), making this route of invasion a more likely scenario than the alternative. Currently, habitat and ecological characteristics of the coast of South Africa seem appropriate for *Girella* species (ecologically similar species such as Chubs, Galjoen, Knifejaws, and Stone Breams are present), indicating that if this route was indeed used by ancestors of *G. stuebeli,* local *Girella* populations might have existed in Southern Africa but are no longer present.

Parts of this pathway have been argued for four unrelated taxa of reef fishes: the goby *Gnatholepis*, the pygmy angelfish *Centropyge,* the stripey, *Microcanthus strigatus,* and the kyphosid chubs. As mentioned above, both *Gnatholepis* and *Centropyge* have Atlantic members with Indian Ocean close relatives. For the stripey (*Microcanthus strigatus*), Western Australian (i.e. Indian Ocean) populations have been shown to be more closely related to populations in Japan than populations in eastern Australia (Tea et al., 2019). For chubs (genus *Kyphosus*), it has been shown that gene flow occurred between Atlantic and Indian Ocean populations (Knudsen et al., 2019). This gene flow in *Kyphosus* species (and probably also for *Gnatholepis* and *Centropyge*) provides support for the idea that movement from the Indian Ocean to the southern Atlantic Ocean, and successful breaching of the Benguela current have both occurred recently.

5. Conclusion

Girella stuebeli stands out as the only Atlantic member of the Girellidae, a family otherwise restricted to the Indo-Pacific, and provides insights into the evolutionary history of this diverse group of omnivorous and herbivorous fishes. Using phylogenetic relationships of the nibblers (Girellidae) based on hundreds of RAD loci, together with published phylogenies and 16S mitochondrial markers, we confirmed that the first main divergence occurred between *Graus nigra* and the genus *Girella*, approximately 30 Mya, and that *Girella* later diverged into three major clades. Reconstructions of the evolutionary history of the sole Atlantic member of the genus *Girella* that is endemic to the Cabo Verde Islands, *G. stuebeli*, show that it is most closely related to the clade of three species currently found in the Sea of Japan. We evaluated two different hypotheses to explain the current distribution, an Eastern route where migration occurred from Japan to the Indian Ocean, and then from there to the Atlantic, and alternatively a western route where migration occurred from Japan to the tropical Eastern Pacific, followed by a migration across the Atlantic to reach the Cabo Verde Archipelago. At this point, while neither of these two equally lengthy (~20,000 km) routes of migration taken by the ancestor of *G. stuebeli* can be rejected, our results suggest that a western route of invasion, across the Indian Ocean and the southern tip of the African continent is a more likely scenario.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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