Extreme gender flexibility: Using a phylogenetic framework to infer the evolution of variation in sex allocation, phylogeography, and speciation in a genus of bidirectional sex changing fishes (*Lythrypnus*, Gobiidae)

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ARTICLE INFO

ABSTRACT

The genus *Lythrypnus* is a group of marine gobies that exhibit extreme gender flexibility as bidirectional sex changers. The genus consists of 20 described species and several undescribed species that are distributed in the Americas. Five species have been characterized with respect to sex allocation and gonad morphology. The hormonal, morphological, and behavioral aspects of sex change have been studied extensively for one species, *L. dalli*. These data, however, have not been interpreted in an evolutionary context because a phylogenetic hypothesis has not previously been proposed for the genus *Lythrypnus*. We propose the first phylogenetic hypothesis for the genus based on molecular data from three mitochondrial genes (*12s*, *ND2*, and *Cytb*), one nuclear gene (*Rag1*) and one nuclear intron (*S7*). We also include three previously undescribed *Lythrypnus* species. Our results support the monophyly of the genus with *L. heterochroma*, an Atlantic species, as the basal taxon. After the divergence of *L. heterochroma*, there are two main clades, one comprised of species distributed in the Atlantic, the other comprised of species distributed in the Pacific. These data indicate an Atlantic origin for the genus, followed by divergence after the closure of the Isthmus of Panama. Our data also support the monophyly of three previously described species complexes, the *L. rhizophora* complex and *L. dalli* complex in the Pacific, and the *L. mowbrayi* complex in the Atlantic. We mapped patterns of sex allocation within this genus onto the fully resolved and supported topology, and found that sexual plasticity and gender flexibility is likely a synapomorphy for the genus. Overall our results create a well-supported framework to understand the phylogeography of the genus, and to interpret the evolution of sex allocation in *Lythrypnus* gobies.

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1. Introduction

1.1. Gobies of the genus Lythrypnus

The genus *Lythrypnus* is a member of the family Gobiidae (Order Percifomes), a monophyletic group of small-bodied benthic fishes that has been characterized as the second most diverse group of vertebrates (Thacker, 2009). The family is comprised of over 1950 species, in 210 genera (Nelson, 2006). The cryptic nature and small size of individuals in the family (often less than 10 cm in length) has left gobies largely understudied (Thacker, 2003). Several members of the family maintain the ability to change sex once or repeatedly, throughout their life. The Gobiidae is one of only 27 families of teleosts (of 448 families) that exhibit hermaphroditism as a reproductive strategy (Cole, 2010; Nelson, 2006). Members of the family are found worldwide and are only absent from the Arctic and Antarctic (Akihito et al., 2000). They are vital players in reef ecosystems and yet their biology is understudied and phylogenetic hypotheses remain contentious.

Lythrypnus gobies are an intriguing group of marine fishes found exclusively in the Americas, they are distributed among benthic, rocky outcrops, at depths ranging from 3 to 150 m and are usually less than 30 mm in length (Böhlke and Robins, 1960, 1968; Bussing, 1990; Ginsburg, 1939; Greenfield, 1988). Previous research on this genus reveals an interesting reproductive tactic, bidirectional sex change, with individuals existing along a gender gradient from pure females to pure males. Interestingly there is variation between species in the distribution of individuals along this gradient (St. Mary, 2000). This novel strategy has made them captivating to researchers who hope to understand the evolution

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of hormonal sex determination and the selective advantage of such alternative reproductive strategies. However, little is known about the evolution of variation in this sexually flexible strategy, nor the evolutionary history of the genus, as the phylogenetic relationships between species have not been previously studied.

The genus *Lythrypnus* is composed of 20 described species (Böhlke and Robins, 1960, 1968; Bussing, 1990; Ginsburg, 1939; Greenfield, 1988). Ten are distributed in the Western Atlantic, from North Carolina to Brazil, with the majority (seven species) occurring in the Caribbean and one species in Bermuda. The remaining ten species occur in the Eastern Pacific, from Southern California to the Galapagos Islands (Fig. 1). Three species (*L. lavenbergi, L. alphagia*, and *L. solenensis*), are monotypic and apparently rare (i.e. only known from the type specimen). Further, there are many *Lythrypnus* specimens in museum collections whose species designation is unknown (e.g. AMNH I-27196, I-73410, I-215231). We include an additional three undescribed species in this study, and conclude that the genus is likely more diverse than is currently recognized. Of the described species, five have been characterized with respect to patterns of sex allocation inferred by gonad morphology (St. Mary, 2000).

1.2. Extreme sex changers

Traditionally hermaphrodites have been placed into one of two discrete categories, sequential hermaphrodites, starting as one sex and then change to the other at some time point, or simultaneous hermaphrodites, acting as both sexes concurrently. Recent studies of several species of marine fishes have found that these discrete classifications are sometimes incomplete. Bidirectional sex change is a third hermaphroditic strategy that is much more rare. It allows individuals to change sex in either direction multiple times (Kuwamura and Nakashima, 1998; Kuwamura et al., 2007; Manabe et al., 2007; Munday et al., 1998; Sakurai et al., 2009). Thus, an individual can take advantage of reproductive opportunities in unpredictable social and environmental conditions (Munday et al., 2006; St. Mary, 1994). To date, bidirectional sex change has been described in only 12 genera from six families, six of which are in the family Gobiidae (Munday et al., 2010). This unusual strategy has been described in two species of *Lythrypnus* (St. Mary, 1996) and inferred for three additional species based on patterns of gonad tissue allocation to ovarian and/or testicular function (St. Mary, 2000), hereafter referred to as sex allocation. The genus *Lythrypnus* is particularly interesting because, while they appear to be simultaneous hermaphrodites, maintaining both types of tissue in their gonad, they act as only one sex at a time. It takes approximately 14 days for a female to change sex to male when paired with another similar sized female (Rodgers et al., 2005). An individual can switch to the opposite sex multiple times depending on density dependent factors such as local sex ratio (Rodgers et al., 2007; St. Mary, 1993, 1996, 2000). Interestingly, there are significant differences in patterns of sex allocation, sexual dimorphism, and inferred sexual tactics between the five characterized *Lythrypnus* species (St. Mary, 2000).

The reproductive strategies of fishes can be inferred by examining the structure of their gonad and the pattern of tissue allocated
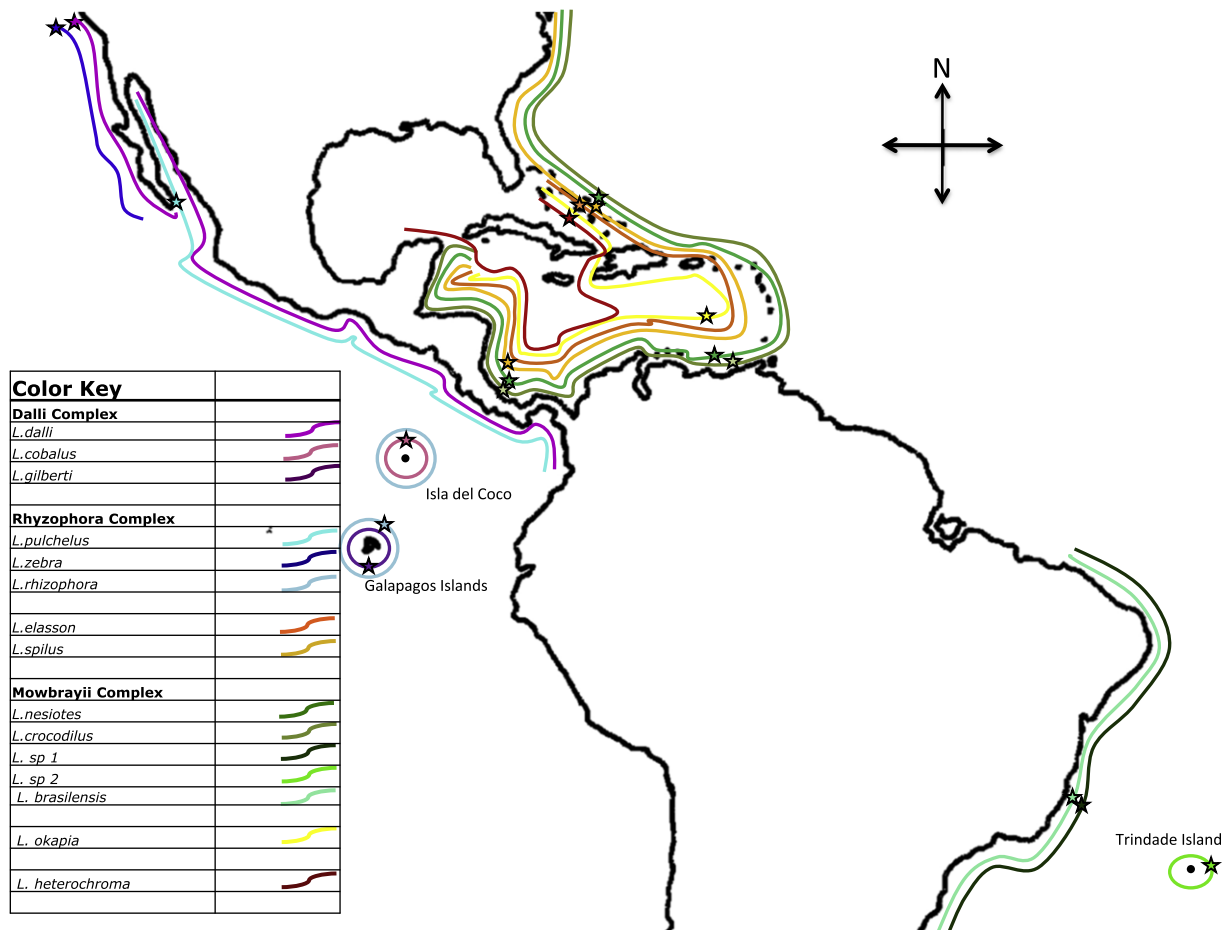

Fig. 1. Species distribution in the Eastern Pacific and Western Atlantic Oceans. The distribution of many species is unclear, particularly in the Caribbean and many are likely more widely dispersed. The stars indicate the approximate locations that the specimens used in this study were collected.

to male or female function (Cole, 1990, 1988). In *Lythrypnus* gobies, gonad morphology has been characterized for *L. dalli*, *L. zebra*, *L. spilus*, *L. nesiotes*, and *L. phorellus*. These five species share similarities with respect to gonad structure and histology. Ovarian tissue is centrally distributed in the gonad and testicular tissue develops from foci on the dorsal and ventral margin of each lobe of the paired gonads, and expands inward (St. Mary, 1998). This differs significantly from any other characterized gobies, and is most likely a synapomorphy for the genus (Cole, 1990; St. Mary, 2000). Individuals from the five species whose sex allocation pattern has been described can be placed in one of six categories. Placement in a category is not static, as an individual can change its allocation to male or female function throughout its lifetime. Individuals can be described as: pure female; strongly female biased hermaphrodites, individuals with less than 5% testicular tissue; weakly female biased hermaphrodites, 5–49% testicular tissue; weakly male biased hermaphrodites, individuals with 50–95% testicular tissue; strongly male biased hermaphrodites, individuals with over 95% testicular tissue, and pure male (St. Mary, 1993). The distribution of individuals across this gradient varies between species (St. Mary, 2000).

Populations of *Lythrypnus dalli*, off Catalina Island, California are composed primarily of pure females and female biased hermaphrodites with some proportion of both strongly male biased hermaphrodites and pure males (St. Mary, 1993). This is the only species to exhibit individuals with the pure male allocation type. *Lythrypnus nesiotes*, distributed in the Atlantic Ocean, is similar to *L. dalli*, in which populations have pure females, strongly female biased hermaphrodites, female biased hermaphrodites and male biased hermaphrodites. The two species differ in allocation to male tissue as *L. nesiotes* does not exhibit any individuals that are pure male, or strongly male biased (i.e. >95% allocation to testis). Both species show sexual dimorphism with respect to size, with males being larger than females and an elongated first dorsal spine in male *L. dalli* (St. Mary, 1993), suggesting that the male strategy may be somewhat stable for some individuals of these species. The remaining three species *L. zebra*, *L. spilus*, and *L. phorellus*, exhibit reduced variation in testicular tissue allocation, and are predominantly female biased with some male biased hermaphrodites. In addition to reduced variation, there is no size dimorphism between male biased and female biased hermaphrodites in these species. While five *Lythrypnus* species have been studied with regard to sex allocation, social factors associated with sex change have been studied in more detail in only two species – *L. dalli* and *L. zebra* (Lorenzi et al., 2006; Rodgers et al., 2007). It has been suggested that predominance of mixed hermaphrodites in *L. zebra*, *L. spilus*, and *L. phorellus*, may be associated with lower densities in natural populations, however it is currently not known whether these species share a more recent common ancestor. The lack of a phylogenetic hypothesis for this group has prevented previous studies from interpreting existing data on sex allocation and sexual strategies in an evolutionary context.

1.3. Phylogenetics

There have been several attempts to propose a phylogenetic hypothesis for the family Gobiidae (Akihito et al., 2000; Thacker, 2003, 2009; Thacker and Hardman, 2005; Thacker and Roje, 2011; Wang et al., 2001). However, the Gobiidae is the second most speciose family of vertebrates, and due to the overwhelming number of species and overall paucity of information on many gobiid genera, most of these analyses are incomplete and lack both resolution and/or statistical support. The phylogenetic affinities of many Gobiid genera have not previously been evaluated and therefore remain tentative. To our knowledge a phylogenetic hypothesis for the genus *Lythrypnus* has also not been previously

proposed. Only recently has a phylogenetic hypothesis for the family Gobiidae been proposed that included any *Lythrypnus* species (Thacker and Roje, 2011). However two of the three *Lythrypnus* species included in the Thacker and Roje study exhibit missing data for one or more loci and therefore the relationship between the members of the genus and its placement within the family remains unclear. It has been suggested that the genus *Lythrypnus* is closely related to *Trimma* or *Priolepis*, based on osteological studies and similarities in gonad morphology (Birdsong et al., 1988), this is supported by molecular data as well (Thacker and Roje, 2011). Overall, several factors have contributed to the lack of information on the natural history and phylogenetic relationships of *Lythrypnus* gobies and related taxa including their cryptic nature, small size, as well as their tendency to live in remote locations.

Lythrypnus gobies are relatively homogeneous with respect to morphology and meristics (Garzon and Acero, 1988). Therefore color and pigmentation patterns are often relied upon to distinguish species. This makes identification of closely related species and the detection of new species problematic due to natural variation within species and with respect to pigmentation patterns. Three species complexes have been described based on morphological characters- one in the Atlantic and two in the Pacific. The *L. mowbrayi* complex, proposed by Böhlke and Robins (1960) and later revised by Greenfield (1988), is based on banding patterns and the lack of prolonged dorsal fin spines and putatively includes five Western Atlantic species: *L. mowbrayi*, *L. phorellus*, *L. nesiotes*, *L. crocodilus*, and *L. brasiliensis*. Two Eastern Pacific assemblages have been proposed based on similarities in banding pattern, the presence or absence of a predorsal crest (Bussing, 1990) and distinctions within neuromast patterns (Ahnelt and Bohacek, 2004). The first assemblage, the *L. dalli* complex, includes *L. dalli*, *L. cobalus*, and *L. gilberti*. The second, the *Lythrypnus rhizophora* complex, includes *L. zebra*, *L. rhizophora*, and *L. pulchellus*. The latter assemblage may also include the remaining four described Pacific *Lythrypnus* species (Ahnelt and Bohacek, 2004).

1.4. Objectives

In order to understand how sexual flexibility has evolved in this group of fishes, a clear evolutionary hypothesis must be put forward. Cole (2010) suggests that sexual plasticity has arisen independently as many as five times in the family Gobiidae. This hypothesis is based on differences in gonad morphology between hermaphroditic gobies but does not include all sexually flexible genera (i.e., *Lythrypnus*). In order to evaluate this hypothesis further, more hermaphroditic genera and more robust phylogenetic analyses must be considered. Here we propose a phylogeny for the genus *Lythrypnus* in order to evaluate existing data on variation in sex allocation, interpret the origin of specific reproductive strategies in an evolutionary context, and to provide a framework for exploring the evolutionary history and biogeographic distribution of this fascinating genus of fishes. In addition, this phylogeny provides a robust framework to distinguish and detect cryptic species. We propose a molecular phylogeny based on multiple loci, including both mitochondrial and nuclear markers, and evaluate the validity of species assemblages that have been previously proposed based on morphological characters.

2. Materials and methods

2.1. Sampling

Tissue samples from 13 of the 20 described species of *Lythrypnus* were obtained from museum collections and two species, *L. dalli* and *L. zebra*, were collected at Catalina Island, CA with hand

Table 1
Lythrypnus samples and sources.

Species	Source	Geographic Location	Accession numbers
Lythrypnus dalli	SFSU	Catlina Island, California	JX024800, JX024832, JX024850, JX024880
Lythrypnus zebra	SFSU	Catalina Island, California	JX024777, JX024804, JX024836, JX024854, JX024884
Lythrypnus cobalus	CAS 205778	Isle del Coco, Costa Rica	JX024775, JX024801, JX024833, JX024851, JX024881
Lythrypnus rhizophora	SIO 00-154	Galapagos Islands	JX024803, JX024835, JX024853, JX024883
Lythrypnus pulchellus	SIO 07-124	Cabo San Lucus, Mexico	JX024778, JX024837, JX024855, JX024885
Lythrypnus gilberti	SIO 00-154	Galapagos Islands	JX024776, JX024802, JX024834, JX024852, JX024882
Lythrypnus elasson	AMNH 249003	Lee stocking, Bahamas	JX024768, JX024785, JX024816,JX024844, JX024864
Lythrypnus heterochroma	AMNH 249001	Lee stocking, Bahamas	JX024766, JX024810, JX024858
Lythrypnus okapia	AMNH 255525	Venezuela	JX024772, JX024792, JX024824,JX024847, JX024872
Lythrypnus spilus 1	AMNH 249006	Lee stocking, Bahamas	JX024767,JX024781, JX024811, JX024843, JX024859
Lythrypnus spilus 2	AMNH 248999	Boco del Toro, Panama	JX024782, JX024812, JX024860
Lythrypnus spilus 3	AMNH 249000	Boco del Toro, Panama	JX024813, JX024861
Lythrypnus spilus 4	AMNH 249001	Boco del Toro, Panama	JX024783, JX024814, JX024862
Lythrypnus spilus 5	AMNH 249002	Boco del Toro, Panama	JX024784, JX024815, JX024863
Lythrypnus nesiotes 1	AMNH 249004	Lee stocking, Bahamas	JX024770,JX024790, JX024821, JX024846,JX024869
Lythrypnus nesiotes 2	AMNH 255522	Panama	JX024791, JX024823, JX024870
Lythrypnus nesiotes 3	AMNH 255521	Isla Iguana, Sucre Venezuala	JX024822, JX024871
Lythrypnus crocodilus 1	AMNH 249007	Sucre Venezuela	JX024769, JX024786, JX024817,JX024851, JX024865
Lythrypnus crocodilus 2	AMNH 249012	Morde Aguera, Venezuela	JX024789, JX024818, JX024866
Lythrypnus crocodilus 3	AMNH 255523	Panama	JX024788, JX024819, JX024867
Lythrypnus crocodilus 4	AMNH 249009	Isla Iguana, Sucre Venezuala	JX024787, JX024820, JX024868
Lythrypnus brasiliensis 1	CIUFES 2013	Iiha Escalvada, Guarapari Brazil	JX024771, JX024793, JX024825,JX024848, JX024873
Lythrypnus brasiliensis 2	CIUFEES 2014	Iiha Escalvada, Guarapari Brazil	JX024794, JX024826, JX024874
Lythrypnus sp 1.1	CIUFES 2016	Iiha Escalvada, Guarapari Brazil	JX024773, JX024795, JX024827, JX024875
Lythrypnus sp 1.2	CIUFES 2017	Iiha Escalvada, Guarapari Brazil	JX024796, JX024828, JX024876
Lythrypnus sp 2.1	CIUFES 2010	Trindade Island, Brazil	JX024774, JX024798, JX024829, JX024877
Lythrypnus sp 2.2	CIUFES 2011	Trindade Island, Brazil	JX024797, JX024830, JX024878
Lythrypnus sp 2.3	CIUFES 2012	Trindade Island, Brazil	JX024799, JX024831, JX024879
Lythypnus sp 3.1	AMNH 255524	Venezuela	JX024805, JX024838, JX024886
Lythrypnus sp 3.2	AMNH 255525	Venezuela	JX024806, JX024839, JX024887
Lythypnus sp 3.3	AMNH 255526	Venezuela	JX024807, JX024840, JX024888
Trimma benjamini	TCN T03929	Palau, philipeans	JX024764, JX024779, JX024808, JX024841, JX024856
Priolepis hipoliti	AMNH 249011	Venezuela	JX024765, JX024780, JX024809, JX024842, JX024857
Risor ruber	NA	NA	EU380937, AY846561, AF491116, AF391495

nets on scuba (Table 1). Of the remaining seven *Lythrypnus* species, *L. alphigena*, *L. lavenbergi*, and *L. solanensis* are known from a single holotype specimen and therefore were unavailable for inclusion in this study. However, three undescribed species were included in the analysis, from here on referred to as *L. sp 1* (Atlantic, mainland Brazil), *L. sp 2* (Atlantic, Trindade Island Brazil), and *L. sp 3* (Atlantic, Venezuela), in order to evaluate the species status of these specimens and to increase sampling of *Lythrypnus* taxa.

The genera *Trimma* and *Priolepis* have been proposed as close relatives to *Lythrypnus* (Birdsong et al., 1988; Cole, 2010; St. Mary, 1998; Thacker and Roje, 2011). A representative from each group, *Trimma benjamini* and *Priolepis hipoliti,* were therefore selected as outgroups for this study. A third outgroup *Risor ruber* was selected based on its proposed relationship to the *Lythrypnus*, *Trimma* and *Priolepis* clade (Thacker, 2009).

2.2. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from fin clips or muscle tissue using the Qiagen DNEasy blood and tissue kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocols. Sequences from three mitochondrial genes (*Cytb*, *12s*, and *ND2*), a nuclear gene (*Rag*) and a nuclear intron (*S7*) for 16 *Lythrypnus* species and a single representative from *Trimma* and *Priolepis*, were used for phylogenetic analyses. Sequences from *Risor ruber* were available from public databases for *12s*, *ND2*, *Cytb*, and *Rag1* loci. Sequences were amplified using published primers or designed specifically from our own data (Table 2). Amplification was performed in 25 μl reactions consisting of 22 μl of 1.1XTaq Reddy Mix (Thermo Scientific, Foster City, CA), 1 μl each of the forward and reverse primers (20 μM), and 1 μl of DNA. Amplification of

Table 2
Primers for the three mitochondrial and two nuclear loci.

Primer	Sequence	Source
CYB_1	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	This study
CYB_2	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	This study
GOBYL4040	GCCCATACCCCWAAYATGTTGGT	Thacker and Hardman (2005)
GOBYH5513	GAGTAGGCTAGGATTTTWCGAAGYTG	Thacker and Hardman (2005)
12sA	AAACTGGGATTAGATACCCCACTAT	Hillis et al. (1996)
12sB	GAGGGTGACGGGCGGTGTGT	Hillis et al. (1996)
*Rag1*_F1	CTGAGCTGCAGTCTACCATAAGATGT	López et al. (2004)
*Rag1*_R811	TCATAGCGCTCTAGGTTCTCC	Neilson and Stepien (2009)
*S7*RPEX1F	TGGCCTCTTCCTTGGCCGTC	Chow and Hazama (1998)
*S7*RPEX2R	AACTCGTCTGGCTTTTCGCC	Chow and Hazama (1998)

12s, *ND2*, *Cytb* and *Rag1* occurred under the following PCR profile: 35 cycles of 95 °C for 30 s, 50–55° C for 30 s, and 72 °C for 45 s. The first intron of *S7* was amplified as follows: 35 cycles of 94 °C for 60 s, 60 °C for 45 s, and 72 °C for 120 s. The PCR products were

visualized on 1% agarose gels stained with ethidium bromide and viewed under UV light. If two or more bands were present, the pre-ferred product was cut from the gel and purified using Qiagen gel extraction kit (Qiagen Inc., Valencia CA). If there was a single band the PCR product was purified using Qiagen PCR purification kit fol-lowing the manufactures protocol. The resulting products for *12s*, *ND2*, *Cytb*, and *Rag1* were directly sequenced using Big Dye chem-istry (Applied Biosystems, Foster City, CA). The *S7* intron was cloned prior to sequencing using the pGEM vector system (Prome-ga, Madison, WI) and was sequenced using the T7 and SP6 standard primers. Sequences are deposited in GenBank (Table 1).

2.3. Phylogenetic analyses

The resulting sequences were aligned using Clustal as imple-mented in Sequencher 4.1.2 (GeneCodes Corp., Ann Arbor, MI) and edited and aligned by eye using the software Se–Al (http://tree.bio.ed.ac.uk/software/seal/). Phylogenetic relationships were assessed using Maximum Parsimony (MP), Neighbor Joining (NJ), and Bayesian Inference (BI) methods implemented by PAUP (Swof-ford, 2002) and MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The five loci were analyzed separately, and statistical support val-ues for all nodes were estimated using 2000 bootstrap replicates for NJ and MP analysis, while Bayesian posterior probabilities were estimated via consensus from 10,000 trees. Model selection for BI was estimated using MrModeltest 2.3 (Nylander, 2008). Agreement between data sets was analyzed using the Shimodaira–Hasegawa test as implemented in PAUP. The concatenated data set was ana-lyzed using the same methods as individual loci.

2.4. Divergence time estimation

We used two approaches to estimate divergence time among major lineages including a molecular clock approach for *Cytb* and a penalized likelihood approach implemented in the software r8s (Sanderson, 2002). *Cytb* is commonly used for phylogenetic recon-struction and divergence time estimation. Rocha et al. (2005) esti-mated a substitution rate of 1.93–2.17% per million years for *Cytb* in a group of tropical gobies. Due to the phylogenetic affinity be-tween *Lythrypnus* gobies and the tropical gobies investigated by Rocha et al. (2005) we estimated average pairwise distances for the *Cytb* gene in PAUP and inferred divergence times, assuming a range of substitution rates of 1.93–2.17% per million years.

The second approach, penalized likelihood described by Sander-son (2002) combines parametric and non-parametric methods and provides a robust estimation of sequence divergence (Ruber and Zardoya, 2005). This approach does not assume a molecular clock and requires a calibration based on the fossil record for at least one node. Bajpai and Kapur (Bajpai and Kapur, 2004) described the earliest fossils for the Gobiidae from the early Eocene (52–56 mya). Therefore we set the age of the node for Gobiidae to 53 million years. Goby fossils are often small and rarely preserved, therefore these time estimations should be considered minimum ages only (Thacker et al., 2008). A second time constraint was ta-ken from geological data. The Atlantic Pacific split in *Lythrypnus*, hypothesized by phylogenetic analysis, suggests that these two clades diverged in association with the formation of the Isthmus of Panama, which began 15 mya with final closure 3 mya (Coates et al., 1992; Jackson et al., 1996; Lessios, 2008). We therefore set a constraint at this node between 3 and 15 mya. *12s* and *Rag1* were the only loci for which the appropriate outgroups for calibrations nodes were available. The tree topologies for these two individual genes, as well as a concatenated *Rag1/12s* tree were used for R8s analysis.

3. Results

3.1. Samples and sequencing

A total of 16 *Lythrypnus* species, including three previously undescribed species, and two of the three outgroups, *Priolepis hipoliti* and *Trimma benjamini* were sequenced for 5 loci (Table 3). Any ambiguous sites in the alignment were excluded from further analyses. There were no indels present in *ND2*, *Cytb*, or *Rag1*, and indels were excluded from *S7* and *12s* data sets. Removal of indels did not result in significant changes to tree topology compared to analyses with indels included. However, removal of indels did re-sult in higher support for several nodes. We were unable to amplify *S7* for *L. heterochroma*, *ND2* for *L. heterochroma* and *L. pulchellus*, or *Cytb* for *L. rhizophora* and *L. dalli*. Plots of sequence divergence for transitions and transversions as well as for first, second, and third codon positions indicated no evidence for saturation in the two protein coding sequences *Cytb* and *ND2* (data not shown).

3.2. Phylogenetic analysis of the concatenated dataset

Congruency between data sets was evaluated using the Shimo-daira–Hasegawa test in PAUP. Four of the five genes, *12s*, *Cytb*, *Rag1* and *S7* were congruent when taxa with missing data were ex-cluded (Table 4). However, the *ND2* locus was not statistically con-gruent, and therefore the initial analysis using a concatenated data set was based on the *12s*, *Cytb*, *Rag1* and *S7* only. When the *ND2* lo-cus was included in the concatenated analysis, the topology was identical with respect to all deep nodes. The only differences that occurred were in the resolution of sister species within two species complexes. However, inclusion of all five loci in the concatenated data set resulted in higher support at deep nodes and an overall topology that is supported by the weight of the evidence from analysis of individual loci. Therefore, we present the topology in-ferred from the complete data set, with inconsistencies at terminal splits depicted as polytomies to remain conservative (Fig. 2).

The concatenated dataset supports the genus *Lythrypnus* as a monophyletic assemblage, and the Atlantic species, *L. heterochro-ma*, is supported as the basal taxon with bootstrap values of 100/100/100 for MP/NJ/BI analyses respectively (and hereafter, Fig. 2). After the divergence of *L. heterochroma*, the remaining taxa group into two main clades defined by geographic distribution and ocean basin. Species distributed in the Atlantic, including *L. spilus*,

Table 3
Summary of sequence information for all loci and concatenated data.

Locus	Fragment length	Variable sites	Parsimony informative sites	AIC model
12s	350	40	127	GTR + I + G
ND2	476	31	267	GTR + I + G
Cytb	327	22	130	HKY + I + G
S7	686	133	225	HKY + G
Rag1	743	112	128	HKY + G
All 5 Loci	2582	342	831	GTR + I + G

Table 4
Results for the Shimodaira–Hasegawa test with *p*-values. No significant difference was found for these four loci.

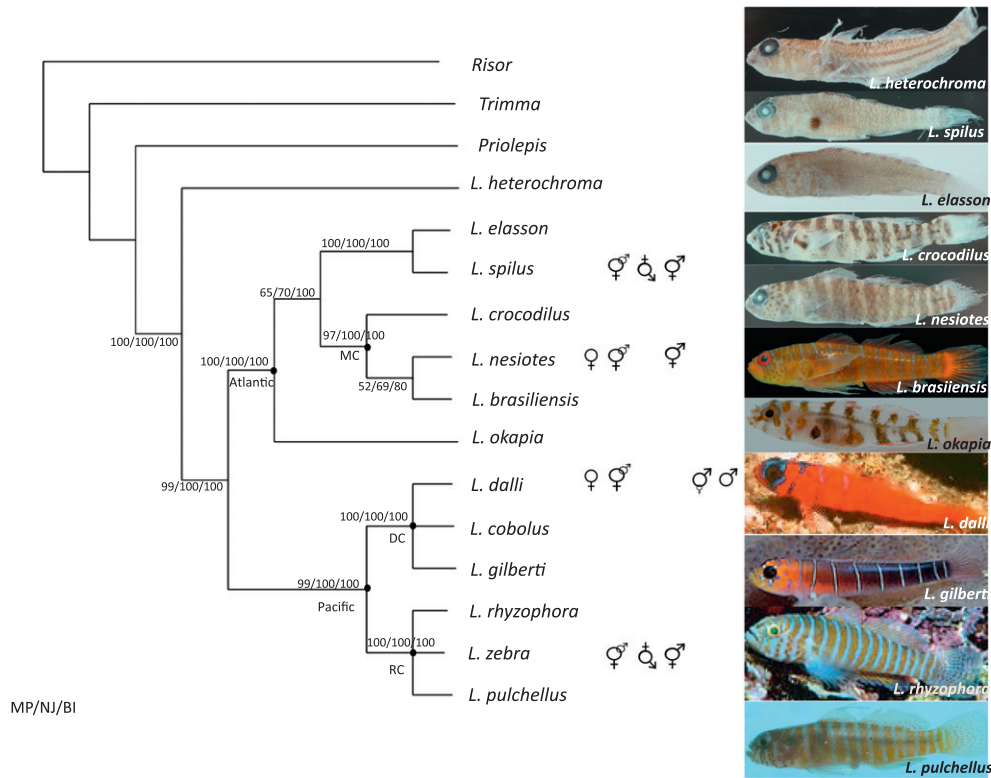| Gene	−ln L	Diff. −ln L	SH (*p*)	wtd-SH
12s	8688.91742	(best)		
Rag1	8692.69156	3.77414	0.7131	0.6801
S7	8717.23182	28.3144	0.1412	0.1078
Cytb	8721.59698	32.67956	0.1051	0.0717

Fig. 2. Molecular phylogeny for the genus *Lythrypnus*. This hypothesis is based on 2 nuclear (*S7* and *Rag1*) and 3 mitochondrial (*Cytb, ND2*, and *12s*) loci and including 13 of the 20 described *Lythrypnus*. Nodes of interest are marked. MC indicates the origin of the *L. mowbrayi* complex, DC the origin of the *L. dalli* complex, and RC the origin of the *L. rhizophora* complex. Sex allocation type is mapped onto the phylogeny and symbolized as follows: ♀ = pure female ♀ = strongly female biased hermaphrodite (<5% testicular tissue) ♀ = weakly female biased hermaphrodite (5–49% testicular tissue) ♂ = weakly male biased hermaphrodite (50–95% testicular tissue) ♂ = strongly male biased hermaphrodite (>95% testicular tissue) ♂ = pure male. Picture of *L. brasiliensis* taken by Raphael M. Macieira. Pacific species *L. dalli, L. rhizophora,* and *L. gilberti* taken from fishbase.org. All other pictures were taken by the authors of this study.

L. elasson, *L. crocodilus*, *L. nesiotes*, *L. brasiliensis*, and *L. okapia* group into a well supported clade with support values of 100/100/100. The remaining taxa, consisting of *L. dalli*, *L. cobalus*, *L. gilberti*, *L. rhizophora*, *L. pulchellus*, and *L. zebra* exhibit geographic distributions in the Pacific, and form a well supported clade as the sister taxon of the Atlantic clade with support values of 99/100/100. Within these two clades three previously described complexes form well supported clades including the *L. mowbrayi* complex (MC), the *L. dalli* complex (DC), and the *L. rhizophora* complex (RC). The MC has high support in all analyses, 97/100/100. In all three analyses *L. nesiotes* and *L. brasiliensis* are sister taxa and form a clade with *L. crocodilus* (52/69/80). In all analyses, a second Atlantic clade, composed of *L. elasson* and *L. spilus* (100/100/100), is sister to the MC and *L. okapia* is supported as basal to the Atlantic clade (65/70/99). The DC is supported as a monophyletic assemblage with 100% statistical support in all analyses. *L. dalli* is sister to *L. cobalus,* and *L. gilberti* is supported as sister to this clade (61/99/100). The RC is also supported as a monophyletic clade (>98%) with *L. zebra* as sister to the clade formed by *L. rhizophora* and *L. pulchellus* (61/99/100). However these two clades are drawn as polytomys due to inconsistencies with the *ND2* dataset.

3.3. 2b Phylogenetic analyses of individual loci

For individual loci, the gene trees estimated by MP, NJ, and BI were mostly identical in topology, and Bayesian analyses resulted in higher support values. There were some differences in topology between loci at shallow nodes, however, the major splits at deeper nodes were strongly supported in all gene trees. Inconsistencies between data sets occurred at tree tips within supported clades

including the previously described DC, MC, or RC species assemblages. For example, there were no analyses from single loci, nor any combined analysis that supported paraphyletic species complexes of the Atlantic or Pacific clades after the divergence of the basal taxon *L. heterochroma*. In addition there were no topologies that conflicted with the monophyly of the three species assemblages described above.

In the *12s* gene tree the genus *Lythrypnus* is recovered as a monophyletic group and *L. heterochroma* is supported as the basal taxon with all support values >90%. The Atlantic/Pacific split is also strongly supported with >98% for all analyses. The MC has 65/93/91 statistical support, while the DC, and RC were supported with >98% support and >92% support values respectively for all analyses. Within the MC, *L. brasiliensis*, *L. crocodilus*, and *L. nesiotes* form a polytomy. *Lythrypnus elasson* and *L. spilus* form a clade with over 98% support in all analyses and are sister to the MC. Finally *L. okapia* is supported as the basal Atlantic taxon with 83/90/99 bootstrap support. While the DC is supported as monophyletic, there is weak support (60/56/__) for *L. gilberti* as sister to *L. dalli* and *L. cobalus*. In the BI analysis this complex is a polytomy. The RC was more resolved with moderate support (>70%). for *L. zebra* as basal to sister species *L. rhizophora* and *L. pulchellus*.

The *S7* gene tree has a very similar topology to *12s*. The genus *Lythrypnus* is monophyletic with 100% bootstrap support in all analyses and there is 100% support for both the Atlantic and Pacific clades. The MC is monophyletic with >98% support. Both the DC and the RC are also well-supported clades, with all bootstrap support of 100%. Like the *12s* gene tree, *L. spilus* and *L. elasson* for a clade with100% support that is sister to the MC. Unlike *12s Lythrypnus okapia* is not supported as basal to the Atlantic clade. Instead it

forms a polytomy with the MC and the *L. elasson/L. spilus* clade. This result does not contradict that of *12s*, but provides no support for that relationship. Resolution within the MC also differs between the *12s* and *S7* gene trees. *S7* supports *L. nesiotes* and *L. brasiliensis* as sister species (51/75/95) and together this clade is sister to *L. crocodilus*. While the DC and RC are supported as monophyletic assemblages, the internal relationships were unresolved with the *S7* data alone.

The gene tree for *Rag1* also supports a monophyletic *Lythrypnus* (>99%) and *L. heterochroma* as the basal *Lythrypnus* taxon with >98% bootstrap supports. The MC is monophyletic with 74/84/99 bootstrap supports and there is strong support for two Pacific clades, the RC and DC (>80%). The topology of the MC differs from both *12s* and *S7*. *Lythrypnus crocodilus* and *L. brasiliensis* are sister species (99%) and *L. nesiotes* is sister to this clade (99%). In both BI and ML *L. okapia* is weakly supported as basal to the MC and forms a polytomy with the MC and the *L. spilus/L. elasson* clade in MP (__/56/84). The internal topology for the DC and RC also differed from the previous two loci. The RC supports *L. rhizophora* and *L. zebra* as sister species forming a clade with *L. pulchellus* (73/70/99). The DC is a polytomy in MP analysis and in both NJ and BI there is weak support for *L. dalli* as sister to *L. gilberti* forming a clade with *L. cobalus* (__/70/69). *L. elasson* did not form a clade with *L. spilus*, in MP it forms a polytomy within *Lythrypnus* with the Pacific and Atlantic clades. In ML it is basal to the Atlantic clade, with weak bootstrap support of 52 and in BI it is basal to *Lythrypnus*, excluding *L. heterochroma*. This relationship differs from all other genes.

The *ND2* gene tree is less resolved then the topologies derived from *12s*, *Rag1* and *S7*. The Atlantic and Pacific clades are recovered in all analyses, but with varying statistical support. For example the BI analysis indicates 100% BPP for both the Atlantic and Pacific clade, yet the MP and NJ have weak or no support at these nodes. Both ML and BI support a monophyletic Atlantic clade (__/60/57). In all analyses there is high support for the two Pacific complexes, the DC has 100% support in all analysis and the RC has >91% support in all analyses. Like, *S7*, *12s*, and *Rag 1*, *L. elasson* and *L. spilus* are sister taxa within the Atlantic clade with support values >98. The MC is supported in all analyses (56/58/99) with the exclusion of *L. nesiotes*, which forms a polytomy with the MC, the *L. spilus/L. elasson* clade and *L. okapia*.

The topology produced from the *Cytb* data for MP and NJ are generally unresolved with only three supported nodes. The BI topology was more resolved, but most nodes are weakly supported (<60). Those nodes with support are the clade that includes the Pacific species (55/59/99). Within the Pacific, the RC and the DC are supported in the MP and NJ analyses but not by BI. Overall, the relationships based on this locus were lacking in resolution and statistical support, and in addition may have been affected by missing data for two taxa. However, inclusion in the concatenated data set did contribute to slightly increased statistical support.

There was no strong support for any internal nodes incongruent with the concatenated data set and overall there was a high level of similarity between gene trees at most nodes. We therefore combined all five genes into a single dataset to contribute to the overall resolution and support for the phylogenetic relationships of the *Lythrypnus* gobies (Fig. 2). Internal nodes within monophyletic species assemblages conflicting or equivocal topologies are indicated as polytomies to interpret the proposed phylogenetic hypothesis conservatively.
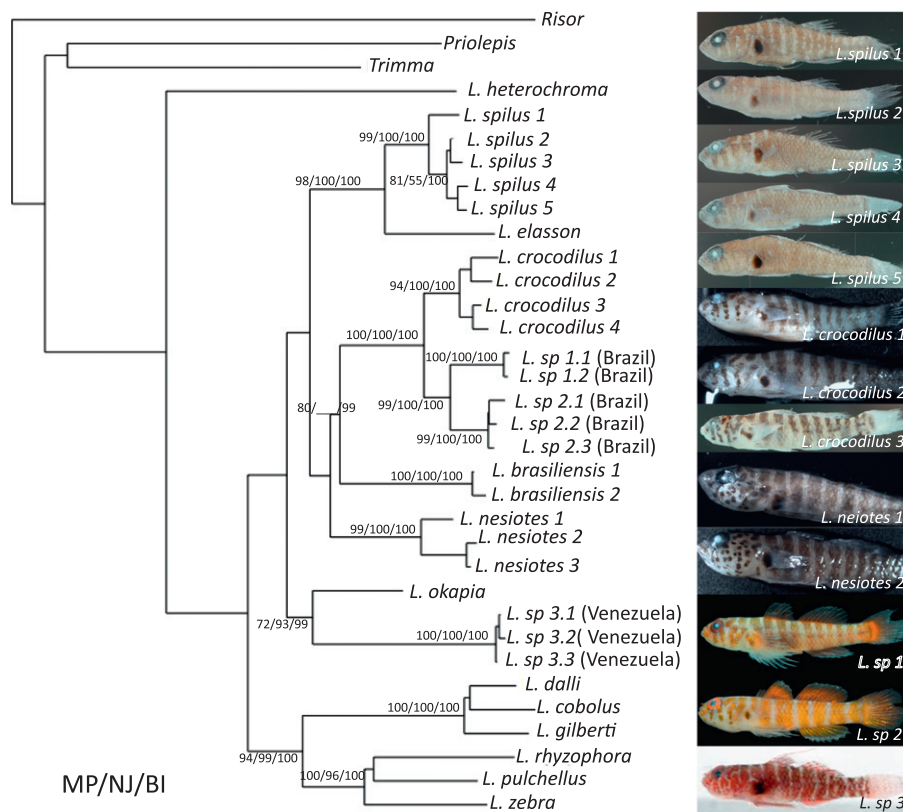

Fig. 3. Phylogram based on *Rag1* and *12s* loci illustrating relationships within the genus *Lythrypnus* and including three undescribed species. Multiple individuals of *L. nesiotes*, *Lspilus*, and *L. crocodilus* were also included to highlight facial pattern variation. *Lythrypnus spilus*, *L. sp 3*, *L. nesiotes* and *L. crocodilus* were photographed by the authors of this paper and *L. sp 1* and *L. sp 2* by Raphael M. Macieira.

Table 5

Age estimates for the Atlantic/Pacific split using a molecular clock and penalized likelihood approaches. The molecular clock approach assumed 1.93–2.17% sequence divergence/million years (based on calibrations from Rocha et al. 2005). The penalized likelihood approach was implemented in r8s for the two loci for which data are available spanning calibration nodes (*12s* and *Rag1*). Using 2 calibration nodes, the age of 6 nodes of interest were estimated. Note the estimated divergence time for the Atlantic/Pacific split using r8s is consisting with the molecular clock analysis.

Molecular clock			
Gene	Avg. pairwise distance of Atl/Pac split	Age: 1.93%/my	Age: 2.17%/my
Cytb	21.83%	11.31	10.06
R8s			
Node/split	RAG 1 Node age	*12s* Node age	*12s* + *Rag1* (GTR + I + G)
Outgroups	23.39	38.9	32.18
Basal node	10.11	21.98	18.45
Atlantic/Pacific	6.49	13.94	12.09
DC/RC	6.04	8.98	9.52
L. spilus/MC	4.09	7.79	7.03
L. brasiliensis/L. crocodilus	2.01	6.58	5.7

3.4. Undescribed and morphologically variant individuals

There were three undescribed species included in this phylogeny, *L. sp 1* (mainland Brazil), *L. sp 2* (Trindade Island, Brazil), and *L. sp 3* (Venezuela). A tree was reconstructed based on 1106 base pairs from the *12s* and *Rag1* loci (Fig. 3). Several individuals (n = 2–3) of each of the first two species mentioned above form reciprocally monophyletic clades with sequence divergence comparable to or greater than other sister taxa. For example, at the *12s* locus (460 bp) there are 22 bp differences between *L. sp1* and *L. sp2* and *L. sp1* differs from *L. crocodilus* by 29 bp. This is similar to the difference observed between sister species *L. elasson* and *L. spilus*, who differ by 17 bp. There is similar divergence observed between *L. sp 3* and *L. okapia* with 34 bp differences. The addition of these undescribed taxa to our phylogenetic analysis results in consistent support for all deep nodes indicated by the concatenated and individual locus analyses.

The two Brazilian species, *L. sp 1* and *L. sp 2* are sister species and form a clade with the Caribbean species *L. crocodilus* (100/100/100). In both MP and BI analyses *L. brasiliensis* is supported as sister to the clade including *L. crocodilus, L. sp 1*, and *L. sp 2* (100/___/100) and *L. nesiotes* is supported as basal to the MC (100/___100). In NJ *L. nesiotes* and *L. brasiliensis* form a polytomy with the *L. crocodilus/L. sp 1/L. sp 2* clade. In all three analyses *L. sp 3* is sister to *L. okapia.* This clade is the basal to the Atlantic clade.

We observed significant inter- and intraspecific variation in facial patterns composed of bars and spots in several Atlantic species. Multiple individuals were included in Fig. 3 for *L. spilus, L. nesiotes,* and *L. crocodilus* to determine if there was phylogenetic signal associated with the observed variation.

3.5. Divergence time estimations

Two methods for estimating divergence times in *Lythrypnus* were used, the molecular clock approach and a penalized likelihood (PL) approach implemented in r8s. Using a substitution rate of 1.93% and 2.17% sequence divergence in the *Cytb* locus (following Rocha et al., 2005), the molecular clock approach yielded a range for the Atlantic–Pacific split between 11.31 and 10.06 my (Table 5). The penalized likelihood analysis using the two loci for which we had data for taxa spanning calibration nodes, *12s* and *Rag1*, the divergence estimates were between 13.94 and 6.49 million years, for *12s* and *Rag1* respectively, which is in agreement with the *Cytb* molecular clock estimate. The PL approach also indicated that the Atlantic MC diverged from the *spilus/elasson* clade 4–7.8 mya, In the Pacific, the RC and the DC clades split 6–9 mya, and basal taxon *L. heterochroma* diverged between 10 and 20 mya. Finally, the PL approach indicates that *Lythrypnus* split from the rest of Gobiidae approximately 23–38 mya (Table 5).

4. Discussion

The topology proposed here allows us to explore the relationships between biogeography, sex allocation and patterns of speciation within in the genus *Lythrypnus*. This is the first fully resolved and supported hypothesis detailing the evolutionary history of the group. This study achieved a high level of taxon sampling that included species from the Atlantic, Pacific, and multiple representatives from all previously described species complexes. In addition, we use this topology to identify several new species as well as document a range of facial pattern variation within a single species.

4.1. Phylogeography

Our results fully support *Lythrypnus* as a monophyletic assemblage. None or our analyses from single gene trees or the concatenated data set, suggest any alternative topology. This result differs from a recent phylogenetic hypothesis for the family Gobiidae proposed by Thacker and Roje (2011) which included only three *Lythrypnus* species, and concluded that the genus is paraphyletic. In their analysis *L. elasson* did not group with *L. zebra* and *L. dalli*, rather they recovered a topology indicating that *L. elasson* is more closely related to *Priolepis hipoliti*. A comparison of our *L. elasson ND2* sequence with that of Thacker and Roje (genbank # HQ909579.1) indicates that these sequences could not be from the same species, with over 150 bp differences (of 561 bp). This is far more than the difference observed between any *Lythrypnus* species we sequenced for this locus. *Lythrypnus* species are small, cryptic and hard to identify and juvenile *L. elasson* looks very similar to *P. hipoliti*. Due to the observed sequence difference between our data and that of Thacker and Roje, and the conflicting placement of *L. elasson* as sister to the *Priolepis* clade, is likely that the individual sequenced by Thacker and Roje was a *Priolpis* species and not *Lythrypnus.*

Lythrypnus heterochroma, supported as the basal member of the genus, is distributed in the Atlantic, indicating that the genus *Lythrypnus* originated in the Atlantic Ocean. The subsequent divergence of the Atlantic and Pacific clades between 6 and 13 mya pre-dates the final closure of the Isthmus of Panama and is likely due to early geological events associated with its formation. *Lythrypnus* gobies are associated with benthic rocky habitats, and reproduce using demersal nests guarded by dominant male. It is likely that these life history characteristics contributed to the early separation of Atlantic and Pacific populations before final isthmus formation.

The remaining *Lythrypnus* species are split into two major clades separated by the Isthmus of Panama. All analyses based on individual loci and the concatenated data set support this topology. Clearly, the Isthmus of Panama played a central role in the ini-

tial diversification of the genus, it does not however appear to have played a driving role in radiation of the genus. Most of the diversity exhibited by this group arose after Atlantic/Pacific separation, indicating that *Lythrypnus* is a relatively young genus. The Isthmus of Panama is estimated to have begun forming approximately 15 mya, with complete separation between the two bodies of water approximately 3 mya (Coates et al., 1992; Jackson et al., 1996; Lessios, 2008). The gradual formation of the isthmus over a period of about 12my separated populations of marine organisms leading to numerous, relatively recent, speciation events (Bernardi et al., 2004). Before complete closure, islands and sedimentary basins formed in the seaway connecting the two oceans. This began to gradually change the geology of the area and the oceanic currents, temperature, salinity and nutrient distribution along this waterway (Coates and Obando, 1996; Lessios, 2008).

Though we were unable to collect samples of *L. mowbrayi* and *L. phorellus* for this study, the three species representing the *L. mowbrayi* complex, *L. nesiotes*, *L. crocodilus*, and *L. brasiliensis* are included and do form a monophyletic clade with robust support in all individual gene trees and the concatenated data set. Two undescribed species, *L. sp 1* and *L. sp 2* are also members of this clade. *Lythrypnus brasiliensis*, which occurs in southern Brazil, is estimated to have diverged from its sister taxon approximately 2–5 mya. The Amazonian barrier, where the Amazon River flows into the Atlantic Ocean, has proven to act as a barrier for vicariant isolation and speciation for many marine organisms including reef fishes (Joyeux et al., 2001; Rocha, 2003). There is some discrepancy between estimated dates for the establishment of the Amazonian barrier but it is believed to have occurred between 11 mya and 1.6 mya (Rocha, 2003). Joyeux and colleagues (2001) noted that many southern Brazilian endemic reef fishes were small, benthic, and demersal spawners. They postulated that these characteristics made it difficult to transverse the Amazonian barrier. *Lythrypnus* gobies share these characteristics, and the barrier created by the freshwater outflow from the Amazon may have effectively separated a continuous population along the Brazilian coast.

Interestingly, while the Brazilian species exhibit distributions that are isolated and distant from the remaining congeners, they are not monophyletic. Two Brazilian species are sister to *L. crocodilus*, which is distributed in the Caribbean. The concatenated data indicate that *L. nesiotes* and *L. brasiliensis* are sister, consistent with two separate episodes of speciation resulting in disjunct Brazilian taxa. *Lythrypnus sp 2* has been found only on the island Trindade in Southern Brazil. This island is estimated to be 3–3.5 myo, which roughly coincides with the age of the *L. crocodilus/L. sp 2* split 2.97 mya (Rocha et al., 2002). *Lythrypnus sp 1* appears to be more wide spread and is found along the coastline, but its range is not known.

There is a significant amount of overlap in the distribution of Caribbean *Lythrypnus* species, however there is no overlap in the distributions of sister taxa except for one species pair- *L. spilus* and *L. elasson*. It is clear that sympatric speciation has not been a driving force in Atlantic *Lythrypnus* diversification. Rather vicariant speciation, driven in large part by the Amazonian barrier, has been a major force in *Lythrypnus* speciation.

There are no apparent barriers separating sister taxa *L. spilus* and *L. elasson*, the only sister taxa to occur in sympatry based on our phylogenetic hypothesis. According to one study they are often collected together and prefer heterogeneous rocky outcrops and vertical walls (Greenfield and Johnson, 1999). One notable difference is the range of distribution. *Lythrypnus spilus* has been recorded as far north as N. Carolina while the northern limit for *L. elasson* is Florida. Taylor and Hellberg (2005) suggest that speciation in marine environments often occurs on smaller geographic scales with no apparent barrier and that factors such as habitat specialization, ecological specialization, or differences in coloration

might lead to assortative mating and ultimately speciation. Though it appears that *L. elasson* and *L. spilus* often occupy similar habitats, their cryptic nature makes intensive studies of microhabitat preferences exceedingly difficult. In order to evaluate factors associated with speciation, a more detailed analysis of habitat preference and general life history would be necessary.

In the Pacific there are two major clades that have been previously described as the *L. dalli* complex (Bussing, 1990) and the *L. rhizophora* complex (Ahnelt and Bohacek, 2004). Our results indicate that both of these assemblages are monophyletic. The tentative inclusion of the remaining Pacific species in the *L. rhizophora* complex by Ahnelt and Bohacek (2004) could not be evaluated with this study, as these species were absent from analysis. These two clades diverged from one another approximately 6.04–9.52 mya, shortly after the divergence of the Atlantic/Pacific clades, approximately 6.49–13 mya. This relatively rapid speciation is likely due, in part, to the approximately concurrent formation of the Galapagos Islands 5–10 mya. Each of these complexes has one species that is endemic to the Galapagos/Isla del Coco. Both the Galapagos and the Isla del Coco harbor a high level of endemic gobies. The islands distance from the mainland is too great for most gobiid larva to disperse (Robertson, 2001), thus populations are effectively isolated from gene flow.

As seen in the Atlantic, sister species in the Pacific do not occur in sympatry. It is interesting that after the initial separation of the two Pacific species assemblages, representatives from each assemblage converged on the same reefs with overlapping distribution patterns. Both clades exhibit species distributed in Southern California, the Gulf of California through Central America, the Isla del Coco, and the Galapagos Islands. This suggests that constraints on larval dispersal and founder effects contributing to speciation patterns were similar in these two clades. The distance between both the Isla del Coco and the Galapagos Islands with the mainland, coupled with larval retention and the low dispersal potential of gobiid larva, may have effectively isolated populations on those islands contributing to speciation (Robertson, 2001; Taylor and Hellberg, 2005). In the *L. rhizophora* clade the Baja California peninsula separates *L. pulchellus* and *L. zebra*. This land mass has been shown to act as an effective barrier, isolating populations and contributing to allopatric speciation in several fishes (Bernardi et al., 2003; Bernardi and Lape, 2005).

4.2. Sexual plasticity

Lythrypnus species display high levels of variation in sex allocation patterns (St. Mary, 2000). This study allows us to infer common ancestry associated with variation in sex allocation patterns in *Lythrypnus* gobies.

St. Mary (2000) described the sex allocation pattern of five *Lythrypnus* species (four of which are included in this phylogeny), and inferred that sex change in both directions is probable for all five species based on similarities in sex allocation patterns and gonad structure. The distribution of these five taxa in the *Lythrypnus* phylogeny indicates that sexual plasticity is ubiquitous throughout the genus. The species that have been described as sexually plastic include representatives from distinct clades in both the Atlantic and Pacific. *Lythrypnus zebra* and *L. spilus* have been described as maintaining a similar, intermediate allocation pattern and are found on opposite sides of the Isthmus of Panama and in different clades. *Lythrypnus dalli* and *L. nesiotes,* described as maintaining two unique strategies are also found on opposite sides of the Isthmus and in different clades. This suggests that the ability to change sex in both directions is most likely a characteristic of all *Lythrypnus* species, and that any *Lythrypnus* species that may be found to display a different strategy is likely to be a derived character state. The basal species *L. heterochroma* has not been characterized with

respect to patterns of sex allocation, these data are necessary to infer whether this novel reproductive strategy is a true synapomorphy, and whether the ancestral state is similar to the four derived taxa that have been described.

Because this phylogeny does not evaluate the relationship of *Lythrypnus* in the larger Gobiidae family, the strategy used by its close relatives is unknown. It has been suggested that the genera *Trimma* and *Priolepis* are closely related to the genus *Lythrypnus*. While both genera differ significantly from *Lythrypnus* with respect to gonad structure, at least three *Trimma* species have been described as bi-directional sex changers, and it has been proposed as likely in at least three *Priolepis* species (Cole, 1990; Manabe et al., 2007; Munday et al., 2010; Sakurai et al., 2009). Additionally, some aspects of gonad morphology in *Trimma* and *Priolepis* are more similar to *Lythrypnus* than other hermaphroditic gobies (Cole, 2010). However, spermatogenic tissue in *Trimma unisquimis* is restricted to the medio-lateral wall of the ovary and expands outward-a pattern that appears to be typical of the entire genus (Cole, 1990, 2010). This represents a striking difference compared to gonad structure in *Lythrypnus* gobies, which have two origins of spermatic tissue on the dorsal and ventral portion of the gonad (Cole, 2010; St. Mary, 2000).

In addition to the ubiquity of sexual plasticity in *Lythrypnus* gobies, data from this study provide further support for St. Mary's hypothesis (1998) that the novel gonad structure seen in *Lythrypnus* is a synapomorphy for the group. However, St. Mary (2000) also proposes that there are at least three distinct sex allocation patterns in *Lythrypnus*. *Lythrypnus dalli* is characterized as maintaining one type with populations consisting of primarily pure females, strongly female-biased hermaphrodites and pure males. *Lythrypnus nesiotes* is described as maintaining a second type consisted primarily of pure females and strongly female-biased hermaphrodites with male-biased individuals (mean allocation to male tissue >75%). *Lythrypnus zebra*, *L. spilus*, and *L. phorellus* are described as exhibiting a third strategy with most individuals characterized as female biased (<10%male tissue), and a significant numbers of individuals with intermediate allocation (10–90% male). We found no evidence for phylogenetic signal, or common ancestry, as the source similarities in the latter sexual strategy. In fact, it is possible that sexual strategies vary among populations within a species, or seasonally, as well as with local density. The relationship between population density and sex change in fishes has been well documented (Caselle et al., 2011; Lutnesky, 1994; Robertson and Gustavo, 1982). One hypothesis for the evolution of bi-directional sex change in fishes is the "risk of movement model" (Avise and Mank, 2009; Munday, 2002; Munday et al., 1998). This model predicts that the risk of movement from one reef to another for small cryptic fishes such as gobies is high. Fitness is therefore increased if an individual maintains the ability to change sex, taking advantage of all reproductive possibilities. Though no direct correlation is made between population size and risk of movement it can be assumed that at lower densities the frequency at which an individual would have to leave an area in search of a mate would be greater when compared to high densities. Populations that are found in lower densities would therefore be expected to maintain a more hermaphroditic strategy in order to decrease time of sex change upon the arrival of a new mate.

The two species with the widest variety of sex allocation types, *L. nesiotes* and *L dalli*, are also found in much higher densities than *L. zebra*, *L. spilus*, and *L. phorellus* (St. Mary, 2000). Though Wiley 1976 did note that *L. zebra* was often as abundant as *L. dalli* in its southern range. Maintaining a proportion of both gonad types is advantageous for quick sex change when faced with uncertain environmental and social conditions. There is no phylogenetic relationship between species exhibiting similar sexual strategies, eliminating an alternative explanation to the hypothesis that there is a correlation between reduced population density and maximum sexual flexibility. Therefore, it remains likely that variation in gonad allocation is density dependent.

The only remaining alternative hypothesis is that the ancestral condition is similar to that observed in *L. zebra*, *L. spilus*, and *L. phorellus*, and that the strategy seen in *L. dalli* and *L. nesiotes* are derived tactics. In order to evaluate this hypothesis, more data on allocation in the basal taxon, *L. heterochroma*, is necessary. In addition experiments directly testing the role of population density on sex allocation in *Lythrypnus* gobies need to be conducted.

4.3. Examination of morphological plasticity and undescribed species

Of the twenty described species of *Lythrypnus*, three are known from a single specimen and were not included in this study. Thirteen described species are included in addition to three undescribed species, Inclusion of the undescribed species provided additional support for the Atlantic/Pacific split and the monophyly of described species complexes were upheld. *Lythrypnus sp2* is known from several specimens collected on Trindade Island and *L. sp1* is known from several specimens collected off the coast of southern Brazil. *Lythrypnus sp3* is known from 3 specimens collected in Venezuela. This study supports the species status of these three undescribed species, they are morphologically different from the three Atlantic species that were not represented in this study, *L. phorellus*, *L. minimus*, and *L. mowbrayi*; and genetically distinct from the 13 taxa included in this study (Fig. 3). These three new *Lythrypnus* species, along with additional undescribed specimens in museum collections indicate that this group is likely much more diverse than the 20 described species.

Lythrypnus species are extremely difficult to identify. This is due, in part, to their small size but also due to the high level of morphological variation that can be found in a single species and the morphological similarity between closely related species. Meristic similarities make color and facial pigmentation important when distinguishing between species (Böhlke and Robins, 1960; Garzon and Acero, 1988; Greenfield, 1988).

In this study, we noticed a high degree of morphological plasticity within species for *L. spilus*, *L nesiotes*, and *L. crocodilus*. Five *L. spilus* individuals, three *L. nesiotes*, and four *L. crocodilus* were sequenced for two loci (*Rag1* and *12s*). Two of the five *L. spilus* individuals were collected in the Bahamas and the remaining three in Panama. While the two populations were morphologically different (e.g. they were originally assigned to different species) they were genetically indistinguishable with 0–2 bp differences between individuals at the *12s* locus. *Lythrypnus crocodilus* and *L. nesiotes* are morphologically very similar, difficult to distinguish from one another, and collected on the same reefs yet are distinct at all molecular loci investigated. Both species have spotty facial patterns. In *L. nesiotes* these are circular and most often form two distinct rows, though sometimes the rows are less uniform and spots appear globular. *Lythrypnus crocodilus* has three dark bands radiating from the eye. Sometimes they are continuous and sometimes broken, somewhat similar to *L. nesiotes*. These slight differences between species pose a problem for identification in the field. All *L. nesiotes* (n = 3) sequenced formed a monophyletic group with sequence differences between 0–2 bp at the *12s* locus. All *L. crocodilus* sequences (n = 4) differed from each other by 5 bp or less. These two species differ from each other by 44 bp. Though similar in facial pattern and morphology, these two taxa are clearly distinct species. It appears that there are two clades within the *L. mowbrayi* complex whose facial patterns may aid in identification in the field. The "bar faced" clade includes *L. crocodilus*, *L. sp 1* and *L. sp 2*. All members of this smaller clade have bar like pattern on their faces. *L. crocodilus* is found in the Carrabin and *L. sp1* and *L. sp2* are found in Brazil. The "spot faced" clade includes *L. nesiotes*

and *L. brasiliensis* are characterized by a pattern of spots on the cheeks. Facial markings appear to be a good character to superficially infer species and might be usual for identifying closely related species in the field.

5. Conclusions

Lythrypnus species have been used as a model for sex determination, and sex change. The novel reproductive tactic used by these fishes and variation within the genus, make them useful as study organisms. Remarkably, this growing body of knowledge has never been interpreted in an evolutionary context, until now. Knowledge of the biogeography, evolutionary history, and distribution of variation in the genus adds depth and context to previous works.

The genus *Lythrypnus* originated in the Atlantic and was separated into two major clades by the formation of the Isthmus of Panama. The distribution of *Lythrypnus* in both the Atlantic and Pacific is intriguing. Though many species overlap in distribution and are often collected together, *L. elasson* and *L. spilus* are the only sister taxa that occur in overlapping ranges. After separation into distinct clades, the *L. rhizophora* complex and the *L. dalli* complex in the Pacific, and the "spot faced" and the "bar faced" clades in the Atlantic, appear to have been shaped by similar environmental pressures and geographic features which caused parallel distribution patterns and species radiation.

In addition to phylogenetics this study evaluated the distribution of variation in sex allocation throughout the genus. Interestingly all major clades and four smaller clades had at least one member that has been described as sexually plastic. It therefore appears that sexual flexibility and bidirectional sex change are ubiquitous throughout the group. While there is variation in sex allocation between species, this study found no trends in variation were inferred by common ancestry. In other words, sexual flexibility appears to have arisen in the stem lineage leading to the genus, or the lineage leading the Atlantic/Pacific split, depending the pattern of sex allocation in the basal taxon, *L. heterochroma*. This still leaves the question of the source of observed variation in the genus. More work needs to be done on the effects of population density on sex allocation to evaluate the hypothesis that density is a contributing factor to differences in sex allocation. It may be that populations that are densely distributed (i.e. more individuals per m^2) exhibit more pure sex biased individuals, whereas populations that are sparsely distributed maintain more intermediate individuals as a hedge-betting tactic to offset the reduced probability of encountering a mate.

Another interesting question that these data will help to evaluate is that of the origin of sexual flexibility in the family Gobiidae. Though the aim of this study was not to evaluate the placement of *Lythrypnus* in the Gobiidae phylogeny, it provides data for future phylogenetic studies and critical data necessary for answering questions about the origin of sexual plasticity in a broader context.

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