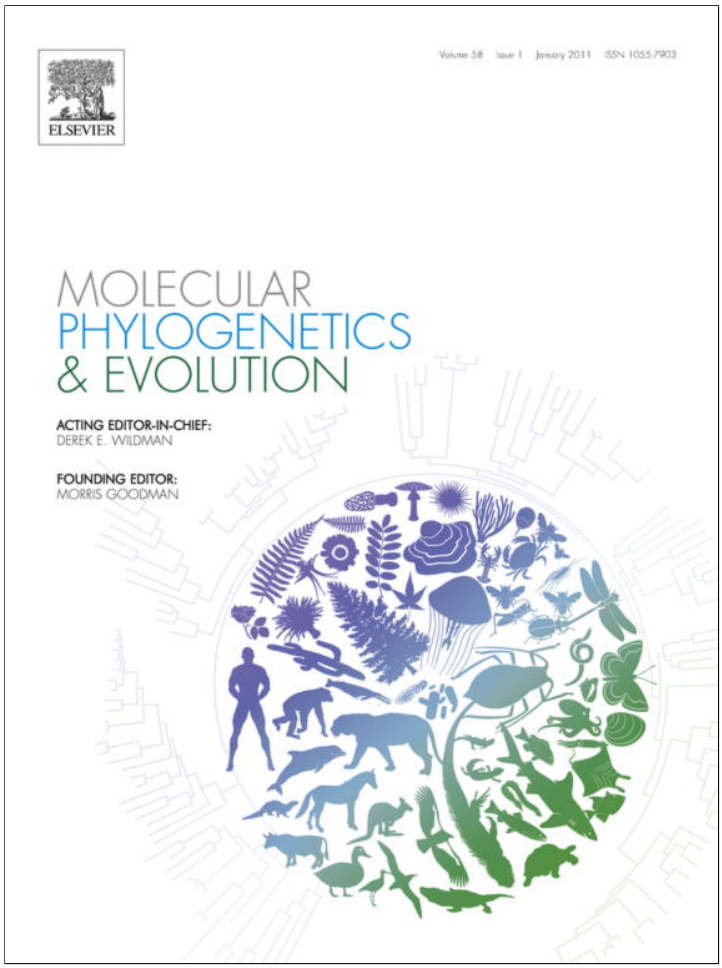

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Species tree estimation and the historical biogeography of heroine cichlids

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ABSTRACT

Heroine cichlids are major components of the fish faunas in both Central America and the Caribbean. To examine the evolutionary patterns of how cichlids colonized both of these regions, we reconstructed the phylogenetic relationships among 23 cichlid lineages. We used three phylogenetically novel nuclear markers (Dystropin b, Myomesin1, and Wnt7b) in combination with sequence data from seven other gene regions (Nd2, Rag1, Enc1, Sreb2, Ptr, Plagl2, and Zic1) to elucidate the species tree of these cichlids. The species examined represent major heroine lineages in South America, Central America, and the Greater Antilles. The individual gene trees of these groups were topologically quite discordant. Therefore, we combined the genetic partitions and inferred the species tree using both concatenation and a coalescent-based Bayesian method. The two resulting phylogenetic topologies were largely concordant but differed in two fundamental ways. First, more nodes in the concatenated tree were supported with substantial or 100% Bayesian posterior support than in the coalescent-based tree. Second, there was a minor, but biogeographically critical, topological difference between the concatenated and coalescent-based trees. Nevertheless, both analyses recovered topologies consistent with the Greater Antillean heroines being phylogenetically nested within the largely Central American heroine radiation. This study suggests that reconstructions of cichlid phylogeny and historical biogeography should account for the vagaries of individual gene histories.

1. Introduction

Molecular phylogenetics has revolutionized how we think about biogeography. Because of their frequent isolation within discrete drainages separated by terrestrial and saltwater environments, freshwater fish have been critical to our understanding of historical biogeography in areas such as Central America and the Caribbean (Rosen, 1975, 1978; Briggs, 1984). The heroine cichlids are one of the dominant freshwater fish groups that inhabit these regions (Miller, 1966). Yet, the phylogenetic relationships among these fish are far from resolved. For instance, previous phylogenetic analyses have provided alternative views on whether the three native cichlid species in the Greater Antilles (GA) are the sister group to or nested within a clade that contains all of the Central American (CA) heroines (Chakrabarty, 2006a,b; Hulsey et al., 2006; Concheiro-Pérez et al., 2007; Higham et al., 2007; Rican et al., 2008; Hulsey et al., 2010a,b; Lopez-Fernandez et al., 2010). One possible reason for this is that previous analyses have relied on concatenated data sets dominated by mitochondrial sequence data. To further explore the evolutionary relationships among heroine cichlids in this dynamic biogeographic region, we examined 10 genes for 23 species in a species tree framework.

The CA cichlid fauna is comprised of over 100 heroine species (Miller, 1966) none of which occur in the GA. Early attempts to resolve the taxonomy of this group resulted in almost all species classified into the genus '*Cichlasoma*' (Kullander, 1983). Efforts have since been made to clarify the taxonomy (Concheiro-Pérez et al., 2007), but without a firm phylogeny to guide taxonomic revision, such attempts are as likely to hinder as aid efforts to communicate evolutionary relationships. The rampant morphological convergence in these species has also made elucidating relationships among these fish difficult (Winemiller et al., 1995; Hulsey, 2006; Hulsey et al., 2008, 2010a,b). A few small monophyletic heroine groups such as *Nandopsis*, that contains the only three cichlid species endemic to the GA, are phylogenetically and geographically well defined (Chakrabarty, 2006b). However, the lack of phylogenetic resolution among most heroine groups is responsible for the continuing ambiguity of relationships for many species like '*Cichlasoma*' *trimaculatum* and '*Cichlasoma*' *salvini*. A robust estimation of phylogeny using multiple genetic loci with sampling of the numerous major heroine groups inhabiting GA, CA, and South America (SA) should provide an evolutionary framework to help resolve taxonomic confusion and more rigorously test historical biogeographic hypotheses for these fishes.

It is clear that the larger heroine clade containing both CA and GA cichlids are nested within the Neotropical radiation of mostly SA cichlids (Farias et al., 2001; Lopez-Fernandez et al., 2010). However, the pathways by which heroine cichlids colonized these two

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regions from SA are uncertain (Fig. 1). This uncertainty is due in part to the fact that the geology of this region is one of the most complicated on earth (Donnelly, 1988). Additionally, the phylogeny for these cichlids remains unresolved. Heroines could have colonized CA via islands in the Caribbean. Islands such as Cuba and Haiti could have provided refuges or stepping-stones for SA heroines to subsequently colonize mainland CA areas. As they drifted east to their current position, the islands in GA are thought to have provided a land-bridge between CA and SA approximately 30 million years ago (Iturralde-Vinent and MacPhee, 1999). Consistent with this hypothesis of a GA land-bridge, a number of phylogenetic studies have recovered a sister group relationship between all major radiations of CA heroine cichlids and the small clade of three *Nandopsis* species that are the only cichlids native to the GA (Hulsey et al., 2006; Concheiro-Pérez et al., 2007). Alternatively, heroines could have invaded CA first and subsequently moved from there to GA islands such as Cuba and Haiti. Because mainland areas tend to have many more species than islands, it is often thought that mainland areas primarily serve as sources of faunas on islands like Cuba and Haiti in the GA (Hedges, 1996; Nicholson et al., 2005). If this were the pathway for the first cichlids dispersing to the GA, we might expect the clade of three GA cichlids to be nested well within a clade of only CA cichlids. Another possibility is that heroines could have invaded CA first and then lineages that either re-invaded or remained in SA could have colonized GA. If GA cichlids were nested within CA heroine lineages but closely related to SA heroine lineages this hypothesis would be viable. A robust phylogeny could help to differentiate among these three alternative biogeographic scenarios.

Interestingly, the three GA cichlids are not the only Caribbean fish that putatively are the sister group to a more species rich CA fish clade. Phylogenetic evidence also indicates that the GA poeciliid genera *Girardinus* and *Quintana* form the sister clade to the majority of CA poeciliid genera (Hrbek et al., 2007). Similarly, sister group relationships exist between synbranchid eels from Cuba and the Yucatán Peninsula (Perdices et al., 2005). There is also a split between fish in the genus *Rivulus* that provides another example of an old sister group divergence between the GA and CA faunas (Murphy et al., 1999). The basal divergence between two extant gars *Atractosteus tristoechus* (from Cuba) and *A. tropicus* (in mainland CA) offers yet another putative example of this biogeographic connection (Wiley, 1976). In combination, these results suggest that large Caribbean islands like Cuba and Haiti might have served as a source for fish groups colonizing CA. Therefore, if these phylogenetic relationships reflect similar routes of fish movement between GA and CA, determining the relationship of *Nandopsis* to all of the heroines in CA could provide further evidence for a general biogeographic pathway for faunistic exchange between CA and GA.

There have been a number of attempts at resolving the broad relationships among heroine cichlids (Roe et al., 1997; Martin and Bermingham, 1998; Hulsey et al., 2004, 2006; Chakrabarty, 2006a; Concheiro-Pérez et al., 2007; Higham et al., 2007; Rican et al., 2008; Lopez-Fernandez et al., 2010). All of these studies were based overwhelmingly on mitochondrial DNA and were analyzed with concatenated sequences. Mitochondrial sequence data is likely not inherently bad at reconstructing phylogenetic relationships. But, the reliance on a single set of linked loci could be generally problematic because incomplete and stochastic sorting of genes is likely ubiquitous (Maddison, 1997). Single gene trees, whether they are estimated using loci from the mitochondrial or nuclear genome, might be expected to deviate substantially from the actual species tree topology (Maddison and Knowles, 2006).

Recently, it has also become clear that analyses that include both nuclear and mitochondrial loci but concatenate the sequence data can lead to inconsistent phylogenetic results and poor estimates of the consensus species tree topology (Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007). New methods that incorporate coalescent theory can account for the discordance between gene trees and potentially provide more robust inference about the true species tree. Like concatenation, coalescent-based approaches do assume that each gene tree represents the relationships between orthologous genes and that there has been no horizontal gene transfer or hybridization between individuals from different species (Maddison and Knowles, 2006). But, unlike concatenation, these methods analyze genetic loci individually and then estimate the species tree from the results of the independent analyses (Heled and Drummond, 2009). These methods potentially offer powerful means to more accurately estimate the species tree. However, it is empirically unclear whether inferences about biogeography of long divergent species derived from concatenated estimation of a species tree often differ substantially from coalescent-based estimates of the species tree.

We examined the phylogenetic relationships among exemplars of major lineages of heroine cichlids from SA, CA, and the GA. We first generated phylogenies from 10 loci that were assumed to be physically unlinked in the genome and that therefore should represent independent estimates of the species phylogeny. To obtain the species tree for these species, we utilized both concatenation of sequences from the ten gene regions and *BEAST, a recently developed coalescent-based approach to reconstruct the species tree (Heled and Drummond, 2009). This allowed us to compare the phylogenies from these two approaches of combining gene regions and provided a robust framework to ask if the trees were more consistent with any of the three hypothesized scenarios of how heroine cichlids first colonized both GA and CA.

2. Materials and methods

2.1. DNA Isolation and sequencing

Specimens sequenced included cichlids that were both collected from the wild and obtained commercially (Table 1). Gene sequences from one mitochondrial and nine nuclear gene regions were obtained from these 23 Neotropical cichlid lineages (Table 2). The species *Nandopsis tetracanthus* from Cuba and *N. haitiensis* from

Fig. 1. The possible colonization paths (shown with arrows) that heroine cichlids could have taken to invade the Greater Antilles (GA) and Central America (CA) from South America (SA). If (A) the cichlids in the GA are sister to all the cichlids in CA, it is feasible that heroines first invaded GA (A1) and then moved from there to CA (A2). Alternatively (B), heroines could have invaded CA first (B1) and then subsequently moved from there to GA (B2). Or (C), heroines could have invaded CA first and lineages that either re-invaded or remained in SA could have colonized GA.

Haiti that represent two of the three named species of *Nandopsis* were examined. The only cichlid that occurs in the GA that was not included in our analysis was *N. ramsdeni*, but this species, that is native to eastern Cuba, has been recovered as sister to *N. tetracanthus* (Chakrabarty, 2006a). Therefore, our inclusion of two-thirds of the GA cichlid species and their recovery as a monophyletic group in all previous analyses suggest our sampling approach for the GA should be phylogenetically and geographically robust. Our remaining specimens largely represented exemplars from most of the currently named genera of heroine cichlids. Three SA species including *Cichla temensis* and two heroines, *Hoplarchus psittacus* and *Heros* sp., were sequenced to serve as outgroups as they have been repeatedly recovered outside of the largely CA heroine clade (Farias et al., 2001; Smith et al., 2008; Lopez-Fernandez et al., 2010).

Sequences of the mitochondrial Nd2 (NADH dehydrogenase 2) gene were generated using primers in (Kocher et al., 1995) and were analyzed for all of these species as a contrast to the cytochrome b (cyt b) gene analyzed in previous studies. The protein coding genes Enc1 (ectodermal-neural cortex 1), Plagl2 (pleomorphic adenoma gene-like 2), Ptr (protease III), Rag1 (recombination-activating gene 1), Sreb2 (superconserved receptor expressed in brain 2), and Zic1 (zinc finger protein of the cerebellum 1) were sequenced using primers in Li et al., (2007). Sequences for Wnt7b (wingless int 7b) were generated from primers in Fraser et al., (2009). The Dystrobrevin1b (Dystb) and Myomesin1 (Myom) nuclear gene partitions were sequenced using degenerate primers designed from alignments of conserved sections of each gene (Dystrobrevin 1: ENSGACT00000015081; Myomesin 1: ENSGACG00000004209) in the stickleback, *Gasterosteus aculeatus*, and shotgun sequencing of Lake Malawi cichlids described in Loh et al., (2008). The primers designed to amplify the Dystb gene region were Dystbfor 5′-GCGCATTGCAGACTTTGATCT-3′ and Dystbrev 5′-TGCTGCTGTTKCCAGATGCCTAAT-3′. The primers used to amplify Myom were Myomfor 5′-CCTCTGACAAAGACGTAGGCTG and Myomrev 5′-CTTCCAGACTCACATTGTTGAT-3′. Primers for these three phylogenetically novel nuclear loci were generated from conserved protein-coding regions. Therefore, the sequences for each locus were expected to contain some protein coding sequence but were considered as possibly spanning intron sequence as well.

For sequencing, total genomic DNA was isolated from fin clips. Amplifications were carried out in an Eppendorf DNA thermocycler using standard methods. Thermal cycling conditions for all regions sequenced consisted of an initial hot start of 94 °C (2 min), then 35 cycles of 94 °C (1 min), 54 °C (1 min) and 72 °C (1 min). A final incubation of 72 °C for 4 min was added to ensure complete extension of amplified products. Subsequently, the PCR products were electrophoretically separated from unincorporated primers and dNTPs using electrophoresis in low melting point agarose gel with ethidium bromide (1 mg/μl) added and run in Tris–acetate buffer (pH 7.8). Positively amplified DNA was then purified using an enzymatic combination of 1 μl of Exonuclease I (10.0 U/μl) and 1 μl shrimp alkaline phosphatase (2.0 U/μl) per 10 μl of PCR product. The PCR products were used as templates for sequencing reactions performed at the High-Throughput DNA Sequencing Facility at the University of Washington. Complete gene sequences were assembled from sequencing reactions generated for both forward and reverse primers using the program Sequencher version 4.1(Gene Codes, Ann Arbor, MI). For analyses, sequences were aligned using Clustal X (Thompson et al., 1997) and codon positions were defined using MacClade 4.0 (Maddison and Maddison, 2000). All sequences are available on Genbank (Table 2).

Table 1
The species examined, their accession numbers in the University of Tennessee Fish Collection (UTFTC), and their collection locality. The native region that species are endemic to is given for all commercially obtained specimens. Species were obtained both commercially and collected from the wild. State names are given for collections from Mexico in order to clarify localities.

Species	UTFTC	Collection locality
'Cichlasoma' pearsei	2874	Rio San Pedro, Chiapas, Mexico
'Cichlasoma' salvini	2607	Rio Sarabia, Veracruz, Mexico
'Cichlasoma' istlanum	2266	Commercial (endemic to CA)
'Cichlasoma' trimaculatum	2759	Rio de los Perros, Oaxaca, Mexico
'Cichlasoma' uropthalmus	2369	Lago de Illusiones, Tabasco, Mexico
Astatheros macracanthus	2602	Rio de los Perros, Oaxaca, Mexico
Caquetaia kraussi	2551	Commercial (endemic to SA)
Cichla temensis	2158	Cinaruco River, Venezuela
Herichthys cyanoguttatus	2443	Rio Conchos, Tamaulipas, Mexico
Herichthys labridens	2688	Media Luna, San Luis Potosi, Mexico
Heros sp.	2157	Cinaruco River, Venezuela
Hoplarchus psittacus	2161	Cinaruco River, Venezuela
Nandopsis haitiensis	2267	Commercial (endemic to GA)
Nandopsis tetracanthus	2178	Commercial (endemic to GA)
Parachromis managuense	1059	Lago Yojoa, Honduras
Paraneetroplus bulleri	2775	Rio Sarabia, Veracruz, Mexico
Petenia splendida	2374	Rio Candelaria, Campeche, Mexico
Rocio octofasciata	2789	Rio Tonala, Veracruz, Mexico
Theraps irregulare	2438	Commercial (endemic to CA)
Thorichthys ellioti	2841	Lago Catemaco, Veracruz, Mexico
Thorichthys meeki	2137	Lago de Illusiones, Tabasco, Mexico
Vieja bifasciata	2033	Rio Grijalva, Chiapas, Mexico
Vieja hartwegi	2537	Rio Grijalva, Chiapas, Mexico

2.2. Gene tree reconstruction

For the phylogenetic analyses, the protein coding gene regions were partitioned into three codon sites and introns were treated as a single partition. ModelTest 3.06 (Posada and Crandall, 1998) was used to identify the best model of molecular evolution for each partition. Bayesian analyses were executed to find approximations of the maximum likelihood tree using MrBayes 3.0 (Ronquist and Huelsenbeck, 2003). We ran three separate Bayesian analyses of each of the ten gene regions for 1,000,000 generations with four Markov chains in each run. We sampled trees from the Markov Chain Monte Carlo (MCMC) search algorithm every 1000 generations. At the end of each analysis, the log-likelihood scores were plotted against generation time to identify the point at which log likelihood values reached a stable equilibrium. Because the log likelihood values appeared to reach a plateau after 50,000 generations for all loci, the sample points prior to generation 100,000 in each run were discarded as burn-in samples. The remaining samples from the three independent runs for each gene were used to produce a majority rule consensus tree representation of the 10 individual gene trees.

2.3. Species tree reconstruction

To generate a species phylogeny from the individual gene trees, we first analyzed the data as a single concatenated dataset in Mr. Bayes 3.0 (Ronquist and Huelsenbeck, 2003). For the concatenated data set, each gene region was partitioned by codon position for exons and single partitions for introns, with optimal models of molecular evolution assigned for each partition. The concatenated data set was run using the same conditions and models as for the individual gene trees. For the concatenated analyses, we ran four separate Bayesian analyses for 10,000,000 generations with four Markov chains in each run. We sampled trees from the Markov Chain Monte Carlo (MCMC) search algorithm every 1000 generations. A plot of the likelihood values versus generation for these concatenated phylogenies suggested likelihood values reached a stationary distribution after 800,000 generations. Therefore, we discarded the first 1,000,000 generations as burn-in samples. To ensure the searches had adequately converged, we also examined

Table 2
The species examined and their Genbank numbers for the 10 genes sequenced. Genbank numbers of previously generated sequences are provided in full. Only the last three of the Genbank accession numbers generated for this study are reported as they all began with HQ427-.

Species	Nd2	Dystb	Myom	Wnt7b	Rag1	Enc1	Sreb2	Ptr	Plagl2	Zic1
'Cichlasoma' pearsei	GU946264	−554	−531	−646	GU595408	−577	−669	−623	−600	−692
'Cichlasoma' salvini	GU946259	−548	−525	−640	GU595402	−571	−663	−617	−594	−686
'Cichlasoma' istlanum	−489	−541	−518	−633	−500	−564	−656	−610	−587	−679
'Cichlasoma' trimaculatum	GU946241	−544	−521	−636	GU595403	−567	−659	−613	−590	−682
'Cichlasoma' uropthalmus	GU946237	−555	−532	−647	−509	−578	−670	−624	−601	−693
Astatheros macracanthus	GU946244	−533	−510	−625	−496	−556	−648	−602	−579	−671
Caquetaia kraussi	GU946246	−535	−512	−627	GU595400	−558	−650	−604	−581	−673
Cichla temensis	−490	−549	−526	−641	−504	−572	−664	−618	−595	−687
Herichthys cyanoguttatus	GU946250	−536	−513	−628	−498	−559	−651	−605	−582	−674
Herichthys labridens	GU946251	−543	−520	−635	−502	−566	−658	−612	−589	−681
Heros "common sp."	−491	−550	−527	−642	−505	−573	−665	−619	−596	−688
Hoplarchus psittacus	−492	−551	−528	−643	−506	−574	−666	−620	−597	−689
Nandopsis haitiensis	GU946254	−538	−515	−630	GU595405	−561	−653	−607	−584	−676
Nandopsis tetracanthus	GU946263	−553	−530	−645	GU595406	−576	−668	−622	−599	−691
Parachromis managuense	−493	−546	−523	−638	GU595411	−569	−661	−615	−592	−684
Paraneetroplus bulleri	GU946260	−545	−522	−637	−503	−568	−660	−614	−591	−683
Petenia splendida	GU946261	−547	−524	−639	GU595412	−570	−662	−616	−593	−685
Rocio octofasciata	GU946252	−537	−514	−629	GU595401	−560	−652	−606	−583	−675
Theraps irregulare	GU946257	−540	−517	−632	GU946257	−563	−655	−609	−586	−678
Thorichthys ellioti	−495	−542	−519	−634	−501	−565	−657	−611	−588	−680
Thorichthys meeki	−494	−552	−529	−644	−507	−575	−667	−621	−598	−690
Vieja bifasciata	HQ529492	−534	−511	−626	−497	−557	−649	−603	−580	−672
Vieja hartwegi	HQ529493	−539	−516	−631	−499	−562	−654	−608	−585	−677

the effective sample size of the likelihoods of the phylogenies remaining post-burn using Tracer v1.5 (Drummond and Rambaut, 2007). Generally, values over 200 for the effective sample size is considered adequately converged (Drummond and Rambaut, 2007). The samples remaining after the burn-in was removed from the four runs were then used to produce a majority rule consensus tree that was subsequently rooted with *Cichla temensis*.

To estimate the coalescent-based species tree, all ten phylogenetic partitions were analyzed simultaneously using *BEAST (Heled and Drummond, 2009). The program *BEAST is a coalescent-based Bayesian method that determines the likelihoods of gene trees in a given species tree to find the most likely containing species tree. Each gene region was partitioned as for the analyses above. The topologies for the partitions, codon sites + introns (if present), in each of the 10 individual genetic regions were then linked in the program BEAUti (Drummond and Rambaut, 2007). The ModelTest 3.06 estimated shape and transition parameters for each region were likewise designated in BEAUti. All molecular clock estimates for the gene regions were examined using the uncorrelated lognormal model. The ploidy level of Nd2 was analyzed as "mitochondrial" and the remaining genes were designated as the default "nuclear autosomal". A preliminary analysis of 20,000,000 generations did not converge. Therefore, the analysis was run five times for 100,000,000 generations each to ensure convergence of the analysis with trees sampled every 1000 generations. The first 30,000,000 generations from each run were discarded as burn-in samples after ensuring the likelihood scores reached a plateau observed using the program Tracer v1.5 (Drummond and Rambaut, 2007). We also examined the effective sample size of the likelihoods of the phylogenies remaining post-burn using Tracer v1.5 (Drummond and Rambaut, 2007). The posteriors from all five runs were then combined to provide the consensus estimate of the coalescent-based species tree. For all individual genes, the concatenated, and the coalescent phylogenetic analyses, posteriors above 95% were considered as evidence for substantial support at a node.

3. Results

All newly generated sequences have been submitted to Genbank (HQ427489–HQ427693). The protein coding loci Nd2 (1047 bp), Enc1 (810 bp), Plagl2 (672 bp), Ptr (705 bp), Rag1 (1013 bp), Sreb2 (987 bp), and Zic1 (849 bp) were monomorphic in their sequence lengths. The loci Dystb (404–408), and Wnt7b (568–672) showed limited variability in their sequence lengths. The Dystb sequence was all non-coding. The first 210 bp of the Wnt7b region is a protein-coding region that was followed by approximately 360 bp of intron. The gene Myom (394–636) was highly variable largely because the species *Nandopsis uropthalmus* exhibited a large (~100 bp) indel that was not present in other species. Two protein-coding regions totaling 210 bp in length bracketed this Myom intron region.

The Nd2 mitochondrial gene exhibited the greatest amount of sequence divergence of any of the genetic loci examined. However, the three new loci examined here for the first time in a phylogenetic context, Dystb, Myom, and Wnt7b, exhibited greater uncorrected sequence divergence than any of the other nuclear loci examined (Table 3). For three of the 10 gene regions, a *Nandopsis* species was one of the pair of heroine species that showed the greatest amount of pair-wise sequence divergence. However, for five of the comparisons either *Astatheros macracanthus* or *Rocio octofasciata* were one of the two heroine species that exhibited the greatest amount of sequence divergence.

None of the gene trees produced strictly bifurcating topologies and generally the gene trees were incongruent (Fig. 2). However, a few relationships were recovered consistently in most of the gene trees. *Herichthys cyanoguttatus* and *H. labridens* were never supported as paraphyletic with posterior support. In every gene tree except Ptr, *Nandopsis haitiensis* and *N. tetracanthus* were found to be monophyletic. Similarly, the sister grouping between *Astatheros macracanthus* and *Rocio octofasciata* was only supported as paraphyletic with posterior support in the Wnt7b and Sreb2 gene trees. *Thorichthys meeki* and *T. ellioti* were similarly recovered together or closely related except in the Myom, Rag1, and Sreb2 gene trees.

Several other relationships were present in most of the gene trees. For example, *'Cichlasoma' istlanum* and *'C.' trimaculatum* often grouped with each other as well as with *Parachromis managuense*, *Petenia splendida*, and *'C.' uropthalmus*. The species *'Cichlasoma' pearsei*, *Theraps irregulare*, *Vieja hartwegi*, and *V. bifasciata* also were commonly found together in many of the gene trees. *Hoplarchus psittacus* was frequently recovered outside of the CA heroines. *Heros* sp. was supported (>50% posteriors) as sister to the remaining

Table 3
Maximum percent sequence divergence for each of the 10 genetic loci used to construct phylogenetic relationships. The species *Cichla temensis*, *Hoplarchus psittacus*, and *Heros* sp., were not included in the below measurements of maximum sequence divergence.

Gene region	Maximum% sequence divergence	Maximally diverged species
Nd2	16.8	*Caquetaia kraussi* vs. *Thorichthys ellioti*
Dystb	3.2	*Parachromis managuense* vs. *Herichthys labridens*
Myom	3.0	*Herichthys labridens* vs. *Astatheros macracanthus*
Wnt7b	2.8	*Thorichthys ellioti* vs. *Rocio octofasciatum*
Enc1	1.9	*Nandopsis haitiensis* vs. *Astatheros macracanthus*
Rag1	1.7	*Caquetaia kraussi* vs. *Theraps irregulare*
Sreb2	1.4	*Parachromis managuense* vs. *Nandopsis tetracanthus*
Plagl2	1.3	*Thorichthys meeki* vs. *Rocio octofasciatum*
Ptr	1.3	*Petenia splendida* vs. *Rocio octofasciatum*
Zic1	0.9	*Thorichthys ellioti* vs. *Nandopsis tetracanthus*

heroines only in the Nd2 and Dystb gene trees. *Caquetaia kraussi* and *'Cichlasoma' salvini* had different affinities in almost every gene tree. None of the gene trees, including the mitochondrial gene Nd2, recovered *Nandopsis tetracanthus* and *N. haitiensis* as the sister group to the entire clade of CA heroines. No single set of phylogenetic relationships was recovered in every single one of the 10 gene trees.

The concatenated tree recovered a strictly bifurcating set of generally strongly supported relationships for all of the CA heroines (Fig. 3). The likelihood scores for the first concatenated tree search had an effective sample size of 1914.5 suggesting the MCMC algorithm was effectively searching tree space. Virtually every single node except those at the base of the CA heroine clade were supported with very high (>95%) to 100% Bayesian posteriors. The two *Thorichthys* species formed a monophyletic clade that grouped with *C. salvini*. The two monophyletic *Herichthys* species were sister to a clade composed of '*Cichlasoma' pearsei*, *Paraneetroplus bulleri*, *Theraps irregulare*, and the two *Vieja* species. *Heros* sp. was recovered as the sister to the entire clade when *Cichla temensis* was utilized as the outgroup. Although it was only supported with 83% posterior probability support, *Astatheros macracanthus* and *Rocio octofasciata* were recovered as sister to the entire clade of CA heroines that included *Nandopsis*. The GA *Nandopsis* were recovered as sister to *Parachromis managuense*, *Petenia splendida*, *'Cichlasoma' uropthalmus*, *C. istlanum*, and C. *trimaculatum*. Albeit with not exceedingly high support (84% posterior probability), all of these species were found to be sister to the SA heroine species *Caquetaia kraussi*.

The likelihood scores for the first coalescent tree search had an effective sample size of 246.5 suggesting the *BEAST analysis effectively searched the available tree space. The coalescent tree was very similar to the concatenated tree (Fig. 3), but there was one major topological difference. In the coalescent tree, the two *Nandopsis* species were nested outside of the clade that included species such as *Petenia splendida* and *Parachromis managuense* as well as *Caquetaia kraussi*. Also, although a number of the relationships in the coalescent tree were supported with 100% Bayesian posteriors, node support values for many nodes in this phylogeny were less than 90% and tended to be lower than that found in the concatenated analysis. For example, *Astatheros macracanthus* and *Rocio octofasciata* were again recovered as sister to the entire clade of CA heroines, but only with 74% posterior probability. Likewise, only 52% of the phylogenies supported the placement of *Nandopsis* in this coalescent tree reconstruction. However, *Nandopsis* was clearly not recovered as the sister group to all CA heroines.

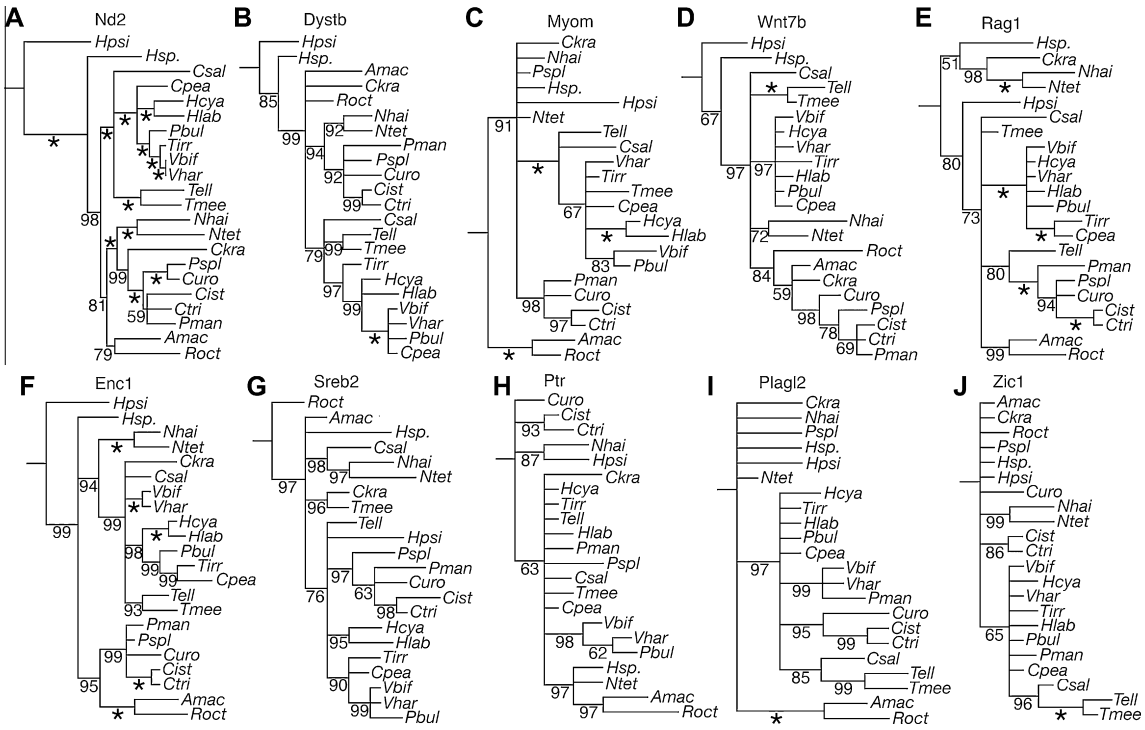

Fig. 2. The 10 individual gene trees recovered for the 23 cichlid species examined. The gene trees for the mitochondrial Nd2 (A) gene and 9 gene regions from the nuclear genome Dystb (B), Myom (C), Wnt7b (D), Rag1 (E), Enc1 (F), Sreb2 (G), Ptr (H), Plagl2 (I), and Zic1 (J) are depicted. The names of all species examined were shortened to save space. The two GA heroines examined were *Nandopsis haitiensis* (*Nhai*) and *N. tetracanthus* (*Ntet*). The species *Caquetaia kraussi* (*Ckra*) is native to SA. The CA heroines examined include *Astatheros macracanthus* (*Amac*), '*Cichlasoma*' salvini (*Csal*), '*C.*' *uropthalmus* (*Curo*), '*C.*' *pearsei* (*Cpea*), '*C.*' *istlanum* (*Cist*), '*C.*' *trimaculatum* (*Ctri*), *Herichthys cyanoguttatus* (*Hcya*), *H. labridens* (*Hlab*), *Parachromis managuense* (*Pman*), *Paraneetroplus bulleri* (*Pbul*), *Petenia splendida* (*Pspl*), *Theraps irregulare* (*Tirr*), *Thorichthys ellioti* (*Tell*), *T. meeki* (*Tmee*), *Vieja bifasciata* (*Vbif*), and *V. hartwegi* (*Vhar*). The SA heroine species *Hoplarchus psittacus* (*Hpsi*) and *Heros* sp. (*Hsp.*) are generally considered out-groups to the CA and GA heroine cichlids. *Cichla temensis* was not depicted because of the long branches between it and the other species. Posterior probabilities for clades are depicted at the node. Clades recovered in 100% of the post-burn-in trees are represented with an asterisk (*).

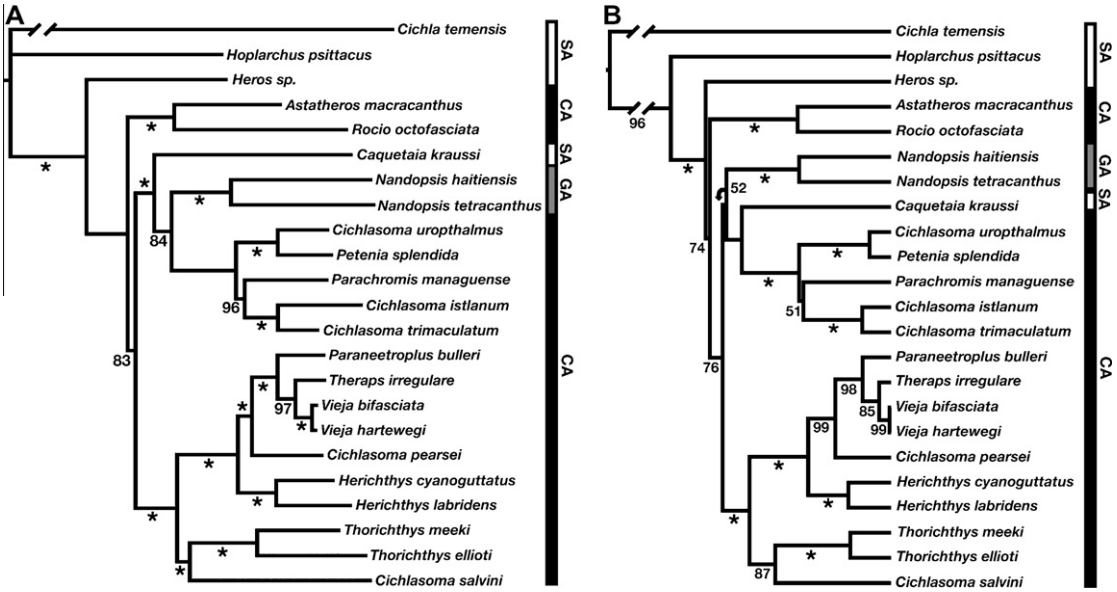

Fig. 3. The concatenated (A) and coalescent-based species trees (B). Posterior probabilities for clades are depicted at the node and an asterisk (*) was depicted at nodes with 100% support. The long recovered branch lengths leading to *Cichla temensis* were graphically shortened. Colored blocks were used to illustrate the three large geographic regions the heroine cichlids inhabit: South America (SA; white), Central America (CA; black), and the Greater Antilles (GA; gray).

4. Discussion

The individual gene trees of the CA heroines were generally discordant. None of the analyses of single gene trees recovered identical consensus topologies. The many factors influencing the discordant gene topologies among these cichlid species should be examined in more detail. However, because of the stochastic sorting of nuclear genes, we should not expect numerous gene trees of a diverse group like the CA heroines to be identical (Maddison, 1997). The three new genetic loci examined here for the first time for cichlids in a phylogenetic context, Dystb, Myom, and Wnt7b, exhibited greater sequence divergence than any of the other genetic partitions examined (Table 3). This is largely due to the fact that each region included intron sequence. Importantly, the variability present in these markers suggests they could be useful in future studies of cichlid phylogenetics.

Mitochondrial genes such as Cytb have been fundamental to our molecular understanding of heroine phylogenetic relationships to date. This may be problematic for several reasons. Some have suggested that mitochondrial genes introgress more readily than nuclear genes (Chan and Levin, 2005; Bachtrog et al., 2006; Bossu and Near, 2009). This could be true, but often this inference is based largely on discordance between mitochondrial and nuclear genes (Mims et al., 2010). Our results and those of others suggest there is likely to be substantial phylogenetic discordance between any two genomically unlinked loci (White et al., 2009). Therefore, whether the mitochondria are particularly poor at resolving phylogenetic questions is unclear. However, it is clear that phylogenetic analyses including only a few nuclear loci concatenated with genes like Cytb have likely recovered topologies disproportionately influenced by mitochondrial divergence and gene trees. Mitochondrial genes like Cytb as well as Nd2 exhibit approximately 10 times greater variability than nuclear loci containing similar numbers of base pairs (Table 3). As the number of variable nuclear genes utilized in phylogenetic studies increases, it will be feasible to evaluate whether mitochondrial data concatenated with nuclear genes have greatly mislead our understanding of phylogenetic relationships within groups such as heroine cichlids.

Interestingly, the topological differences between our concatenated and coalescent-based phylogeny were relatively minor (Fig. 3). However, the overwhelming support for virtually every node in the concatenated tree is likely providing inflated posteriors for the relationships recovered using this approach (Kolaczkowski and Thornton, 2009). The phylogenetic variation among the individual gene trees certainly does not instill this level of confidence (Fig. 2). The coalescent-based species tree assembled from the gene trees using *BEAST provides less support for many of the relationships recovered, and these posteriors could more accurately reflect the level of uncertainty in these nodes (Leaché, 2010). Further analyses of the phylogenetic relationships among these and other CA heroines with additional genetic loci are clearly going to be necessary if we want to assemble an unambiguous species tree and stabilize the taxonomy of this group.

Although there were substantial similarities, the two species trees obtained here do differ topologically from the most recent densely sampled concatenated phylogeny of CA heroines (Lopez-Fernandez et al., 2010). In neither of our trees were *Nandopsis* and *Caquetaia* recovered as sister groups. '*Cichlasoma*' *salvini* was found here to have close affinities with *Thorichthys* as was originally indicated in several studies using only cytochrome *b*. However, in both Lopez-Fernandez et al., (2010) and Chakrabarty (2006a), the species '*C.*' *salvini* groups with the amphilophine heroines such as *Petenia splendida*. Nevertheless, most of the relationships recovered in our analyses were similar to these previous studies. This similarity leaves open the question of whether deep coalescence is a process that is highly problematic for estimating a phylogeny in groups such as the heroine cichlids that likely diverged on the order of 15–30 million years ago (Hulsey et al., 2010a,b).

Generally, coalescent methods likely perform better when multiple sequences from multiple individuals within a species are utilized to reconstruct phylogenies (Maddison and Knowles, 2006). The use of multiple sequences from multiple individuals provides more robust estimates of intraspecific polymorphism and effective population sizes that are important in determining how genes coalesce among lineages (Degnan and Rosenberg, 2006). Although we utilized multiple loci, our study utilized only one individual per species and this could have limited the effectiveness of *BEAST in reconstructing the best estimate of the species tree. However, the individual gene tree phylogenies (Fig. 2) suggest that a coalescent

approach that explicitly incorporates the independent sorting of gene trees while reconstructing the species trees is likely necessary in order to recover the best estimate of the heroine species phylogeny. Additionally, despite our lack of intraspecific sampling, our exploration of the coalescent-based species tree provided an informative contrast to the concatenated species tree. In the future, it would be interesting to compare coalescent-based heroine phylogenies generated using a single individual per species to phylogenies reconstructed with multiple individuals per species.

Despite methodological differences, there were several relationships that were recovered in both the concatenated and coalescent-based methods. For instance, the Cuban cichlid *Nandopsis tetracanthus* and the Hispaniolan cichlid, *N. haitiensis* are clearly recovered as sister groups (Fig. 3). This suggests heroine cichlids likely only invaded the GA one time especially if *N. ramsdeni* that was not examined in this study is the sister species to *N. tetracanthus* (Chakrabarty, 2006a). Furthermore, the phylogenetic placement of the *Nandopsis* species could have important implications for estimating the timing of diversification of all heroine cichlids. The only known heroine cichlid fossil is *Nandopsis woodringi* (Cockerell, 1923), and it is found on the Caribbean island of Hispaniola (Haiti). This fossil is likely from the upper or middle Miocene (minimum age ~15 million years) (Tee-Van, 1935; Chakrabarty, 2006b). This fossil could be useful as a calibration for the minimum age of divergence between *N. tetracanthus* and *N. haitiensis*. The geological separation of Cuba and Haiti between 20 and 25 million years ago might also be used to calibrate their divergence (Chakrabarty, 2006a). These external age estimates for *Nandopsis* could be useful to estimate the age of all of the heroine cichlids especially if the placement of this genus within the larger CA heroine phylogeny can be further resolved.

The *Nandopsis* clade was not placed as the sister group to all other CA heroines. None of the 10 gene trees examined here had strong support for this relationship (Fig. 2). Despite Nd2 being linked physically to Cytb in the mitochondrial genome, Nd2 did not even resolve the two *Nandopsis* as sister to all other cichlids in the CA heroine clade. This could indicate that the results from Cytb are simply due to the vagaries in this genetic partition or problems in how phylogenetic algorithms reconstruct the position of the GA clade using this gene. Regardless, the clade that was composed of *Rocio octofasciata* and *Astatheros macracanthus* was recovered in both species trees as the sister group to the clade that includes the GA cichlids, the SA species *Caquetaia kraussi*, and the remaining representatives of the CA heroines. This phylogenetic result is most consistent with heroine cichlids invading CA before they invaded the islands of the Caribbean. However, if one considers 95% posterior support in a concatenated Bayesian analysis as strong support for a clade's position in a phylogeny, we did not recover overwhelming support for this relationship. Likewise the coalescent based reconstruction only recovered 74% of phylogenies with *Rocio octofasciata* and *Astatheros macracanthus* as sister to the remaining CA + GA heroines and *Caquetaia.*

Interestingly, the close relationships of *Nandopsis* and *Caquetaia* found in both the coalescent and concatenated species trees leaves open the possibility that the GA did serve as a stepping stone between SA and CA for a subset of heroine cichlids examined here. Based on our results, *Nandopsis* could have arrived in Cuba and Haiti via SA and subsequently given rise to the clade containing a subset of CA heroines like *Petenia splendida* and *Parachromis managuense*. However, our phylogenetic results are also consistent with the lineage that gave rise to *Nandopsis* originating in CA, colonizing the GA and then finally moving into SA.

The complex geology of Central America and the Caribbean has likely structured the biogeography of groups like heroine cichlids that occur in CA, SA, and GA (Rosen, 1978; Donnelly, 1988; Hedges, 1996). Often, our understanding of geology and the distributions of organisms can be used to reciprocally illuminate our understanding of one another. The GA could have provided a land-bridge between CA and SA approximately 30 million years ago (Iturralde-Vinent and MacPhee, 1999). However, geologists remain uncertain about both the timeframe and movement of many land masses in this region such as Cuba and the Chortis block that underlies CA (Keppie and Morán-Zenteno, 2005). Understanding the phylogenetic placement of *Nandopsis*, the only cichlid fishes endemic to GA, is important to understanding both the biogeography of other groups as well the geology of this region. Our results do not support the hypothesis that *Nandopsis* is the progenitor of all CA heroines. However, in order to more rigorously test the biogeographic pathways fish and other organisms used to colonize both the GA and CA, we will need more thorough geologic reconstructions of this region, further phylogenetic analyses, and better estimates of the timeframe of divergence for many groups including heroine cichlids.

A transition from single gene to multi-gene analyses in molecular phylogenetic analyses (Maddison and Knowles, 2006; White et al., 2009) and reconstructions of historical biogeography is well underway. With the availability of fully sequenced genomes, the ability to examine numerous nuclear loci should no longer be limiting in phylogenetic studies (Hulsey, 2009). The incorporation of more genomically independent partitions will also allow more biologically realistic models of molecular evolution to be incorporated into phylogenetic studies. This is important because the utilization of multiple loci in the fields of molecular ecology and phylogeography has revealed the need to more accurately model the relationships among gene trees at different loci (Pluzhnikov and Donnelly, 1996; Felsenstein, 2006; Maddison and Knowles, 2006). However, the optimal combination of sequencing strategy and biological parameterization for resolving deeper phylogenetic questions is not yet clear. Because of the variability we recovered among the phylogenetic inferences drawn from the 10 single gene regions examined in the heroine cichlids, accounting for discordance in gene histories seems critical. Our understanding of heroine phylogeny and the biogeographic patterns inferred from these trees will remain incomplete if we do not account for different patterns of gene coalescence when reconstructing species trees.

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