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Phylogenetic relationships of Middle American cichlids (Cichlidae, Heroini) based on combined evidence from nuclear genes, mtDNA, and morphology

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ABSTRACT

Heroine cichlids are the second largest and very diverse tribe of Neotropical cichlids, and the only cichlid group that inhabits Mesoamerica. The taxonomy of heroines is complex because monophyly of most genera has never been demonstrated, and many species groups are without applicable generic names after their removal from the catch-all genus *Cichlasoma* (sensu Regan, 1905). Hence, a robust phylogeny for the group is largely wanting. A rather complete heroine phylogeny based on *cytb* sequence data is available [Concheiro Pérez, G.A., Říčan O., Ortí G., Bermingham, E., Doadrio, I., Zardoya, R. 2007. Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae) based on sequences of the cytochrome *b* gene. Mol. Phylogenet. Evol. 43, 91–110], and in the present study, we have added and analyzed independent data sets (nuclear and morphological) to further confirm and strengthen the *cytb*-phylogenetic hypothesis. We have analyzed a combined *cytb*-nuclear (RAG1 and two S7 introns) data set of 48 species representing main heroine lineages to achieve further resolution of heroine higher taxonomic levels and a combined *cytb*-morphological data set of 92 species to stabilize generic taxonomy. The recovered phylogenies supported the circumamazonian—CAM—Heroini (sensu Concheiro Peréz et al., 2007) as a monophyletic group, that could be divided into six main clades: (1) australoheroines (the southernmost heroine genus *Australoheros*), (2) nandopsines (the Antillean genus *Nandopsis*), (3) caquetaines (including the north western Amazonian genera *Caquetaia* and *Heroina*), (4) astatheroines (including *Astatheros*, *Herotilapia* and *Rocio*), (5) amphilophines (including *Amphilophus* and related genera), and (6) herichthyines (including *Herichthyis* and related genera). Nuclear and mitochondrial data partitions arrived at highly congruent topologies. Suprageneric relationships were influenced mainly by the nuclear signal, as well as the most basal phylogenetic position of *Australoheros* within CAM heroines. The new phylogeny of the tribe Heroini provides robust framework to stabilize the taxonomy of the group and for future comparative studies on these morphologically and ecologically diverse freshwater fishes. Morphology was mostly informative at the genus level and aid in determining the monophyly and composition of heroine genera. Upon acceptance of all putative genera, as recovered in this study, the Heroini would be with 35 genera the most genus-rich clade of Neotropical cichlids.

1. Introduction

Heroine cichlids are secondary freshwater fishes that constitute an important component of the Neotropical fish fauna, especially in Mesoamerica, where they make up some 25% of the freshwater fish diversity (Bussing, 1985). They are one of the few groups of freshwater fishes that are distributed from southern South America to North America (where they cross the trans-Mexican volcanic Belt), and also are the only cichlids in the Greater Antilles. Heroine cichlids show a wide diversity of morphologies, as well as ecological and behavioral adaptations (e.g. Bussing, 1985; Martin and Bermingham, 1998; Miller et al., 2005). Moreover, they constitute a model system to study biogeography of the Neotropical region (Concheiro Pérez et al., 2007), and for instance, the Midas cichlid complex (*Amphilophus* sp.) living in crater lakes in Nicaragua has been proposed to be a model system to study sympatric speciation (Barluenga et al., 2006).

The taxonomical and nomenclatural history of the tribe Heroini is inextricably connected with that of the genus *Cichlasoma* Swainson, 1839. After the revision of Regan (1905) most heroine species were assigned to the genus *Cichlasoma*. However, Kullander (1983) recognized that *Cichlasoma* was an unnatural catch-all group, and restricted it to 12 morphologically very similar species closely

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related to the genus *Aequidens* Eigenmann & Bray, 1894 (but a sizeable portion of Mesoamerican heroines was still left in *Cichlasoma* in Kullander, 2003). At present, the genus *Cichlasoma*—somewhat ironically—is the type of Cichlasomatini. Further work (summarized in Kullander, 1998) elucidated the generic taxonomy of the Cichlasomatini, but Heroini were almost completely left out, and this chaotic situation has changed little since then. At present, the difficulty with assigning generic names to many heroines hinders evolutionary studies with these taxa, and necessarily requires resolving with confidence phylogenetic relationships among species and genera.

The monophyly of the tribe Heroini as well as its sister group relationship with the tribe Cichlasomatini, forming together the subfamily Cichlasomatinae are well supported based on both morphological and molecular grounds (Kullander, 1998; Farias et al., 1999, 2000, 2001). However, phylogenetic relationships and generic allocation of most species within the tribe Heroini are highly contentious, and far from being understood (Miller, 1966, 1996; Miller et al., 2005; Kullander, 1998, 2003; Concheiro Pérez et al., 2007).

In spite of many morphological studies published on heroine cichlids (Appendix 1), none to date has analyzed morphological characters in combination with molecular data. On the other hand, early studies utilizing molecular data were based mostly on mitochondrial (mt) cytochrome *b* (*cytb*) gene sequences, and were characterized by relatively sparse taxon samplings (Roe et al., 1997; Martin and Bermingham, 1998; Hulsey et al., 2004; Říčan and Kullander, 2006; Chakrabarty, 2006). The most inclusive molecular study (Concheiro Pérez et al., 2007) to date on heroine phylogenetic relationships included virtually all heroine lineages, but was based solely on *cytb*. Concheiro Pérez et al. (2007) showed that heroines could be divided into a paraphyletic stem lineage of Amazonian genera (referred to as the Amazonian Heroini; AM) and a monophyletic lineage (termed Circumamazonian Heroini; CAM) including all Middle American, Antillean and trans-Andean heroine cichlids as well as three *cis*-Andean South American genera, namely *Caquetaia*, *Heroina* and *Australoheros*. Moreover, the majority of Mesoamerican heroines could be placed into either one of two large suprageneric clades, the amphilophines and the herichthyines (Concheiro Pérez et al., 2007). This study set a sound starting point towards resolution of heroine phylogeny and stabilization of generic taxonomy. However, the *cytb*-based phylogeny of heroines (Concheiro Pérez et al., 2007) is characterized by rather short internodes, which require further confirmation both by analyzing more mt sequence data to find additional synapomorphies, as well as by examining independent data sets. In this regard, it is well known that phylogenetic analyses of combined mt and nuclear sequence data provide resolution not achieved by each type of data separately (e.g. Brower et al., 1996; Wiley et al., 1998; Rüber et al., 2004; Farias et al., 2000; López-Fernández et al., 2005a). Thus far, however, only two studies (Chakrabarty, 2006; Higham et al., 2007) have used nuclear sequence data to address phylogenetic relationships of Heroini, although with reduced taxon samplings. Phylogenetic studies based on morphology could be very informative together with molecular sequence data in further supporting monophyly of the different genera.

Given the urgent need of having a robust phylogeny of Mesoamerican cichlids as framework for the wealth of comparative studies ongoing on these taxa, the main goal of the present study was to strengthen and test the previous hypothesis on the phylogenetic relationships of CAM heroine cichlids based on *cytb* data (Concheiro Pérez et al., 2007) by analyzing two additional independent (nuclear and morphological) data sets using a thorough generic sampling. Two nuclear markers were studied to increase phylogenetic resolution both at deeper levels (*RAG1* gene), as well as at lower taxonomic levels (two introns of the S7 gene). In addition, phylogenetic analyses of a rather complete morphological data set were performed with the particular goal of stabilizing heroine genera.

2. Materials and methods

2.1. Taxon sampling

In order to further resolve the phylogeny of heroines, a nuclear (RAG1 and S7 introns) sequence data set, which included 48 species representing all major lineages of CAM heroines as well as most genera was compiled, and analyzed in combination with a mt *cytb* gene sequences of the same 48 species, most taken from Říčan and Kullander (2006) and Concheiro Pérez et al. (2007), and four newly sequenced species ('*Heros*' *beani*, *Theraps bocourti, Theraps irregularis* and *Theraps nourissati*).

In order to test monophyly of the different genera and stabilize heroine taxonomy at this level, a morphological data set was gathered based on an extensive review of literature coupled with a thorough study of museum specimens (Appendix 2). A total of 97 CAM heroine species representing all putative and established CAM heroine genera, as well as all type species of established CAM heroine genera were included in the phylogenetic analyses based on morphology combined with *cytb* gene sequence data (Říčan and Kullander, 2006; Concheiro Pérez et al., 2007; this paper).

In all phylogenetic analyses, geophagines, cichlasomatines and Amazonian heroines were used as outgroup taxa.

2.2. Generic placement of heroine species in this study

The present nomenclatural treatment of the more than 100 Mesoamerican heroine cichlid species most of which were formerly referred to as *Cichlasoma* (Regan, 1905) is both chaotic and frustrating (Kullander, 1983, 2003; Concheiro Pérez et al., 2007). To deal with the complex nomenclature of CAM heroines, we used the following approach. Monophyletic lineages including type species of established genera were considered valid, and their species composition was adjusted to keep the genera monophyletic. Established genera, which we found as non-monophyletic were restricted to include only the type species and the monophyletic lineage to which it belongs. The remaining lineages excluded from these previously non-monophyletic genera, and those monophyletic lineages without applicable generic names were propose to be named as new genera (we refer to these putative new genera as '*Heros*' species groups) if their monophyly was found to be a significantly better hypothesis than competing hypotheses found in the literature.

2.3. Molecular methods

DNA was extracted from small pieces of muscle or gill (10–25 mg) using the DNeasy™ Tissue Kit (QIAGEN). The complete *cytb* gene was PCR amplified in four species as previously described (Concheiro Pérez et al., 2007). The 3′ half of the RAG1 gene (1.5 kb) was PCR amplified with primers RAG1F1 5′-CTG AGC TGC AGT CAG TAC CAT AAG ATG T-3′ and RAG1R1 5′-CTG AGT CCT TGT GAG CTT CCA TRA AYT T-3′ (López et al., 2004). Two introns of the S7 ribosomal protein-coding gene were PCR amplified using primers S7RPEX1F 5′-TGGCCTCTTCCTTGGCCGTC-3′ and S7RPEX2R 5′-AACTCGTCTGGCTTTTCGCC-3′ for S7 gene intron 1 and primers S7RPEX2F 5′-AGCGCCAAAATAGTGAAGCC-3′ and S7RPEX3R 5′-GCCTTCAGGTCAGAGTTCAT-3′ for S7 gene intron 2 (Chow and Hazama, 1998).

PCR amplification of RAG1 gene was carried out with an initial denaturing step at 95 °C for 1 min, followed by 35–40 cycles of amplification (denaturing at 95 °C for 45 s, annealing at 54 °C for

Table 1
Comparison of phylogenetic performance of the genes used in this study (as assessed in separate analyses with the reduced taxon-sampling)

	Sites	PI sites	In %	CI	RI	PI sites Plus indels	In %	CI	RI
RAG1	1486	66	4.440	0.5984	0.8061	66	–	–	–
S7i1	632	84	13.29	0.5988	0.7473	135	**21.36**	**0.6221**	0.6831
S7i2	871	191	21.92	0.5667	0.7567	296	**33.98**	**0.5898**	0.6604
Cytb	1143	443	38.75	0.2317	0.4386	443	–	–	–

Both introns in the S7 gene include significant amount of information in form of indels (in bold). PI, parsimony informative sites; CI, consistency index; RI, retention index.

60 s and extending at 72 °C for 90 s), with a final extending step at 72 °C for 10 min. PCR amplification of the S7 gene introns was carried out with an initial denaturing step at 95 °C for 1 min, followed by 30–35 cycles of amplification (denaturing at 95 °C for 30 s, annealing at 60 °C for 60 s, and extending at 72 °C for 120 s), with a final extending cycle at 72 °C for 10 min. PCR products were purified either by ethanol precipitation or using Microcon PCR Filter Units (Millipore), and directly sequenced with the corresponding PCR primers using the BigDye™ Terminator Cycle Sequencing Kit v.3.1 (PE Applied Biosystems), and following manufacturer's instructions. Sequencing reaction products were cleaned either by ethanol precipitation or with DyeEx 2.0 Spin Kit (QIAGEN), and run on ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). Chromatograms were assembled and checked by eye for potential mistakes using SeqMan II of the DNAStar software package (http://www.dnastar.com). Edited sequences were aligned using ClustalX software (Thompson et al., 1997), and either the default settings (*cytb* and RAG1) or three different parameter settings (for the S7 introns; gap opening/extension: 10/5, 7/5, 10/10). Aligned sequences were manually adjusted in BioEdit (Biological sequence alignment editor v 5.0.9, http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and sites that shifted relative position in the three alignments (S7), and could not be reconciled by eye were excluded from phylogenetic analyses (Gatesy et al., 1993).

All sequence data newly determined in this study were deposited in GenBank under the Accession Nos. EF436463–EF436466 (*cytb*), EF433005–EF433048 (S7i1), EF433049–EF433086 (S7i2), and EF362572–EF362613 (RAG1).

Table 2
Parameters of ML analyses estimated with Modeltest

	Cytb	RAG1	S7
Model selected by Modeltest (AIC criteria)	TrN+I+G	GTR+I+G	HKY+G
Nucleotide proportions	A = 0.2822	A = 0.2506	A = 0.2640
	C = 0.3896	C = 0.2391	C = 0.1829
	G = 0.0783	G = 0.2761	G = 0.2397
	T = 0.2498	T = 0.2343	T = 0.3133
Assumed proportion of invariable sites (I)	0.4908	0.4923	0
Alpha (G)	0.8995	1.022	0.8441

2.4. Morphological methods

Most of the characters included in Kullander's (1998) morphological matrix were found to be uninformative to resolve phylogenetic relationships among closely related CAM Heroini genera. Therefore, a thorough morphological study of a large number of museum specimens covering all recognized or putative heroine lineages was carried out to seek for additional characters. References to major literature sources from which morphological characters were extracted are listed in Appendix 1. A total of 97 ingroup species (1218 museum specimens) and 31 outgroup (amazonian heroines, Cichlasomatines, and Geophagines) species (135 specimens) were examined (Appendix 2). Character descriptions are provided in Appendix 3, and the morphological character

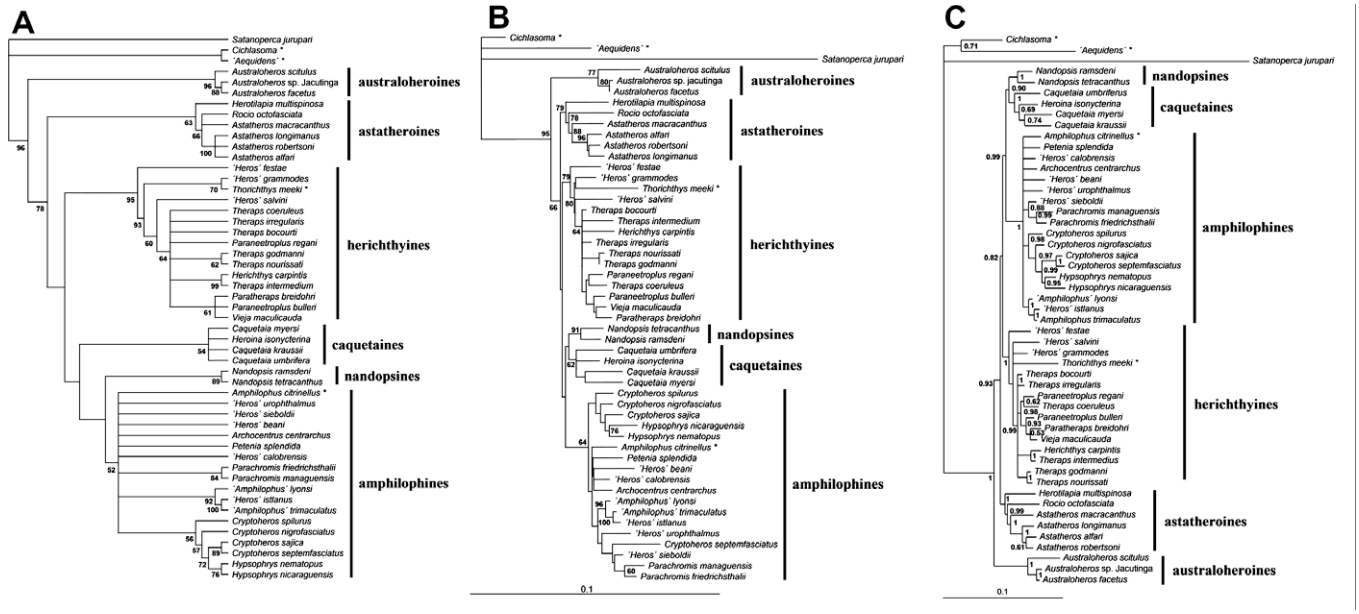

Fig. 1. Phylogenetic analyses of nuclear genes. (A) Strict consensus MP tree based on the nuclear data set excluding indels (PI = 341; *N* = 15,000+; *L* = 1461; CI = 0.5652; RI = 0.6907). (B) ML phylogram (ln L = −13,026.51) inferred under the GTR+I+G model. (C) BI phylogram (ln L = −12,769.69) inferred under the GTR+I+G model. Numbers show bootstrap support (*N* = 1000). Asterisks denote chimerical sequences resulting from combining closely related species to increase taxon overlap between datasets. Major clades are indicated to facilitate tree interpretation and comparisons.

matrix is given in Appendix 4. A total of 81 informative characters were analyzed. Of these, 23 (characters 1–23) were meristic characters, four (characters 24–27) described external, non-meristic characters, 19 (characters 28–46) referred to descriptive internal and external characters (mostly from teeth and jaw morphology) that could not be coded in a quantitative manner, and 35 (characters 47–81) were color pattern characters, which were studied in an ontogenetic perspective (Říčan et al., 2005).

Coding of morphological characters follows recommendations of Campbell and Frost (1993) and Wiens (1995, 1999). Qualitative characters (characters 24–81) were coded using the majority approach. Some characters, which showed more discernible states, were coded using the scaled coding (Campbell and Frost, 1993) under the assumption that traits pass through a polymorphic stage between absence and fixed presence. The scaled method is advantageous in that it allows polymorphisms to act as synapomorphies.

Quantitative characters (characters 1–23) were coded using a modified gap weighting method (GW) of Thiele (1993). Thiele's implementation of gap weighting involves finding, for a given character, the mean value of the trait in each species in the analysis, the range of mean species values among taxa (i.e., the species with the greatest mean value and the species with the lowest), and then dividing this range into smaller ranges or segments equal to the maximum number of character states allowed by the phylogenetic software program (i.e., 32 for PAUP*). States are then assigned to species based on these ranges. An important advantage of the gap-weighting method is that it incorporates information on the distance between states, weighting the changes according to the difference between mean species values (hence the name).

We used the between-state scaling (Wiens, 2001) to weight quantitative multistate characters (i.e., those coded with the modified gap-weighting method (GW); see above) against each other. This weighting scheme assigns transformations between species with fixed, adjacent values of meristic variables (e.g., 13–14 vertebrae or 5–6 anal spines) the same weight in all GW coded characters. The more fixed steps a multistate quantitative character expresses the more information in contains, but all multistate characters are a-priory weighted 1:1 in this method. To weight quantitative multistate characters against qualitative characters we used the between-character scaling (Wiens, 2001). All characters are thus in effect weighted 1:1 to each other irrespective of their method of coding. Changes in binary variables (0–1) thus have the same weight as the whole transformation series of a multistate character.

2.5. Phylogenetic inference

Phylogenetic analyses of the nuclear gene sequence data separately, and combined with *cytb* gene sequence data were performed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference (BI) as implemented in PAUP* (Swofford, 2000), PHYML (Guindon and Gascuel, 2003), and MrBayes

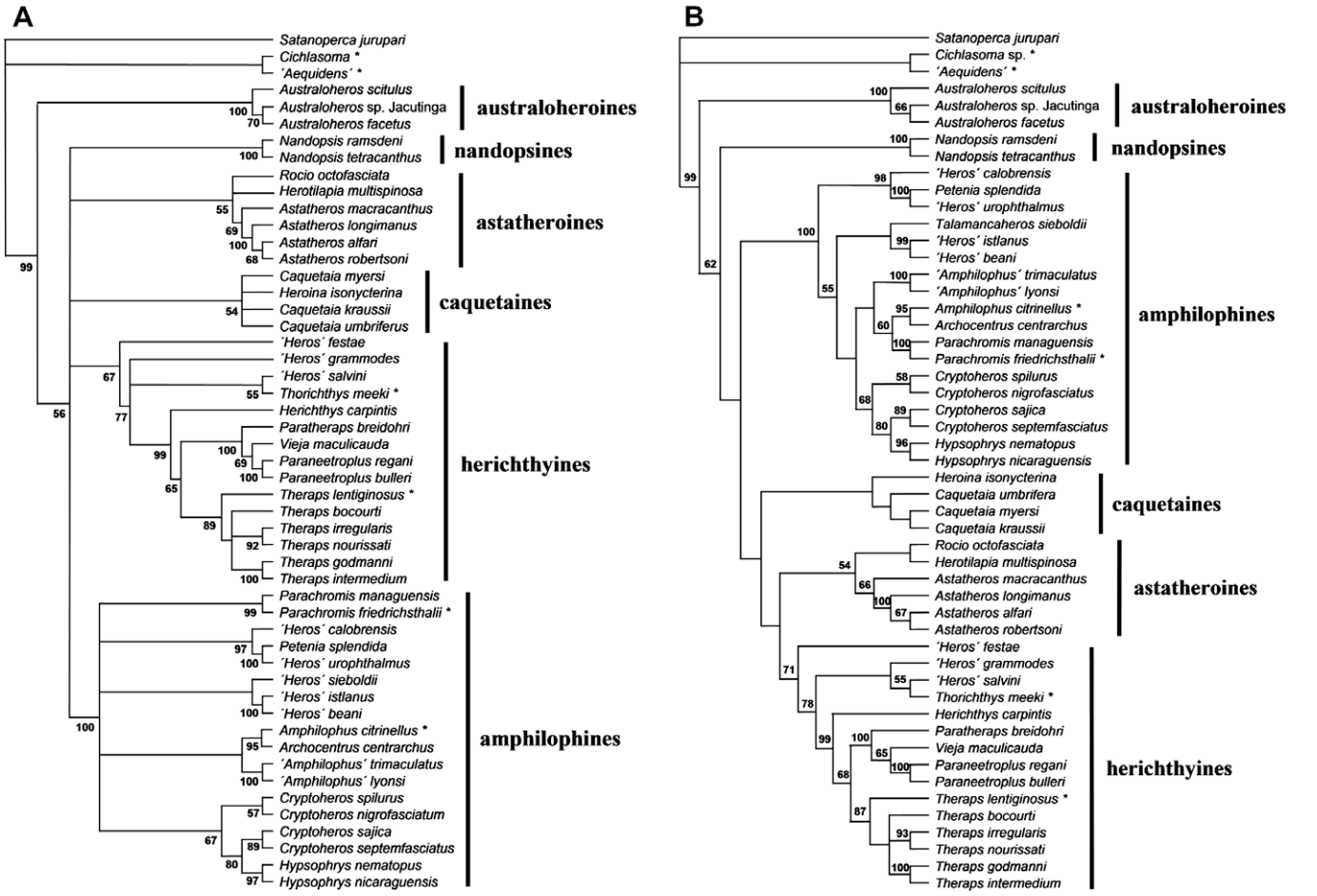

Fig. 2. MP Analyses of all genes combined. (A) Strict consensus MP tree based on the combined molecular data set excluding indels (PI = 784; *N* = 60; *L* = 4635; CI = 0.2956; RI = 0.4770). (B) Strict consensus MP tree based on the combined molecular data set including indels (PI = 915; *N* = 3; *L* = 5341; CI = 0.3288; RI = 0.4866). Numbers show bootstrap support (*N* = 1000). Asterisks denote chimerical sequences resulting from combining closely related species to increase taxon overlap between datasets. Major clades are indicated to facilitate tree interpretation and comparisons.

3.0 (Huelsenbeck and Ronquist, 2001), respectively. MP analyses were conducted with heuristic searches (TBR branch swapping, MULTREES option in effect, and 10 random stepwise additions of taxa). We first extensively sampled the tree space using 1000 random sequence additions and keeping 10 trees per search (commands in PAUP*: hsearch addseq = random nchuck = 10 chuckscore = 1 nreps = 1000). In the next step, we run a search on the saved trees to find all the shortest trees (commands in PAUP*: hsearch start = current nchuck = 0 chuckscore = 0). For the ML and BI analyses, the best-fit model for the different genes was selected with Modeltest 3.06 (Posada and Crandall, 1998) using the Akaike information criterion (AIC). Bayesian analyses were performed using two independent runs of four Metropolis-coupled chains (MCMC) of 5 million generations each, to estimate the posterior probability distribution. The combined sequence matrices were partitioned per gene fragment, and independent model parameters were estimated for each partition. Topologies were sampled every 100 generations and majority-rule consensus trees were estimated after discarding the first 10% generations for the nuclear, and combined gene data sets, respectively. Robustness of clades was assessed using bootstrapping (1000 pseudoreplicates) for the MP and ML analyses, and Bayesian posterior probabilities for the BI analyses.

Phylogenetic analyses of the morphological data set separately and combined with the *cytb* gene nucleotide sequences were performed using MP as implemented in PAUP* (Swofford, 2000; see search strategy above). Robustness of clades was assessed using bootstrapping (1000 pseudo-replications in PAUP*) and the same approach as in the MP searches, with five random sequence additions per bootstrap replication, and saving 10 trees from each random sequence addition. Data exploration further included Bremer support and PBS (partitioned Bremer support) to assess congruence or conflict between the data partitions at each node of interest. Bremer analyses were run with the same parameters as MP searches in PAUP*.

2.6. Tests of alternative topologies

In order to statistically test the significance of our results against competing taxonomies (as found in the literature) and alternative phylogenies, we performed statistical tests of alternative topologies. The tests used maximum parsimony (MP) and included the compare-2 T-PTP test, the Templeton test and the Shimodaira-Hasegawa (SH) as implemented in PAUP* and using default settings. The compare-2 T-PTP test has been run with 500 random additions.

3. Results

3.1. Performance of individual gene datasets

Comparison of phylogenetic performance of individual genes is found in Table 1. Best-fit models and corresponding model parameters as estimated using Modeltest (Posada and Crandall, 1998) are provided in Table 2. The nuclear genes showed much higher values

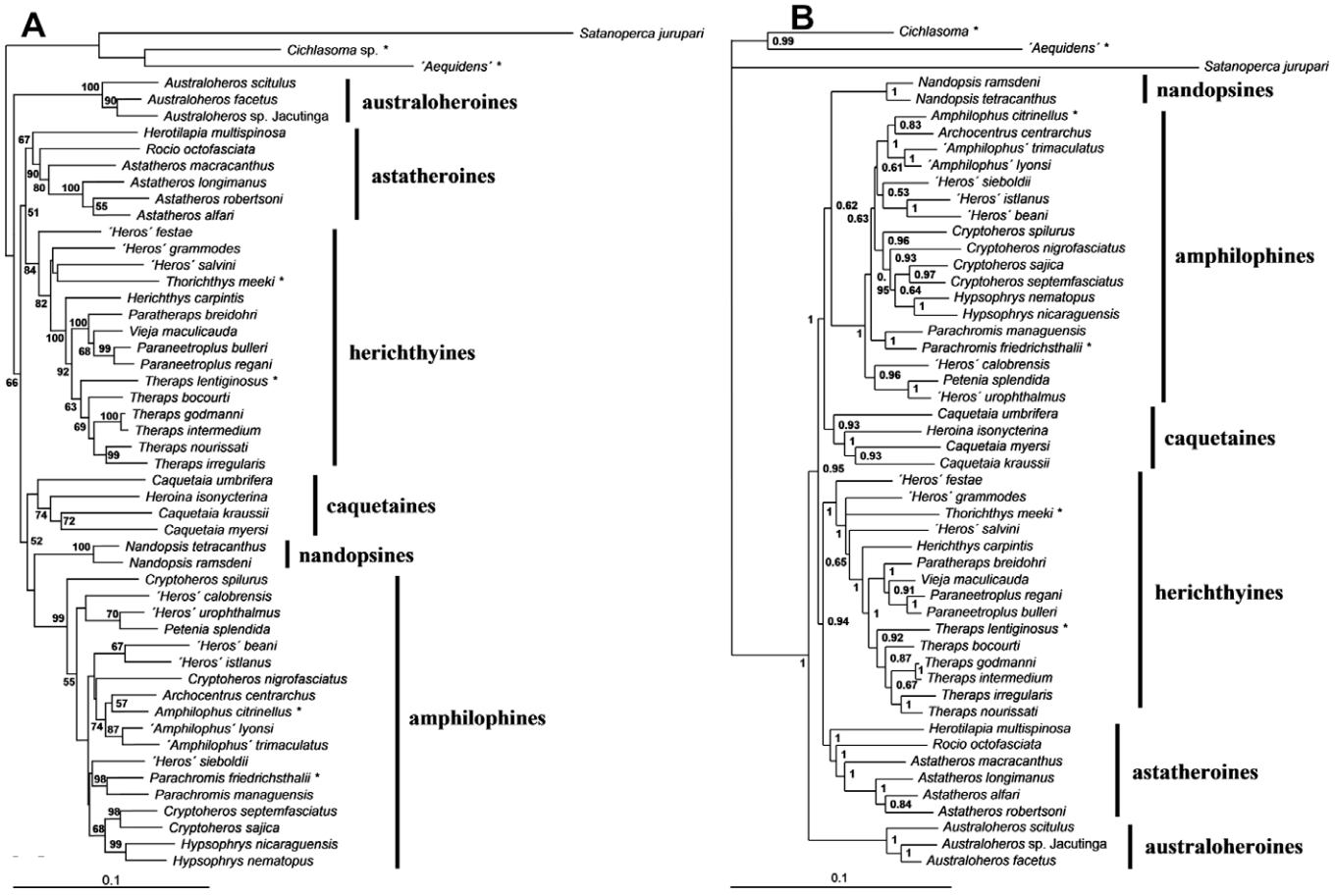

Fig. 3. ML and BI analyses of all genes excluding indels. (A) ML phylogram (ln L = −28,170.07) using the GTR+I+G model. (B) BI phylogram (ln L = −27,594.12) using the GTR+I+G model. Numbers show bootstrap support (N = 1000; ML analysis) and posterior probabilities for BI analysis. Asterisks denote chimerical sequences resulting from combining closely related species to increase taxon overlap between datasets. Major clades are indicated to facilitate tree interpretation and comparisons.

of consistency (CI) and retention (RI) indices, whereas *cytb* gene exhibited more parsimony informative (PI) sites. The proportion of PI sites of the two S7 introns increased when including indels, and approached that of the *cytb* gene. Importantly, including PI indels of the S7 introns into the phylogenetic analyses augmented the CI index (Table 1) and phylogenetic resolution (Fig. 2). Moreover, several indels were found diagnostic even for suprageneric clades (Fig. 6). The RAG 1 gene was very conservative with by far the lowest amount of PI sites.

3.2. Phylogenetic relationships based on nuclear and combined nuclear—mt gene sequence data

In order to confirm and strengthen high-level phylogenetic relationships among heroine main lineages as recovered based on *cytb* gene sequence data (Concheiro Pérez et al., 2007) two additional nuclear markers (RAG1 + S7 introns) have been added and analyzed using MP, ML and BI methods (Fig. 1). The results of these analyses are in very good agreement with the *cytb* phylogeny (Concheiro Pérez

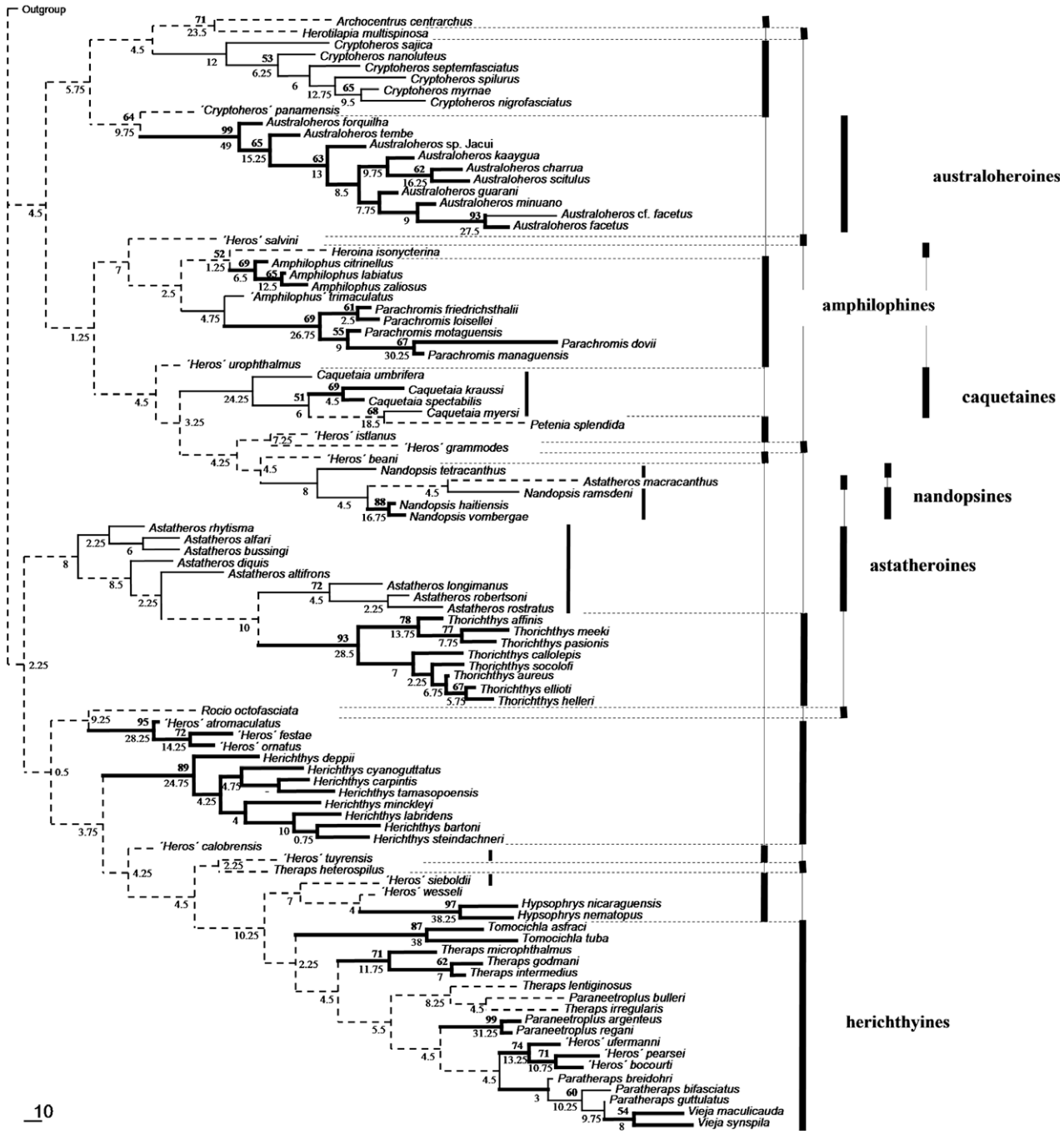

Fig. 4. Maximum parsimony analysis of the 81 informative morphological characters. The analysis resulted in two MP trees (L = 5248; CI = 0.21; RI = 0.67), which differed only regarding the sister taxon of *Herichthys tamasopoensis* (either *H. carpintis* or *H. cyanoguttatus*). The tree shown here is one of the two MP trees (branch lengths shown), with *H. carpintis* as the sister group of *H. tamasopoensis* in agreement with *cytb*-based phylogeny (Concheiro Pérez et al., 2007). Numbers above nodes show bootstrap support (N = 1000). Numbers below nodes show Bremer support. Topology shown with interrupted lines is in conflict with molecular trees (see Figs. 1–3, 5 and 6 and Concheiro Pérez et al., 2007). The phylogeny does not recover the six major clades found in molecular trees (see above). The major clades are however indicated to facilitate tree interpretation and comparisons, since also combined molecular—morphological trees recover the six major clades.

et al., 2007). The only two important differences are in the positions of *Herotilapia* (placed with *Rocio* and *Astatheros*), and of *Caquetaia umbrifera* (placed with rest of *Caquetaia* and *Heroina*) see below).

The combined nuclear–*cytb* data set was analyzed under MP either excluding indels (Fig. 2A) or considering them as a fifth character (Fig. 2B). In addition, the molecular data set was analyzed under ML (Fig. 3A) and BI (Fig. 3B) without considering indels (not implemented in these methods).

The reconstructed phylogenies based on the combined nuclear-*cytb* gene sequence data using the different methods of phylogenetic inference show influence by the nuclear signal (see differences to *cytb* phylogeny above), and divide CAM heroine cichlids into four suprageneric and two generic main clades that received relatively strong support (Figs. 2 and 3). Phylogenetic relationships among the six main clades lacked support, with the exception of the sister group relationship of australoheroines (*Australoheros*) to the remaining CAM heroines, which received moderate support (Figs. 2 and 3). Interestingly, MP and ML/BI phylogenetic analyses differed in the relative phylogenetic position of nandopsines (*Nandopsis*), which in the former was

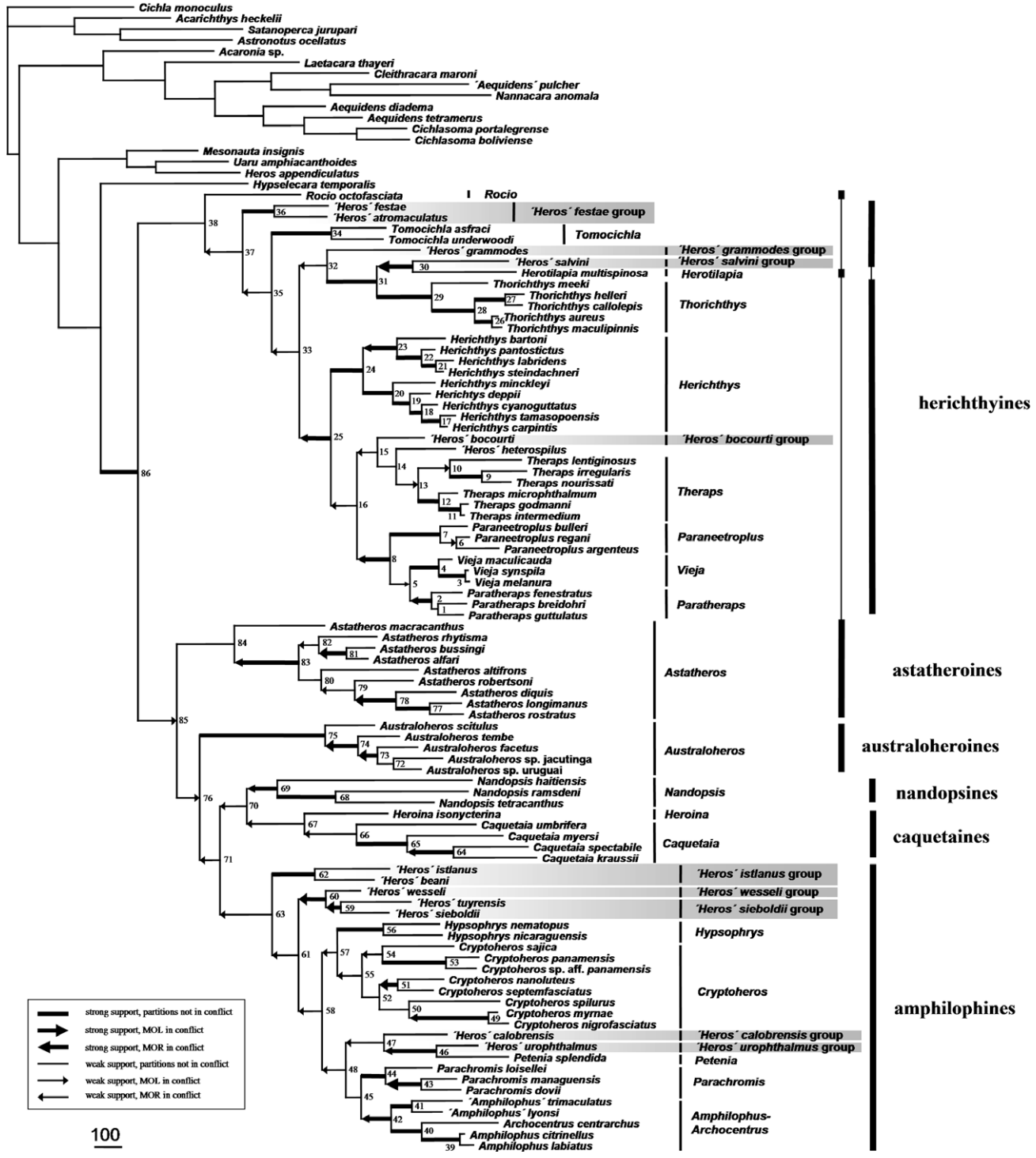

Fig. 5. Maximum parsimony analysis of the mor-*cytb* data set. One of the three MP trees (L = 34415.75) is shown. Numbers show node numbers. See Table 3 for bootstrap support and partitioned Bremer support. Major clades are indicated to facilitate tree interpretation and comparisons. Taxa highlighted in grey represent putative new genera.

recovered as the next lineage branching out after *Australoheros*, whereas in the latter appeared in a more derived position closer to amphilophines and caquetaines (*Caquetaia* + *Heroina*) (Figs. 2 and 3), as in the phylogenies based exclusively on nuclear genes (Fig. 1). Similarly, caquetaines were recovered as sister group of astatheroines and herichtyines (Fig. 2B) and of amphilophines plus nandopsines (Fig. 3) in the ML/BI phylogenetic analyses, respectively.

3.3. Phylogenetic relationships based on morphological characters

The maximum parsimony (MP) analysis of the 81 informative morphological characters recovered two MP trees (L = 5248; CI = 0.21; RI = 0.67; Fig. 4), which differed only regarding the sister taxon of *Herichthys tamasopoensis* (either *H. carpintis* or *H. cyanoguttatus*). The tree in Fig. 4 shows *H. carpintis* as sister group of *H. tamasopoensis* in agreement with *cytb*-based phylogeny (Concheiro Pérez et al., 2007). The recovered tree lacked support and congruence with molecular phylogenies at deeper nodes (cf. Figs. 1–3 and Concheiro Pérez et al., 2007), and the main split of CAM heroines into amphilophines and herichthyines was not supported (Fig. 4). Instead, morphology showed relative resolving power at the genus level, and the main recovered clades supported the following non-monotypic genera: *Cryptoheros* (but including also *C. nigrofasciatus*, and not including *C. panamensis*), *Australoheros*, *Amphilophus* s. str., *Parachromis*, *Herichthys*, and

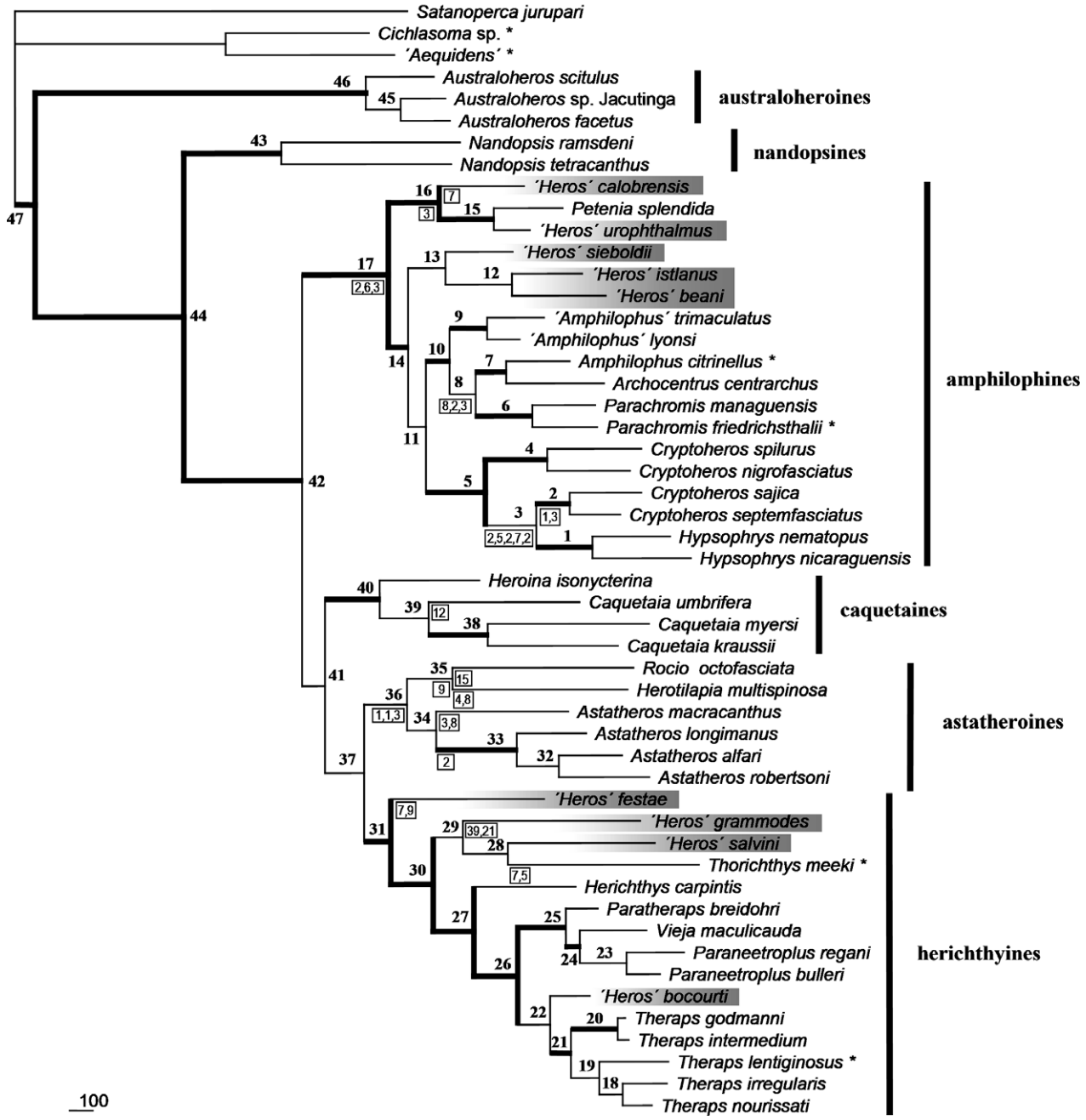

Fig. 6. Maximum parsimony analysis based on all genes (with indels) and morphology (PI = 977; N = 1; L = 30,696.25; CI = 0.3146; RI = 0.4793). Branch lengths are proportional to the number of steps. Values above branches are node numbers corresponding to Table 5 (see for bootstrap support and partitioned bremer support). Values in boxes bellow branches show number and length of unique apomorphic indels in S7 introns (except most single indels). Thick branches identify nodes where all or most data partitions are in agreement (see Table 5). Asterisks denote chimerical sequences resulting from combining closely related species to increase taxon overlap between datasets. Major clades are indicated to facilitate tree interpretation and comparisons. Taxa highlighted in grey represent putative new genera.

Thorichthys. In addition, all monotypic genera with the exception of *Petenia* were found as isolated lineages, not disrupting monophyly of other established genera. The following species were recovered as relatively divergent lineages without immediate close relatives among named genera: '*Heros*' *calobrensis*, '*Heros*' *urophthalmus*, '*Heros*' *salvini*, and to a lesser extent '*Heros*' *sieboldii*, '*Heros*' *tuyrensis*, '*Heros*' *wesseli*, '*Heros*' *istlanus*, '*Heros*' *grammodes*, '*Heros*' *beani* (Fig. 4), which is supported by molecular phylogenies (Figs. 1–3 and Concheiro Pérez et al., 2007). The only intergeneric relationship that showed statistical support was the sister group relationship of *Archocentrus* and *Herotilapia* (which is however not recovered in molecular phylogenies; Figs. 1–3 and Concheiro Pérez et al., 2007). In agreement with molecular data the morphology-based tree (Fig. 4) supported the monophyly and separate status of '*Heros*' *festae* group, as well as the non-synonymy of '*Heros*' *sieboldii* with *Tomocichla*. In conflict with the molecular phylogenies (Figs. 1–3 and Concheiro Pérez et al., 2007), *Petenia* was nested inside *Caquetaia*, *Astatheros macracanthus* was recovered within *Nandopsis*, *Thorichthys* rendered the rest of *Astatheros* paraphyletic, *Vieja* was recovered within *Paratheraps*, and *Theraps* was recovered as non-monophyletic (Fig. 4).

3.4. Generic composition of heroine cichlids based on a combined morphological—cytb phylogeny

Only two of our data sets have a full taxon sampling at the species level (morphology and *cytb*). A full taxon sampling is however important in order to determine monophyly of all genera. A combined analysis of the MOR-*cytb* data set was performed under MP (Fig. 5). PTP congruence test did not reject the null hypothesis of homogeneity of both data sets ($P = 0.9$) and they were thus analyzed simultaneously. Morphological characters were weighted 1:1 with respect to *cytb* gene sequence

Table 3
Results of bootstrap analysis ($N = 1000$) and partitioned bremer support (PBS) for the combined morphology plus *cytb* (mor-*cytb*) data set (see tree nodes in Fig. 5)

Node		1	2	3	4	5	6	7	8	9	10
MP bootstrap		58	94	**100**	**100**	53	65	**100**	**98**	**96**	52
BS		10	40	**71.25**	**74.75**	15.5	16	**117**	58.5	**44.5**	12.5
PBS	Mor	0	−10	**21.25**	**−0.25**	20.5	26	**27**	−31.5	**9.5**	32.25
	Cytb	10	50	**50**	**75**	−5	−10	**90**	90	**35**	−20
Node		**11**	**12**	13	14	15	16	**17**	**18**	**19**	**20**
MP bootstrap		**100**	**100**	–	–	68	68	**100**	**92**	**92**	**99**
BS		**54.5**	**66.25**	12.25	9.5	16.25	15.75	**39.5**	**22**	**25**	**58.5**
PBS	Mor	**14.5**	**11.25**	22.25	9.5	−8.75	−4.25	**4.5**	**7**	**5**	−1.75
	Cytb	**40**	**55**	−10	0	25	20	**35**	**15**	**20**	**60**
Node		**21**	**22**	**23**	**24**	25	**26**	**27**	**28**	**29**	30
MP bootstrap		**100**	**100**	**100**	**99**	**82**	**95**	**100**	**100**	**100**	**74**
BS		**55**	**50**	**78.5**	**41**	30.5	**25**	**88.5**	**100**	**151.75**	20.75
PBS	Mor	**0**	**0**	**−6.5**	**21**	−24.5	0	−1.5	5	**61.75**	−39.25
	Cytb	**55**	**50**	**85**	**20**	55	**25**	**90**	**95**	**90**	60
Node		**31**	32	33	**34**	35	**36**	37	38	**39**	**40**
MP bootstrap		**78**	–	–	**100**	–	**95**	–	–	**100**	**89**
BS		**47.25**	11.5	8.25	**114**	15.5	**37.25**	10.25	5.25	**92.75**	**31.5**
PBS	Mor	**37.5**	−68.5	−17.5	**94**	−9.5	**2.25**	−54.75	5.25	**42.75**	**11.5**
	Cytb	**10**	80	10	**20**	25	**35**	65	0	**50**	**20**
Node		**41**	**42**	43	**44**	45	46	47	48	49	50
MP bootstrap		**98**	**79**	**95**	**86**	–	**99**	55	–	**100**	61
BS		**35.25**	**17**	34.75	**35.5**	7.25	82.5	5.5	0.75	226.55	16
PBS	Mor	**5.25**	**−3**	−25.25	**5.5**	2.25	−17.5	-29.5	-34.25	-33.5	1
	Cytb	**30**	**20**	60	**30**	5	100	35	35	260	15
Node		51	52	**53**	54	55	**56**	57	58	59	60
MP bootstrap		**94**	–	**100**	–	–	**100**	–	–	**97**	**71**
BS		31.5	10.5	**131**	0.5	5.5	**67.75**	5.5	0.75	41.75	10.75
PBS	Mor	−23.5	0.5	**76**	−14.5	−29.5	**17.75**	−29.5	−34.25	−43.25	−24.25
	Cytb	55	10	**55**	15	35	**50**	35	35	85	35
Node		61	**62**	63	64	**65**	66	67	**68**	69	70
MP bootstrap		–	**100**	57	**94**	**75**	–	–	**100**	**95**	–
BS		0.75	**77**	19.75	38.75	**14.5**	3.25	0.75	**144.25**	57.25	0.75
PBS	Mor	−34.25	**82**	−85.25	−11.25	**4.5**	−16.75	−34.25	**−0.75**	−17.75	−34.25
	Cytb	35	**−5**	105	50	**10**	20	35	**145**	75	35
Node		71	72	73	74	**75**	76	**77**	78	79	80
MP bootstrap		–	63	–	**76**	**100**	–	**100**	**84**	–	–
BS		0.75	12	2.25	17.5	**168.75**	5.25	**71.25**	40.25	0.75	5.25
PBS	Mor	−34.25	7	−47.75	−47.5	**18.75**	40.25	**11.25**	−54.75	−34.25	5.25
	Cytb	35	5	50	65	**150**	-35	**60**	95	35	0
Node		81	82	83	84	85	**86**				
MP bootstrap		**99**	–	**99**	–	–	**89**				
BS		37.25	5.25	73.5	9	5.25	**24.5**				
PBS	Mor	−52.75	−54.75	−11.5	9	40.25	**−0.5**				
	Cytb	90	60	85	0	−35	**25**				

BS, total bremer support. Values in bold show strong support in the bootstrap analysis and agreement among the two data partitions as evaluated by PBS.

Table 4
Tests of alternative hypotheses on the morphology (mor), *cytb*, and mor-*cytb* data sets

Test no.	Genus/species group/species	Explanation/alternative hypotheses	Tested data set (length difference)	Compare-2	Test Templeton	Kishino-hasegawa
Tomocichla						
1	*Tomocichla* (sensu Bussing, 1975)	Includes *Heroś sieboldii*	Comb. data (262.75)	0.01*	0.0016*	0.0029*
2	'*Heros*' *sieboldii* group	Monophyletic	Mor data (2.25)	0.41	0.8653	0.8079
3	'*Heros*' *sieboldii*	Part of herichthyines	Comb. data (177.75)	0.01*	0.0120*	0.0132*
4	'*Heros*' *tuyrensis*	Part of herichthyines	Comb. data (158.5)	0.02*	0.0421*	0.0387*
Caquetaia-Petenia						
5	*Caquetaia* (sensu Miller)	Includes *Petenia splendida*	Comb. data (135.5)	0.04*	0.0730	0.1022
6			*Cytb* data (30)	0.02*	0.0451*	0.0431*
7	*Caquetaia* (sensu Kullander, 2003)	Includes *C. umbrifera*	*Cytb* data (9)	0.11	0.6474	0.5702
8			Mor data (28.75)	0.22	0.3033	0.2647
9	'*Heros*' *urophthalmus*	Part of *Amphilophus* s. str.	Comb. data (278.75)	0.01*	<0.0001*	<0.0001*
10	'*Heros*' *salvini*	Part of Amphilophina	Comb. data (166.25)	0.02*	0.0055*	0.0309*
11		Sister group of *Heroś grammodes*	Comb. data (107.50)	0.01*	0.0067*	0.0071*
12		Sister group of *Herotilapia*	Mor data (50)	0.13	0.2786	0.1978
13		Sister group of *Thorichthys*	Mor data (28.75)	0.06	0.3360	0.4625
14	'*Heros*' *grammodes*	Part of amphilophines	Comb. data (108.75)	0.02*	0.0978*	0.1156*
15		Sister to *Heroś istlanus*	Comb. data (136.25)	0.01*	0.0319	0.0130
Amphilophus–Archocentrus						
16	*Amphilophus* (sensu Kullander, 2003)	Monophyletic	Comb. data (572.5)	0.01*	<0.0001*	<0.0001*
17	*Amphilophus*	Trimaculatus group part of *Parachromis*	Comb. data (17)	0.25*	0.6504*	0.5247
18	*Archocentrus*	*A. centrarchus* sister to *Herotilapia*	Comb. data (245.50)	0.01*	0.0021*	0.013*
19	*Archocentrus* (sensu Miller, 1993)	Monophyletic	Comb. data (76)	0.15	0.3288	0.3299
20			*Cytb* data (16)	0.12	0.1508	0.1573
21	*Archocentrus* (sensu Kullander, 2003)	Monophyletic, includes *Heroś panamenesis*	Comb. data (76)	0.09	0.3288*	0.3299*
22			*Cytb* data (18)	0.12	0.0297*	0.0441*
23	*Cryptoheros* (sensu Algayer, 2001)	Monophyletic	Comb. data (0.5)	0.84	0.8813	0.9849
24		Monophyletic	*Cytb* data (5)	0.44*	0.5721	0.5690
25	*Cryptoheros panamensis*	Part of Neetroplus (sister to *N. nematopus*)	Comb. data (114.75)	0.02*	0.0658	0.1121
26			*Cytb* data (16)	0.1	0.1255	0.1202
27		Part of *Cryptoheros*	*Cytb* data (5)	0.42*	0.5973*	0.6043*
28	*Astatheros*	Part of *Amphilophus* s. str.	Comb. data (256)	0.01*	0.0002*	0.0001*
29		monophyl. s. lat.	Mor data (16.25)	0.39	0.9413	0.6368
30	*Astatheros*	Sister group of *Thorichthys*	Comb. data (76.25)	0.23*	0.2256	0.2986
31			*Cytb* data (17)	0.03*	0.2759*	0.2330*
32		Paraphyletic to *Thorichthys*	Comb. data (164.5)	0.01*	0.0137*	0.0308*
33			*Cytb* data (37)	0.01*	0.0003*	0.0003*
34	'*Heros*' *calobrensis*	Part of *Astatheros*	Comb. data (81.5)	0.01*	0.0768	0.2083
35			*Cytb* data (26)	0.01*	0.1868	0.1462
36			Mor data (20.5)	0.36*	0.5377	0.6367
37		Part of *Amphilophus* s. str.	Comb. data (116)	0.04*	0.0766*	0.0836*
38	'*Heros*' *istlanus* group	Part of *Amphilophus* s. str.	Comb. data (123)	0.02*	0.0138*	0.016*
39		Sister group of *Herośurophthalmus*	Comb. data (137)	0.02*	0.0248*	0.0131*
40		Monophyletic	Mor data (13.25)	0.35	0.6774	0.7002
41	*Rocio octofasciata*	Part of *Heroś festae* group	Comb. data (10.25)	0.37	0.5321	0.8645
42			*Cytb* data (7)	0.51*	0.4555*	0.4948*
43	'*Heros*' *festae* group	Part of *Amphilophus* s. str.	Comb. data (340.5)	0.01*	<0.0001*	<0.0001*
44		Part of *Nandopsis*	Comb. data (191.25)	0.01*	0.0108*	0.0099*
45	*Rocio* + '*H.*' *festae* gr. + *Tomocichla*	Monophyletic	Comb. data (31.25)	0.19	0.5291	0.6503
46			*Cytb* data (10)	0.30*	0.6424	0.5080
47			Mor data (30.25)	0.01*	0.3506	0.3156

48	*Vieja* (sensu Kullander, 2003)	Monophyletic (excluding *Heroš tuyrensis*)	Comb. data (224)	0.01*		0.0005*		0.0026*
49	*Vieja* (including *Paratheraps*)	Monophyletic	*Cytb* data (6)	0.46		0.2888		0.2890
50	*Paraneetroplus*	*P. regani* group part of *Vieja* s. lat.	Comb. data (117.75)	0.16		0.0689*		0.087*
51			*Cytb* data (17)	0.09		0.0407		0.0406
52		Monophyletic (including regani group)	Mor data (22.75)	0.23		0.4187		0.3356
53	*Theraps* s. str.	Includes *T. tentiginosus/coeruleus*	*Cytb* data (2)	0.76*		0.7849*		0.7964*
54	*Theraps* s. lat.	Bocourti gr. part of *Herichthys*	Comb. data (133.5)	0.01*		0.0002*		0.0004*
55		Bocourti group part of *Vieja* s. lat.	Comb. data (83.25)	0.01*		0.0026*		0.0031*
56		Heterospilus part of *Herichthys*	Comb. data (122.5)	0.01*		0.0018		0.0011*
57		Heterospilus part of *Vieja* s. lat.	Comb. data (74.5)	0.22		0.0748*		0.0754*
58			*Cytb* data (22)	0.06*		0.0217*		0.0217
59		*Microphthalmus* group part of Vieja s. lat.	Comb. data (134.5)	0.02*		0.0416*		0.0525
60		Incl.*microp.* gr., *bocourti* gr. and *heterospilus*	Mor data (65.5)	0.14*		0.1359*		0.1334*
61		*T. nourissati* part of *Astatheros*	Comb. data (234.75)	0.01*		0.0007*		0.0005*
62	*'Heros' wesseli*	Part of *Theraps* s. lat.	Comb. data (179.50)	0.01*		0.0002*		0.0003*
63	*Nandopsis* (sensu Kullander, 2003)	Non-monophyletic, excludes *N. ramsdeni*	Comb. data (387.25)	0.01*		<0.0001*		<0.0001*
64	*Nandopsis* (sensu Miller, 1966)	Paraphyletic, *Astatheros* sister to *N. ramsdeni*	Comb. data (241.25)	0.01*		0.0004*		0.0002*
65		Monophyl., *ramsdeni* not sister to *tetracanthus*	Comb. data (144.25)	0.01*		0.0064*		0.0050*
66		*Ramsdeni* sister group of bocourti group	Comb. data (477.25)	0.01*		<0.0001*		<0.0001*
67		Monophyletic	Mor data (6.75)	0.57*		0.8311*		0.7834*
68	Amphilophines	Monophyletic	Mor data (235)	0.01*		0.0020*		0.0009*
69	Herichthyines	Monophyletic	Mor data (179.75)	0.01*		0.0083*		0.0083*

"Part of" is tested as "sister group to". *Statistically significant test results (at 0.05). References to alternative topologies are in Appendix 1.

data, while keeping the between-state scaling of the morphological characters as detailed in the Section 2. The MP analysis of the MOR-*cytb* data set (590 PI characters) resulted in three MP trees ($L = 34415.75$; CI = 0.16; RI = 0.51). The difference between the three recovered MP trees was only in the relative phylogenetic position of the genera *Mesonauta*, *Uaru* and *Heros*, all outgroup taxa. The topology of Fig. 5 shows the three genera as a monophyletic group, in agreement with previously published studies (see e.g. Farias et al., 2000). Heroines formed a monophyletic group with respect to outgroup taxa, and the monophyly of CAM heroines (node 86) received high support (Table 3). As expected, bootstrap and Bremer supports for deeper nodes within CAM heroines were generally weak (Table 3). The recovered phylogenetic relationships among CAM heroines based on the mor-*cytb* data set were largely dominated by the *cytb* gene phylogenetic signal (as also evidenced by partitioned Bremer support, PBS, Table 3). Six main clades as in combined molecular analyses (see above) were recovered within CAM heroines (Fig. 5): (1) amphilophines (node 63) with moderate support (Table 3); (2) herichthyines (node 38), albeit without strong support (Table 3), and including *Rocio octofasciata* and *Herotilapia multispinosa*, which were however recovered with relatively strong statistical support as sister group of *Astatheros*, and forming the astatheroines based on the nuclear and nuclear-*cytb* sequence data (Figs. 1–3); (3) *Australoheros* (node 75) with high support (Table 3); (4) *Astatheros* (node 84) with high support except *A. macracanthus* (Table 3); (5) *Nandopsis* (node 69) with high support (Table 3), and (6) *Caquetaia* + *Heroina* (node 67), albeit without strong support (Table 3). The latter four clades were more closely related to amphilophines than to herichthyines (Fig. 3).

The recovered tree showed relatively high resolving power at the genus level (Fig. 3), and the main recovered clades supported the following non-monotypic genera: '*Heros*' *festae* group, *Tomocichla* (but not including *T. sieboldii*), *Thorichthys*, *Herichthys*, *Theraps*, *Paraneetroplus*, *Vieja*, *Paratheraps*, *Astatheros*, *Australoheros*, *Nandopsis*, *Caquetaia*, *Amphilophus* s. str., *Cryptoheros* (including also *C. nigrofasciatus*; see Concheiro Pérez et al., 2007), *Parachromis*, *Amphilophus*, and *Archocentrus*. In addition, all monotypic genera were found as isolated lineages, not disrupting monophyly of other established genera (Fig. 5). Alternative phylogenetic hypotheses pertaining to the genus level taxonomy as found in literature (or as recovered in the phylogenetic analysis of morphological characters; Fig. 4) were tested (Table 4). The ' Heros' species groups in Fig. 5 represent putative new genera based on these results.

3.5. Phylogenetic analyses of a combined morphological and molecular data set

The addition of morphological data to the combined molecular data set into a total evidence analysis under MP rendered a phylogeny (Fig. 6) that is identical in topology to the MP phylogeny based only on the combined molecular data set (Fig. 2B). Tests of alternative phylogenetic hypotheses pertaining to the genus level taxonomy based on this data set are shown in Table 6. The most basal position of *Nandopsis* within the CAM heroine group (instead of *Australoheros*; as in Concheiro Pérez et al., 2007) could not be rejected with our data (Table 6, tests 9–12). A consensus tree of all tree-building algorithms and analyzed data sets is given in Fig. 7 to summarize the common signal of all data sets. Important life-history traits have been mapped onto the consensus tree in Fig. 7 in order to demonstrate cases of probable parallel evolution (Fig. 8A). Traditional taxonomy (the most complex and conflicting cases) has also been mapped onto the consensus tree (Fig. 8B).

4. Discussion

4.1. Data sets and taxon sampling

The results of our study show that the inclusion of two nuclear markers with markedly different substitution rates under an extensive taxon-sampling scheme was crucial to increase resolution and support of heroine cichlid phylogeny. This result was not only due to inclusion of additional sequence data, but also to the fact that the nuclear gene phylogenetic signal was distributed at different taxonomic levels, and was highly congruent with *cytb* gene signal. Moreover, a strong point of the study aimed to maximize resolution was the extensive sampling strategy, which included many more heroine lineages that any previous study thus far. We tested the relative importance of taxon-sampling versus increased number of characters by analyzing a data set which included three additional markers under a limited taxon-sampling of 18 lineages (species), and resulted from combining the present study with that of Chakrabarty (2006). Clade support did not improve with the addition of new sequence data (not shown), in agreement with many previous empirical and simulation studies, which concluded that addition of key taxa is in most cases more important than inclusion of extra data (Hillis, 1996; Graybeal, 1998; Yoder and Irwin, 1999; Zwickl and Hillis, 2002; Hillis et al., 2003).

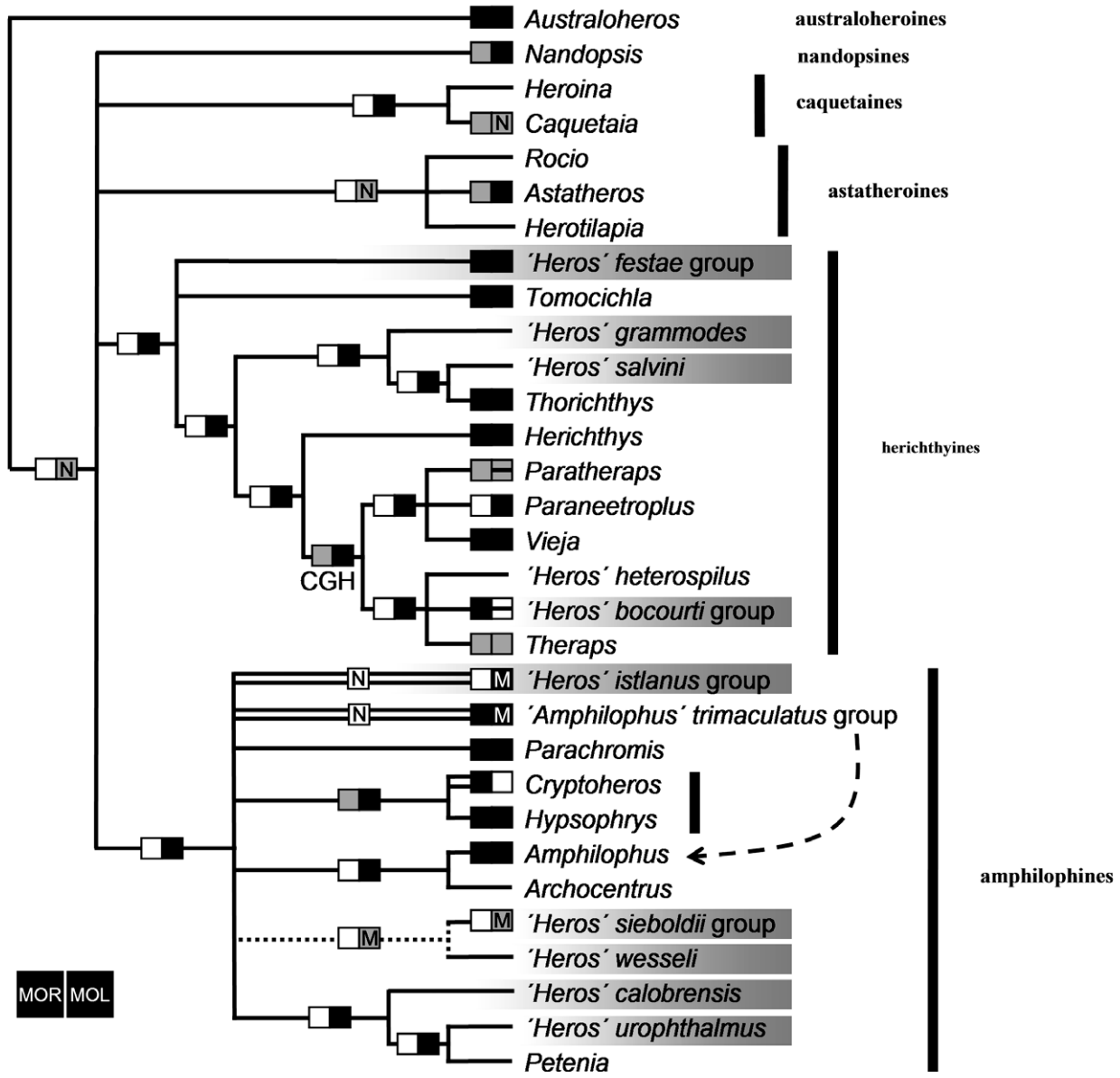

Fig. 7. Consensus tree of all tree-building algorithms and analyzed data sets. Support for nodes in the morphological and molecular partitions is given. A black box demonstrates strong support in all analyses, grey box in some and white box in none (a white box does not necessarily mean conflict, especially in the morphological partition, where resolution and support at deeper nodes is weak). The letters "M" (mitochondrial) and "N" (nuclear) in the molecular partition box show which partition supports the grouping. Boxes at terminal branches test monophyly of genera (monotypic taxa are without boxes at terminal branches). Molecular boxes with a hash mark show genera where too few species were included to enable testing of generic monophyly in nuclear genes. Double lines show genera with questionable monophyly (*Cryptoheros* may be best synonymized with *Hypsophrys*; the monophyly of the '*Heros*' *istlanus* group and of the '*Amphilophus*' *trimaculatus* group is disrupted by a conflict between nuclear and mitochondrial genes). Taxa highlighted in grey represent putative new genera. The '*Heros*' *istlanus* group is the least supported putative genus, held together strongly only by the *cytb* data (clearly requiring further study). The '*Amphilophus*' *trimaculatus* group may be part of *Amphilophus* according to some of our analyses. The placement of the '*Heros*' *wesseli* group as the sister group of the '*Heros*' *sieboldii* group, and the monophyly of the latter is tentative, based solely on the *cytb* data (nuclear data not available for '*Heros*' *wesseli* and '*Heros*' *tuyrensis*). CGH, crown-group herichthyines.

Morphology is by far the most conflicting data partition in our phylogenetic analyses (Fig. 4, Table 5). This result could be attributed to a general lack of phylogenetic information of morphological data at the suprageneric level, likely due to homoplasy and convergent evolution, which are common in cichlids (Kocher et al., 1993; Meyer, 1993; Martin and Bermingham, 1998; Rüber et al., 1999; Rüber and Adams, 2001; López-Fernández et al., 2005a) and found in many instances in our study (see Fig. 8A for a few examples). For instance, in Geophaginae cichlids, phylogentic analyses based on morphological data grouped small-bodied taxa into a monophyletic group, which was rejected based on molecular data (López-Fernández et al., 2005a). Similarly, in our phylogenetic analysis based on morphology (Fig. 4), small-bodied species (with high anal fin meristics; *Archocentrus*, *Herotilapia*, *Cryptoheros*), extreme piscivores (*Petenia*, *Caquetaia*), extreme rheophilic species (*Theraps irregularis*, *Paraneetroplus bulleri*), and genera with a similar benthic life style and morphology (*Astatheros*, *Thorichthys*) were recovered together, respectively, against molecular evidence. In other words, the reconstructed phylogenies support convergent evolution of morpho/ecotypes in different lineages of Middle American heroines (Fig. 8A). Nevertheless, and as expected, there were some common agreements between morphological and molecular evidence, but only at the generic level (e.g. monophyly and relationships within *Herichthys* and *Thorichthys*). Overall, conflicts in the mor-*cytb* analysis (Fig. 5) compared to the total evidence analysis (Fig. 6) were less extensive as judged by PBS (Tables 3 and 5), suggesting some degree of character reinforcement.

4.2. Phylogeny of the tribe Heroini

Nuclear and mitochondrial data partitions arrived at highly congruent topologies, and when combined strongly supported the existence of six main clades of CAM heroines. The monophyly and generic composition of the two largest clades i.e., amphilophines and herichthyines as originally described by Concheiro Pérez et al. (2007) was further supported in the

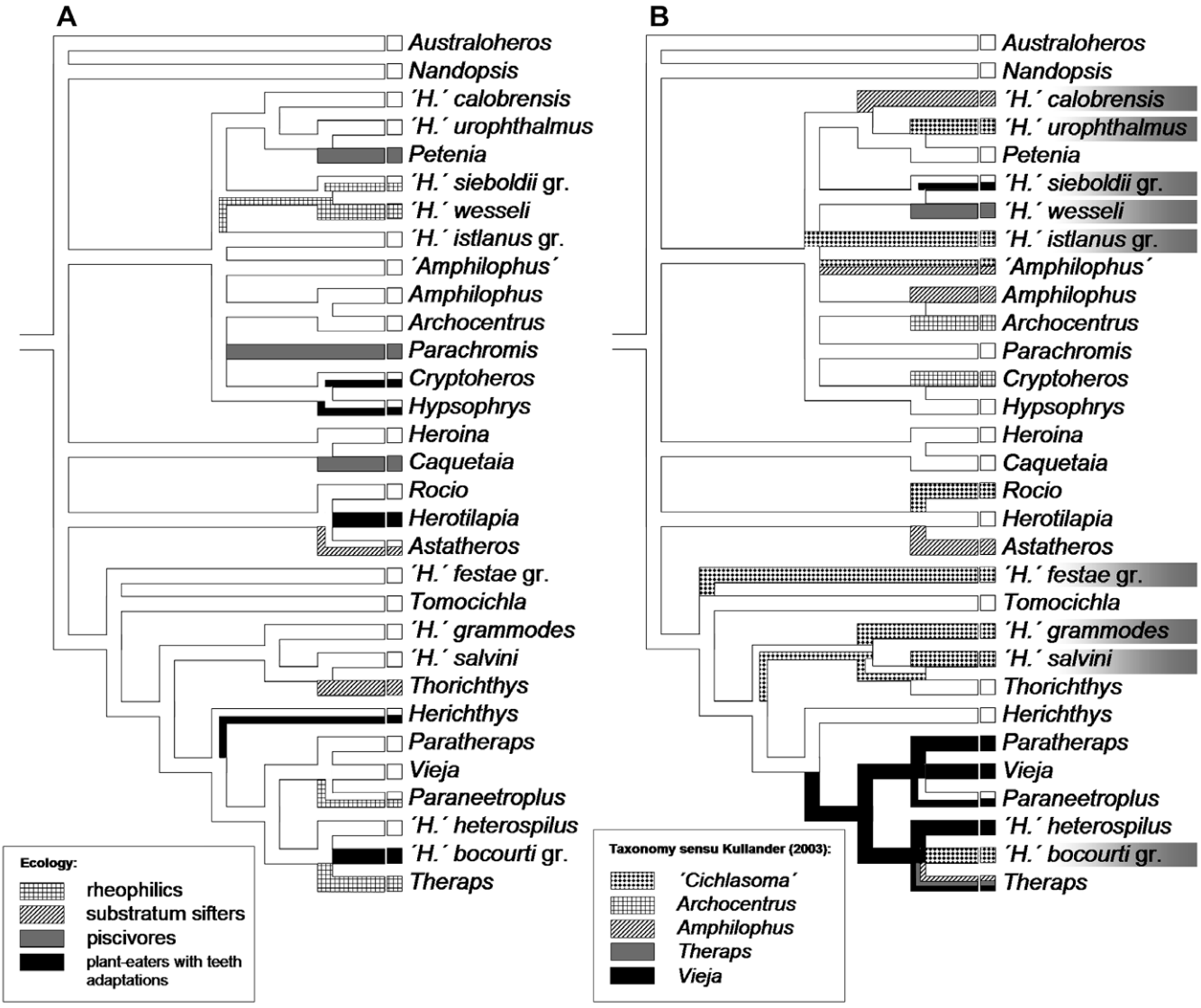

Fig. 8. Important life-history traits and traditional taxonomy mapped onto the consensus tree of all tree-building algorithms and analyzed data sets (see Fig. 7). (A) important life-history traits mapped in Mesquite (Maddison and Maddison, 2004) to demonstrate cases of probable parallel evolution. (B) Traditional taxonomy (the most complex and conflicting cases) mapped in Mesquite (Maddison and Maddison, 2004) onto the consensus tree. Taxa highlighted in grey represent putative new genera.

present study by nuclear genes as well as in the total evidence analyses including also morphology. While there were indications for additional clades in the *cytb* gene phylogenetic analyses, Concheiro Pérez et al. (2007) hesitated about their reality based only on the *cytb* gene. The present study further supports the separate status of *Australoheros* (australoheroines) and *Nandopsis* (nandopsines), and the likely sister group relationship of the former to the remaining CAM heroines. Moreover, a close relationship between *Caquetaia* and *Heroina* (caquetaines), and of *Astatheros* with *Herotilapia* and *Rocio* (astatheroines), and the separate from both main clades was also relatively well supported. Despite our phylogenetic study failed to fully resolve phylogenetic relationships among the six main clades of CAM heroines (Fig. 7), finding first strong evidence that these cichlids can be divided into suprageneric groups is already a big leap forward compared to the stagnation of heroine systematics throughout the last century. In order to achieve higher resolution of deeper nodes in the Heroini phylogeny, future studies will need to incorporate additional sequence data into the phylogenetic analyses while maintaining a thorough taxon-sampling as in the present study.

4.3. Generic diversity among Heroini and Neotropical Cichlidae

Our analyses provide a major insight into the phylogenetic relationships of heroine cichlids, which constitutes a necessary prerequisite for any attempts aimed at solving the highly complex generic taxonomy of the group. The recovered Heroini phylogeny calls for the stabilization of generic taxonomy, and for diagnosability of putative undescribed genera, which will be provided elsewhere (in prep). Several lineages need description as new putative genera, indicating that Heroini would be the most genus-rich clade of Neotropical cichlids. The generic and species diversity of the, predominantly Middle American, heroini is even more striking when considering the area relationship between Middle and South America, the former being only 9.5% of the latter. Middle American Heroini evolved several unique morphologies not encountered or rare in their South American sister group the Cichlasomatini (several cases of rheophily, repeated teeth adaptations for eating plant matter, repeated adaptations for extreme piscivory, the great spectrum in body sizes and shapes; see Fig. 8A), and the Amazonian heroini add to this already wide morphological spectrum the deep bodied and unique

Table 5
Results of bootstrap analysis ($N = 1000$) and partitioned bremer support (PBS) for the total evidence analysis including indels (Fig. 6)

Data/node	1	2	3	4	5	6	7	8	9	10
MP bootstrap	100	86	81	83	96	100	93	54	100	–
BS	110.25	40.5	36.5	40.5	49.25	81.25	39.25	8.25	83	8.25
PBS–mor	15.25	5.5	−13.5	5.5	14.25	31.25	9.25	−46.75	−2	−46.75
RAG1	5	0	0	0	10	10	−5	5	5	5
S7i1	10	0	5	0	−5	0	0	0	10	0
S7i2	20	0	50	0	20	5	15	15	65	15
Cytb	60	35	−5	35	10	35	20	35	5	35
Data/node	11	12	13	14	15	16	17	18	19	20
MP bootstrap	54	100	71	50	100	91	100	91	58	100
BS	18.5	94.75	23.75	17	116.25	60.75	124.75	35	18	120
PBS–mor	−16.5	−15.25	−21.25	−33	16.25	−49.25	−45.25	0	23	25
RAG1	0	−5	−10	0	0	−5	40	0	0	0
S7i1	−10	0	0	5	0	0	25	0	0	0
S7i2	0	10	0	15	0	35	10	0	0	0
Cytb	45	105	55	30	100	80	95	35	−5	95
Data/node	21	22	23	24	25	26	27	28	29	30
MP bootstrap	56	81	100	62	98	95	97	66	–	53
BS	15	33.25	100.25	15	62.25	46.75	56.5	29	2.5	4.5
PBS–mor	5	−11.75	15.25	5	−7.75	26.75	−18.5	19	−2.5	−20.5
RAG1	0	0	0	0	0	−5	15	−5	0	0
S7i1	0	0	0	0	5	0	10	0	0	0
S7i2	0	−5	−15	0	5	0	25	−5	−5	10
Cytb	10	50	100	10	60	25	25	20	10	15
Data/node	31	32	33	34	35	36	37	38	39	40
MP bootstrap	63	52	100	65	–	–	–	73	57	64
BS	26	5.75	140.5	37	37	10	10	30.25	25.25	39.75
PBS–mor	−9	−14.25	−4.5	−8	−8	−30	−30	15.25	−14.75	−55.25
RAG1	0	0	20	5	5	−5	−5	−5	−10	15
S7i1	10	0	30	5	5	−5	−5	0	−5	5
S7i2	25	−10	−10	−20	−20	10	10	5	20	0
Cytb	0	30	105	55	55	40	40	15	35	75
Data/node	41	42	43	44	45	46	47			
MP bootstrap	–	–	100	–	72	100	100			
BS	10	10	216.75	39.75	7.75	204	131			
PBS–mor	−30	−35	6.75	−55.25	−7.25	49	−19			
RAG1	−5	−5	30	15	10	20	60			
S7i1	−5	0	20	5	0	15	20			
S7i2	10	10	80	0	0	0	10			
Cytb	40	40	80	75	5	120	60			
BS		PBS mor		PBS RAG1		PBS S7i1		PBS S7i2		PBS cytb
2516.5		−383.5		205		150		450		2095
100		−15		8		6		18		83

BS, total bremer support. The PBS of individual data sets is expressed also in percent contribution to total BS (below).

Table 6
Tests of alternative hypotheses based on combined molecular and total evidence data sets

Test no	Genus/species group/species	Explanation/alternative hypotheses	Tested data set (length difference)	Compare-2	Test Templeton	Kishino-hasegawa
1	*'Heros' calobrensis group n.gen.*	Part of *Astatheros*	Comb. data (233.5)	0.01*	0.0004*	0.0002*
2		Part of *Amphilophus*	Comb. data (124)	0.01	0.0039*	0.0046
3	*Rocio*	Part of *Andinoheros*	Comb. data (59.75)	0.12*	0.2447	0.2178
4			Molecular data + gaps (16)	0.03*	0.0733*	0.0736*
5			Nuclear genes (13)	0.01*	0.0067*	0.0067*
6	*Archocentrus* (sensu Miller, 1993)	Monophyletic	Comb. data (163.5)	0.01*	0.0005*	0.0007*
7	*Cryptoheros* (sensu Algayer, 2001)	Monophyletic	Comb. data (36.5)	0.01*	0.0780*	0.1366*
8			Molecular data + gaps (10)	0.03*	0.0253*	0.0253*
9	*Australoheros* sister to all CAM heroines	*Nandopsis* sister to all CAM heroines	Comb. data (51.5)	0.01*	0.2407	0.2814
10			Molecular data + gaps (8)	0.24*	0.4675	0.4653
11			Nuclear genes + gaps (11)	0.05*	0.1235	0.1235
12			Nuclear genes (11)	0.03*	0.0870	0.0858
13	*'Heros' istlanus group n.gen.*	Trimaculatus group + *istlanus*	Mor data (63.5)	0.1*	0.1022*	0.0999*
14			Cytb data (34)	0.01*	0.0003*	0.0003*
15		Monophyletic	Nuclear data (6)	0.11	0.0578	0.0578
16			Nuclear genes + gaps (7)	0.12	0.1937	0.1936
17	*Amphilophus*	Trimaculatus group part of *Parachromis*	All genes + MOR (12.25)	0.29	0.5692	0.6122

"Part of" is tested as "sister group of". *Statistically significant test results (at 0.05). References to alternative topologies are in Supplementary Appendix 1.

morphologies of *Pterophyllum*, *Mesonauta*, *Uaru* or *Heros*. Mouthbrooding is the only trait of Cichlasomatini not encountered among Middle American Heroini, but it is present at least in *Heros severus* among South American Heroini. Thus, Heroini appear to be the Middle American counterpart of the South American evolutionary diversification of the Geophagini (López-Fernández et al., 2005a,b).

4.4. Biogeographic implications of Middle American Heroini

Our results show that Middle American Heroini are not a monophyletic group, as already stressed by Concheiro Pérez et al., 2007. Of the major clades, two are *cis*-Andean South American (australoheroines and caquetaines), one is Antillean (nandopsines), and one is entirely Middle American (astatheroines). The two large suprageneric clades (herichthyines and amphilophines) have some basal members in South America or in terrenes classified as South America, but geologically part of Central America (trans-Andean South America), together with more derived Middle American members. Importantly, the areas of distribution of Middle American herichthyines and amphilophines are geographically mutually exclusive around the Motagua Fault in Guatemala, the former being distributed north of it and the latter to the south (Concheiro Pérez et al., 2007), as in other groups such as *Synbranchus–Ophisternon* (Perdices et al., 2005) and some *Rhamdia* (Perdices et al., 2002). Hence, Middle American Heroini diversity results from at least three independent colonizations from South America (amphilophines, herichthyines, astatheroines). The three major heroine clades in Middle America may fulfill the criteria of a radiation, i.e., common ancestry, rapid speciation, phenotype-environment correlation, and trait utility (Schluter, 2000). However, the hypothesis of a radiation of cichlids in Middle America still needs to be tested appropriately (Pybus et al., 2002), as well as whether geographical movement into Middle America triggered shift in diversification rates (Moore and Donoghue, 2007).

According to phylogenetic analyses based on the nuclear gene, combined molecular, and total evidence data sets, *Australoheros* is the most basal CAM heroine clade, and interestingly, it also shows the most peripheral distribution. In some of our phylogenetic analyses, the Antillean *Nandopsis* was placed putatively as the second most basal CAM heroine group. Such relatively basal position of *Nandopsis* would be in agreement with the hypothesis that these cichlids migrated via a land bridge from South America into the Antilles (Iturralde-Vinennt and MacPhee, 1999; Iturralde-Vinennt, 2006), and would reject the phylogenetic hypothesis that Antillean cichlids are deeply nested within the cichlids of Middle America, and originated through a drift vicariance event that took place when the islands became separated from Yucatan (Chakrabarty, 2006). This latter hypothesis was based on a molecular phylogeny (Chakrabarty, 2006) with a rather limited taxon-sampling (e.g. neither the South American *Australoheros* nor *Heroina* or *Caquetaia* were included in the phylogenetic analyses), insufficient to address the biogeographical question at hand.

Following the isolation of the paleo-Amazonas-Orinoco from trans-Andean NW South America due to the rise of the Andes dated at 10.1–11.8 MYA (Lundberg, 1998) no group could probably back colonize *cis*-Andean South America. Based on the results of Concheiro Pérez et al. (2007) and confirmed in this study, invasions of the ancestors of herichthyines, astatheroines, and amphilophines into Middle America were likely concurrent, and took place before the final separation of *cis*- and *trans*-Andean South America, dated at 16–26 Ma (depending on calibration method used; Concheiro Pérez et al., 2007).

5. Conclusions

This study presents the first comprehensive phylogeny of the Middle American Heroini combining both morphological and molecular (mt and nuclear) data. We have for the first time included in the phylogenetic analyses all putative generic lineages as discussed in the literature. Nuclear genes provide more resolution at deeper levels, and arrive at a Heroini phylogeny highly congruent with that based on *cytb* gene data. Morphological characters in combination with *cytb* sequence data provide resolution at the generic level. The total evidence phylogenetic analysis renders a rather resolved phylogeny, which is our best hypothesis for phylogenetic relationships among CAM Heroini. The new phylogenetic framework provided in this study constitutes a major contribution towards stabilization of the taxonomy of Middle American cichlids. Future comparative eco-morphological studies involving heroines should bear on the new phylogeny, whereas previous ones need to be interpreted with caution until revised, since many of the traditionally accepted genera and sister group relationships are found to have no support.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.07.022.

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