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Not a simple case – A first comprehensive phylogenetic hypothesis for the Midas cichlid complex in Nicaragua (Teleostei: Cichlidae: *Amphilophus*)

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ABSTRACT

Nicaraguan Midas cichlids from crater lakes have recently attracted attention as potential model systems for speciation research, but no attempt has been made to comprehensively reconstruct phylogenetic relationships of this highly diverse and recently evolved species complex. We present a first AFLP (2793 loci) and mtDNA based phylogenetic hypothesis including all described and several undescribed species from six crater lakes (Apoyeque, Apoyo, Asososca Leon, Masaya, Tiscapa and Xiloá), the two great Lakes Managua and Nicaragua and the San Juan River. Our analyses demonstrate that the relationships between the Midas cichlid members are complex, and that phylogenetic information from different markers and methods do not always yield congruent results. Nevertheless, monophyly support for crater lake assemblages from Lakes Apoyeque, Apoyo, A. Leon is high as compared to those from L. Xiloá indicating occurrence of sympatric speciation. Further, we demonstrate that a 'three species' concept for the Midas cichlid complex is inapplicable and consequently that an individualized and voucher based approach in speciation research of the Midas cichlid complex is necessary at least as long as there is no comprehensive revision of the species complex available.

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1. Introduction

Cichlid species flocks of the African lakes (Teleostei: Perciformes: Cichlidae) have served for decades as prime model systems for evolutionary biology in general and speciation research in particular (Kocher, 2004; Seehausen, 2006). Along the East African cichlid radiations in Lake Victoria, Malawi and Tanganyika harbour estimated 2000 species (Seehausen, 2006), which all belong to a single major lineage of predominantly Eastern and Southern African cichlids, the Austrotilapiines (Schwarzer et al., 2009). The sheer size of these mega-diverse East African cichlid species flocks as well as the physical size of the three lakes not only holds promises but also constraints as a model system, because it is difficult to sample these systems in toto. Small monophyletic cichlid species flocks endemic to comparatively small lakes, e.g. crater lakes, represent an interesting alternative model system, if the analysis of complete species richness is the focus. Comparatively well known are the monophyletic cichlid flocks of Cameroonian crater lakes with 5–11 species per lake (Schliewen et al., 2001; Schliewen and Klee, 2004; Stiassny et al., 1992; Trewavas et al., 1972). More recently, crater lake species flocks of the Nicaraguan Midas cichlid

complex (genus *Amphilophus*) attracted the attention of evolutionary biologists (Barlow, 1976; Barluenga and Meyer, 2004; Barluenga et al., 2006a; Elmer et al., 2010a; McKaye et al., 2002; Stauffer and McKaye, 2002; Stauffer et al., 2008; Wilson et al., 2000). Both, *Amphilophus* cichlids as well as the Cameroonian crater lake cichlids belong to phylogenetic lineages only distantly related to the East African cichlid radiations and therefore may serve as comparative systems for the analysis of parallel patterns of speciation.

In contrast to the Cameroonian crater lake assemblages, there are no detailed estimates for cichlid species numbers in the Nicaraguan crater lakes, and consequently, there is no established phylogenetic species-level framework for the Midas cichlid species complex. The Nicaraguan lake system is nested within the San Juan River drainage and consists of at least seven crater lakes, which are isolated from the two old rift-lakes Managua and Nicaragua (Fig. 1). Each lake contains several locally endemic Midas cichlid phenotypes or species, which are highly diverse in morphology, color, behavior and ecology (e.g. Barlow, 1973, 1976; Barlow and Rogers, 1978; Elmer et al., 2010b; McKaye, 1977, 1980; McKaye et al., 2002; Wilson et al., 2000; Stauffer et al., 2008). One group of researchers considers only three polymorphic species which are distributed over the whole Lake Nicaragua system including multiple crater lakes, i.e. *A. citrinellus*, *A. zaliosus* and *A. labiatus* (Barlow and Munsey, 1976; Barluenga and Meyer, 2004; Barluenga

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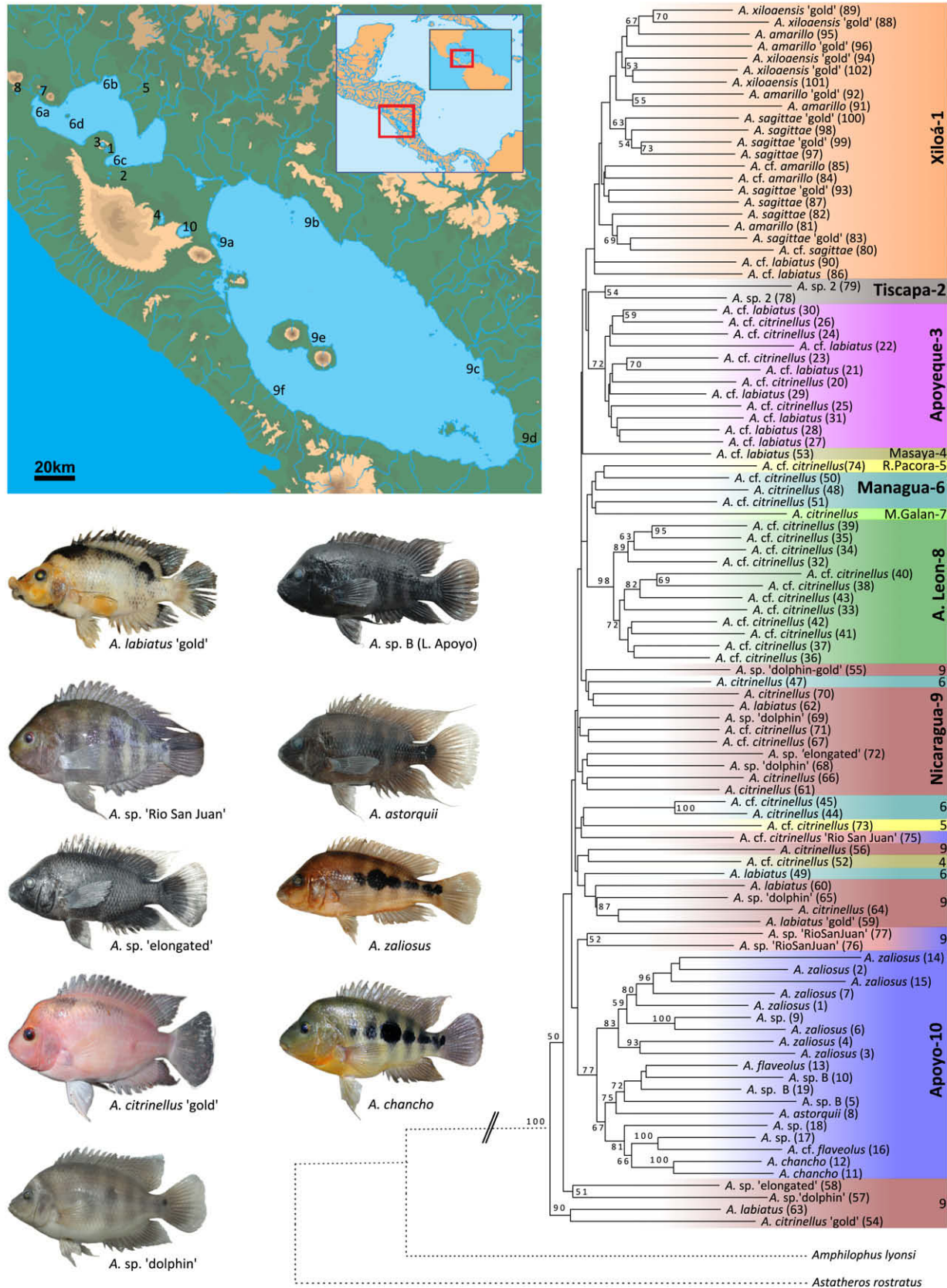


Fig. 1. AFLP NJ-tree with bootstrap supports and sample ID given in brackets, map indicating sample locations and some representative species of the Midas cichlid complex.

et al., 2006a,b; Bunje et al., 2007; Wilson et al., 2000). The opposite view claims that the phenotypic diversity of the Midas cichlid species complex falls into multiple species per lake including the cra-

ter lakes (McKaye et al., 2002; Stauffer and McKaye, 2002; Stauffer et al., 2008). The multi-species concept has now gained wider acceptance as judged from most recent publications (Elmer et al.,

2010a,b; Lehtonen et al., 2010). Depending on the author, the Midas cichlid complex incorporates three and nine species at the moment, but may contain 30 or more species (McKaye et al., 2002; pers. obs.).

All published phylogenetic hypotheses (Barluenga and Meyer, 2004; Barluenga et al., 2006a; Wilson et al., 2000) are based on the aforementioned three species concept (*A. citrinellus*, *A. labiatus* and *A. zalius*) and are built upon mitochondrial haplotype data or few nuclear loci only. This approach has resulted in a debate about the validity of interpretations with regard to the quality of evidence for sympatric speciation in one of the Nicaraguan crater lakes, L. Apoyo (Barluenga et al., 2006b; Schliewen et al., 2006). In particular, it was criticized by Schliewen et al. (2006) that with the given unclear taxon sampling in Barluenga et al. (2006a) including only “*A. citrinellus* Apoyo” and *A. zalius* from L. Apoyo (while there are four described species in L. Apoyo) and with evidence for mitochondrial monophyly alone it cannot be argued that the whole species assemblage of crater L. Apoyo is indeed monophyletic, because mitochondrial monophyly does not necessarily imply species flock monophyly. The null hypothesis of multiple invasion and introgression of mitochondrial lineages into L. Apoyo across species borders remains a valid alternative, which needs to be tested with a fully representative taxon sampling for L. Apoyo and across the whole Midas cichlid flock and the Nicaraguan lakes system and with individualized nuclear multilocus data (Schliewen et al., 2006).

This work presents results from the first, to date most comprehensive survey of the phylogenetic relationships of Nicaraguan Midas cichlids based on 102 specimens representing the phenotypic diversity of the whole species complex, on 2793 AFLP loci from 20 restrictive amplifications as well as a re-analysis of all published mtDNA-data including newly produced control region data. The sampling includes specimens from six crater lakes (Apoyeque, Apoyo, Asososca Leon, Masaya, Tiscapa and Xiloá), several locations around the two large Lakes Managua and Nicaragua as well as from the San Juan River including all formally described (Supplementary Material S1) and several undescribed species from throughout the native range of the species complex.

The study was designed to (1) provide a first comprehensive phylogenetic hypothesis for the Midas cichlid complex, and to (2) test for a monophyletic vs. polyphyletic origin of multiple crater lake assemblages including L. Apoyo. Furthermore, the phylogenetic hypothesis should provide a framework for future taxonomical revisions.

2. Materials and methods

2.1. Taxon sampling and specimen collection

One individual of each, *Amphilophus lyonsi* (aquarium stock) and *Astatheros rostratus* (L. Nicaragua) were chosen as outgroup taxa, their adequacy as a valid outgroup supported by e.g. Concheiro Pérez et al. (2007) or Řičan et al. (2008). All included *Amphilophus* individuals were caught during two field seasons from January to April in 2007 and December to March in 2007/2008. Fishes from crater lakes Apoyo, Apoyeque, Asososca Leon and Xiloá were caught SCUBA diving or snorkeling with harpoon while material from lakes Managua, Masaya, Monte Galan, Nicaragua and Tiscapa was caught with gill nets (40 mm mesh-width).

Where possible, described species were identified according to the most recent species descriptions, or in other cases, where labeled using working names. Fishes from Lakes Managua and Nicaragua with greatly enlarged lips with “moveable subtriangular flap” as presented in the primary description of *A. labiatus* (Günther, 1864) were assigned to that species whereas individuals with

only fleshy but still conspicuously lips were labeled as *A. cf. labiatus*. The addition “gold” to individuals labeling describes fishes that have lost their melanophores as adults and thus their pattern of black bars and spots.

Fishes were anesthetized and killed using an overdose of clove oil and a fin-clip was taken and stored in 96% EtOH for molecular genetic analysis. Each specimen was photographed to document live-coloration and preserved with pinned fins in 4–10% formaline. Individual whole body and tissue vouchers are stored permanently at Bavarian State Collection Munich (ZSM) with their respective collection numbers (Supplementary Material S1).

2.2. Morphometry

A total of 647 adult individuals from eight lakes and two rivers belonging to nine described and several undescribed potential species of Midas cichlids were used to estimate phenotypic variability as reflected in body shape differentiation. Fifteen landmarks were positioned on digital images of carefully preserved fish-bodies, based on a modified protocol from two other studies applying the method to Midas cichlids (Klingenberg et al., 2003; Parsons et al., 2003). For morphometric data acquisition the TPS software package (Rohlf and Marcus, 1993; Rohlf, 2006a,b) was used. Principal components analysis (PCA) based on the partial-warp scores was applied to examine variation in body shape among individuals. PCA is part of the IMP package (Sheets, 2003), and was used after removing non-shape variation via a “Generalised least squares Procrustes superimposition” (GLS). Procrustes superimposition scales specimens to a unit size, translates them to a common location and rotates them to their corresponding landmarks line up as closely as possible, thus removing artificial variation (non-shape variation) between specimens based on differences in size and position on the picture. By plotting the 2nd vs. the 3rd principal component of the partial-warp scores the total morpho-space occupied by the Midas cichlid complex was obtained (Fig. 2). For a more comprehensive description of geometric morphometrics see Zelditch et al. (2004) and the literature aforementioned.

2.3. DNA extraction, PCR and mtDNA- and AFLP genotyping

Genomic DNA was extracted using the Quiagen® DNeasy® 96 Tissue Kit for animal tissues according to the protocol provided by the manufacturer. For a total of 177 individuals from throughout the native range of Midas cichlids in Nicaragua part of the mitochondrial control region was amplified using previously published primers and protocols as well as one newly designed primer: L15995 (Meyer et al., 1994), H00651 (Kocher et al., 1989) and H00834 (5'-ATATACACATGTACGTAAG-3'). PCR conditions were as follows: 15 min at 95 °C; then 39 cycles of 95 °C for 30 s, 58 °C for 90 s and 72 °C for 90 s, followed by 72 °C for 10 min. Sequencing of the ~790 bp long fragment was done at the sequencing service of the Department of Biology of the Ludwig Maximilian University (Munich), using the Big Dye v.3.1 Kit and primers L15995, H00834 and H00498 (5'-GAACCCCTTGCCCGCTAGAAA GAAT-3').

Using a modified protocol of the original AFLP method of Vos et al. (1995) as suggested in Herder et al. (2008), a set of representative 104 individuals was genotyped with 20 selective amplifications using enzymes EcoRI and MseI with three selective bases (Supplementary Material S2). Fragment size was determined on an AB 3130 capillary sequencer with an internal size standard (ROX 500 XL). A bin-set was created in Genemapper® 4.0 with bin-width set to 0.75 bps and rfu threshold to 50 for each selective amplification, ranging between 50 and 500 bp in fragment length. Bins containing inconsistently produced fragments were identified using 10 replicate samples which have been run either twice

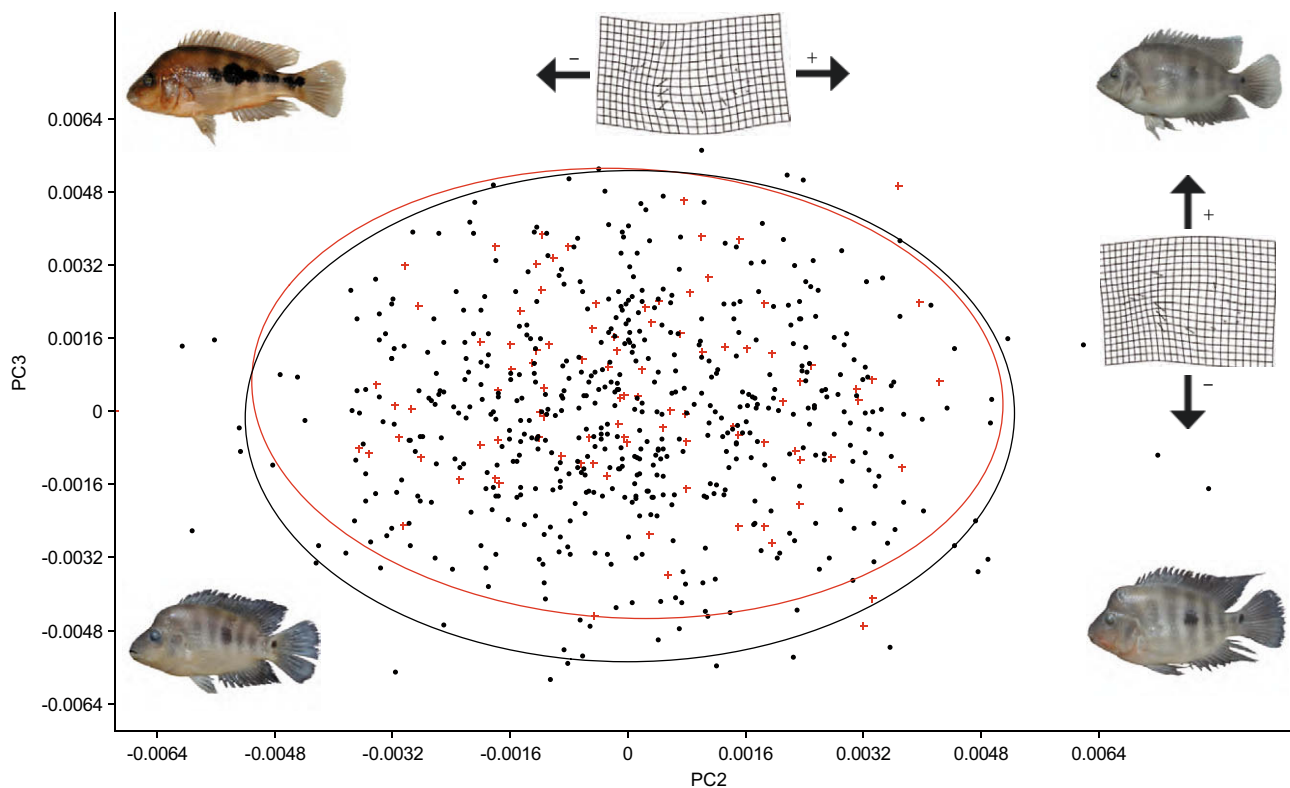


Fig. 2. Plot of the second vs. the third principal component of the partial-warp scores from the geometric morphometric landmark analysis (647 individuals) with deformation grids for each axes and representative individuals. Black ellipse: 95% confidence interval of the whole sample. Red ellipse: 95% confidence interval for the specimens used in the molecular genetic study.

($n = 6$) or three times ($n = 4$). Inconsistent bins were removed before exporting a 0/1 (absence/presence) matrix into a spreadsheet program. In addition, all pairs of neighboring bins whose minimum distance between each other was less than 0.25 bps and also bins containing fragments differing more than 0.65 bps in size were removed (Albertson et al., 1999). Finally, those bins with fragments that differed by more than 20% relative frequency between the two runs were removed. This last step in primary data acquisition decreases rather than increases the likelihood of detection of population structure and was chosen to prune the data set from plate specific effects deriving from the fact that samples were run in two batches. The resulting binary data matrix had 2793 characters.

2.4. Phylogeny reconstruction

Mitochondrial DNA sequence data were combined for phylogenetic analysis with published control-region sequence data from Barluenga and Meyer (2004); (GenBank Accession Numbers AY567011.1–AY567470.1) and Barluenga et al. (2006a); (GenBank Accession Numbers DQ229964.1–DQ230081.1). A first alignment of sequences was conducted by hand using BioEdit version 7.0.5.3 (Hall, 1999), a final alignment was created in ClustalX (1.81) with default settings (Thompson et al., 1997). Several poly-nucleotide stretches were removed from the final alignment as resulting gaps could not unambiguously be aligned: a poly-A stretch (3–4 As) at position 162, a poly-T stretch (11–12 Ts) at position 497, a poly-A stretch (6–8 As) at position 582 and a poly-C stretch (7–8 Cs) at position 701 with nucleotide-positions referring to published sequence DQ229964.1. The pruned final alignment contained 752 sequences and 720 bp including gaps. Haplotype-frequencies were calculated using Collapse 1.2 (Posada, 2004) with default settings, i.e. treating gaps as 5th state. Using the pruned alignment with only one sequence per haplotype, a first median-

joining haplotype network containing all shortest least complex phylogenetic trees (all maximum parsimony or MP trees) was constructed using Network 4.5.10 following Bandelt et al. (1995, 1999) with default settings (epsilon = 0). To reduce complexity and improve clarity, haplotypes in the first network that were directly connected to the central haplotype and did not visualize more distant interconnections with other haplotype-groups were identified, excluded and assembled in a separate alignment. In a second step a separate alignment was created with only the remaining sequences. Further simplification of the second network based on the reduced alignment was achieved by choosing the star-contraction algorithm in Network 4.5.10 with delta set to 1 (contraction radius in number of mutated positions).

For the tree reconstruction based on AFLP data the software package TREECON 1.3b was used (Van de Peer and De Wachter, 1994). The Link et al. (1995) distance measure was chosen to compute a pairwise genetic distance matrix based on the binary AFLP matrix and to generate a neighbor-joining tree with 2000 bootstrap replicates. Link's formula is based on Jaccard's similarity measure and takes into account only shared and unique bands while absent bands are ignored. This is especially important for AFLP data since the absence of a band in the final data matrix may have several more reasons as compared to the presence of a band. The obtained topology was compared with NJ-tree reconstructions based on (1) Nei and Li genetic distance (Nei and Li, 1979) implemented in TREECON 1.3b and (2) to the NJ-tree based directly on Jaccard's similarity measure (Jaccard, 1908) calculated in FAMD (Schlüter and Harris, 2006).

To assess overall robustness of AFLP based phylogenetic hypothesis, and to explore alternative branching-patterns leaf-stability (LS) and lineage-movement (LM) indices for each single taxon and whole clades were calculated in Phyutility v.2.2 (Smith and Dunn, 2008). The LS index measures the consistency of each tax-

on's position across a chosen number of bootstrap replicates. A value of 1 would indicate that the individual's position in the topology is stable and equal in all examined trees. The LM index calculates branch attachment frequencies of selected clades from alternative tree topologies thus identifying where a lineage is falling alternatively to its position in the tree based on the complete (non-bootstrapped) matrix (Smith and Dunn, 2008). Bootstrap replicates were generated with Phylogenetic Computer Tools v. 1.3 (Buntjer, 1997–2001) by first generating 1000 new 0/1 matrices (with 35% loci replaced) and then calculating 1000 distance matrices based on Jaccard's distance coefficient. Neighbor and Consense from the software package Phylip (Felsenstein, 2004) were then used to generate NJ-trees and a consensus tree from 1000 bootstrap replicates.

To test for homoplasy-excess possibly introduced by hybrid taxa, a tree based method as suggested by Seehausen (2004) was applied. The inclusion of a hybrid taxon introduces homoplasy with clades that include the hybrid's parental lineage due to their mosaic composition of the genome. Removal of a hybrid should decrease the amount of homoplasy and thus increase bootstrap support for clades containing hybrid parents or their descendents. Conversely, removal of non-hybrid taxa should not affect bootstrap support of other nodes. To identify potential terminals of hybrid origin and to assess robustness of clade stability each single taxon was excluded manually and bootstrap support values recalculated in TREECON 1.3b from 1000 replicates using the Link et al. algorithm. In addition, Jackknife-Monophyly indices (JMI) were calculated for specimen groups sampled from single lakes as the percentage of occurrence as monophyletic unit in 225 taxon-removal experiments as suggested in Siddall (1995) and Krüger and Gargas, 2006. To that purpose (1) each of the 102 individuals, (2) 100 times 23 randomly chosen individuals and (3) 1–23 random drawn individuals were removed and bootstrap support recalculated.

For visualization of conflicting phylogenetic signal the Link et al. distance matrix was used to create a phylogenetic network based on the neighbor-net algorithm (Bryant and Moulton, 2004) as implemented in SplitsTree (Huson, 1998).

2.5. Inference of genetic structure

Structure 2.2 (Pritchard et al., 2000; Falush et al., 2007) was used to identify the number of differentiated clusters without a *a priori* group designation based on the AFLP data. Structure uses an individual-based Bayesian algorithm to identify genetically homogeneous groups. After an exploratory pilot phase, the admixture-model with correlated allele-frequencies (Falush et al., 2003) was chosen, and α (Dirichlet parameter for the degree of admixture) and λ (parameter of allelic frequencies distribution) were set to be inferred from the data (i.e. not fixed). Each run consisted of a burn-in period of 50,000 followed by 250,000 iterations of data-collecting for posterior-probability estimates. All runs for each single K (number of populations or clusters) were replicated at least 20 times, where K ranged from 1 to 15. Runs that did not converge during the burn-in phase were identified with the help of log. probability vs. iteration plots and removed from further analysis. Complementary, the approach of Evanno et al. (2005) was applied to detect the uppermost hierarchical level of genetic structure by calculating ΔK from the Structure output 'LnP(D)' (see Evanno et al., 2005).

Finally, the structure of genetic diversity was investigated using hierarchical AMOVAs as implemented in Arlequin 3.11 for both, the mtDNA and AFLP data independently. Molecular variance was estimated among and within (1) all lake populations (nine for the mtDNA and six for the AFLP data) and (2) the crater lakes and a 'super-lake' sample consisting of all L. Managua, L. Nicaragua

and Tisma samples. The 'super-lake' samples were pooled together because historically L. Managua was connected to L. Nicaragua via the nowadays swampy Tisma area (e.g. Villa, 1976a). Genetic differentiation between geographically isolated populations (lakes) was estimated using F -statistics on uncorrected p -distances (Weir and Cockerham, 1984) as implemented in Arlequin 3.11, and their significance tested by permutating haplotypes among populations.

3. Results

3.1. Morphometrics

Results from the geometric landmark approach helped to assess the representativeness of individuals included in the genetic analysis based on their position in the complete morpho-space of the Midas cichlid complex. The plot of the morpho-space occupied by all to date described and several undescribed *Amphilophus* species in comparison with the morpho-space of all genetically analyzed specimens demonstrates that the majority of phenotypic variability for our molecular genetic analysis was captured (Fig. 2). The 2nd and 3rd principal axes together explain 32.35% of total variance, PC 2 17.91% and is mainly associated with body-depth as depicted in the corresponding deformation grid. Principal component 3 accounts for 14.41% of total variance and is related to differences in the position of the pectoral and pelvic fins as well as differences in head shape. As it was not the aim of the present work to delimit species or morphotype boundaries, results of further detailed morphological comparisons are not presented.

3.2. Phylogeny

The recovered NJ-phylogeny mirrors the geographic distribution of the Midas cichlid members and demonstrates the complex situation within the *A. cf. citrinellus* complex from lakes Managua and Nicaragua. The NJ-tree based on the Link et al. (1995) distance measure on 2793 AFLP loci (1351 polymorphic for the in-group) reflects the geographic isolation of the crater lake populations (Fig. 1). Independently of which distance-model is applied, the main topology did not differ between the tree estimation methods, only the L. Xiloá species assemblage is not monophyletic using Nei and Li's distance measure (not shown).

Several clades with a strong signal for monophyly were nested within the complex relationships of the great lakes populations: L. Apoyo (JMI 96.5%), L. Apoyeque (JMI 100%) and A. Leon (JMI 100%) all had bootstrap supports above 70%. For L. Apoyo there is a clear separation of *A. zalius* and the remaining species in that lake. Lake Xiloá's species flock also appears monophyletic (JMI 89%) but is not strongly supported with a high bootstrap value.

Lake Nicaragua's species assemblage does not constitute a monophyletic group as it contains the four most basal taxa of the in-group, belonging to four different putative species (Fig. 1). Interestingly, individuals of L. Nicaragua do not cluster according to their geographic or taxonomic identity while in contrast individuals from L. Managua are largely grouped according to their geographic origin within the lake basin – a preliminary indication of restricted gene-flow between populations from opposing shores. Lake Managua's close affinity to L. Nicaragua's fauna is reflected in the phylogeny, as four of the seven analyzed individuals cluster with L. Nicaragua individuals. The remaining three cluster with the sample from M. Galan and one individual from R. Pacora as sister-group to the A. Leon clade, in line with their geographic affinities.

Only two individuals from L. Tiscapa and L. Masaya could be included in the AFLP analysis and while the two L. Tiscapa's samples are the sister-group to the L. Apoyeque clade, individuals from L.

Masaya do not form a unit. Out of the three specimens from the Rio San Juan, two cluster as sister-group to the monophyletic L. Apoyo assemblage and one individual clusters with one Rio Pacora and two L. Managua individuals.

The in-group neighbor-net derived from genetic distances calculated with the Link et al. algorithm (1995) shows the same major monophyletic groups as the NJ-tree but also indicates that there is conflicting signal at the base of each putative intra-lake radiation and especially at the base of the L. Apoyo Assemblage (Fig. 3).

Among all 752 mtDNA control-region sequences included in this study 201 different haplotypes were identified. Due to the high degree of complexity and many cross-linked terminals it was not possible to combine all 201 discovered mtDNA-haplotypes into one readable MP median-joining network. Thus, the first network (Fig. 4) shows only those 79 haplotypes that are connected to the central haplotype 'A' without further complex branching. Except for samples from L. A. Leon all sampled populations contain haplotypes that are closely (≤ 3 mutations) related to the central haplotype 'A'. Almost one quarter of the 752 individuals (22.74%) from eight different lakes and several species (e.g. *A. citrinellus*, *A. labiatus*, *A. amarillo*, *A. sagittae*, *A. xiloensis*) carry the most common haplotype 'A'. As shown in Fig. 5 the relationships within the Midas cichlids based on the mtDNA are complex. Lakes Apoyo and A. Leon contain only private haplotypes whereas all other populations share a variable amount of mitochondrial haplotypes.

3.3. Genetic variation

Investigation of within- and between-lake variation using hierarchical AMOVA with the mtDNA-haplotype data showed that most ($\sim 73\%$) variation is attributable to within lake differentiation, whereas only $\sim 27\%$ can be explained by between-lake differences and only $\sim 5\%$ remain when comparing crater lakes with the 'super-lake' (Lakes Managua, Nicaragua and Tisma lagoon as one group, Table 1). Results from AMOVAs with the AFLP data were comparable to those obtained from the mtDNA: genetic variation was par-

itioned as $\sim 16\%$ among and $\sim 84\%$ within lakes (Table 2). Grouping samples together into the 'super-lake' vs. crater lakes did not deliver any significant variation that would explain those groups.

Comparisons of pairwise genetic distances between Midas cichlid populations from different lakes based on both mtDNA-haplotypes and AFLP loci detected strong differentiation between those populations, partly reflecting the assumed colonization history. Low levels of genetic differentiation between our L. Managua and L. Nicaragua samples expressed in low pairwise F_{st} values derived from the AFLPs reflects the geographic history of the two once permanently connected lakes. Pairwise F_{st} comparisons to the Tisma sample show both old lakes to be genetically close to the population that probably also nowadays connects the two lakes during periods of floodwaters.

Population pairwise F_{st} values for the mtDNA were all significant except for pairs L. Apoyeque/L. Xiloá, L. Managua/Tisma and L. Apoyeque/L. Nicaragua and ranged between 0.003 and 0.774 (Table 3). Population specific F_{st} values were very similar for each lake and ranged between 0.266 and 0.280 (Table 4) indicating that each single lake population contributes equally to the global F_{st} of 0.270. All pairwise F_{st} values from the AFLP data were significant (Table 5) and range from 0.04 to 0.25. Lake-specific F_{st} values were again very homogeneous (0.151–0.173) and show that all groups do equally support the global F_{st} of 0.160 (Table 4).

3.4. Structure analysis of AFLP data

Results from the Bayesian cluster analysis with Structure v2.2 are largely congruent with the findings from our NJ-tree reconstruction and point to the presence of hierarchical genetic structure. Applying Evanno's (2005) method to estimate the number of genetic clusters via ΔK , a strong signal for $K = 2$ was observed, dividing the whole sample into two clusters: Lake Apoyo individuals in one and the remaining in the other cluster with only few admixed individuals in each cluster (Fig. 6). In line with Structure's direct output $\text{LnP}(D)$ (Fig. 7), the highest log-likelihood probabili-

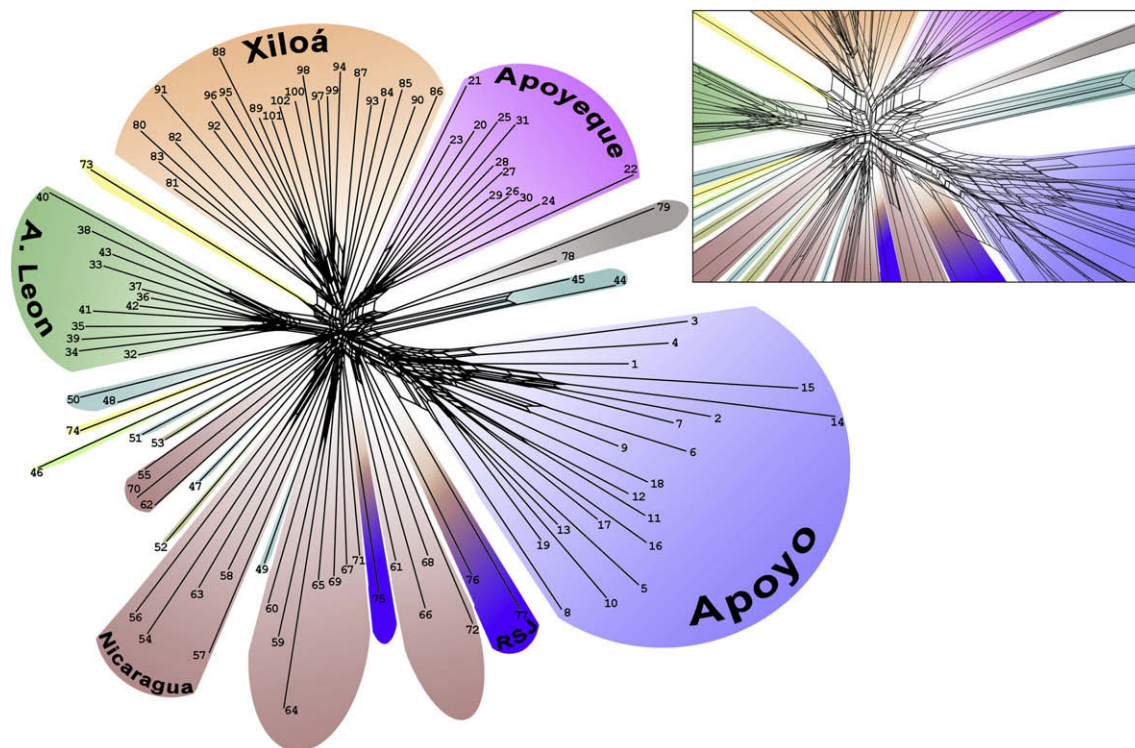


Fig. 3. AFLP neighbor-network based on Link's genetic distances. Sample IDs are those used throughout. Conflicting phylogenetic signal in the center magnified top right.

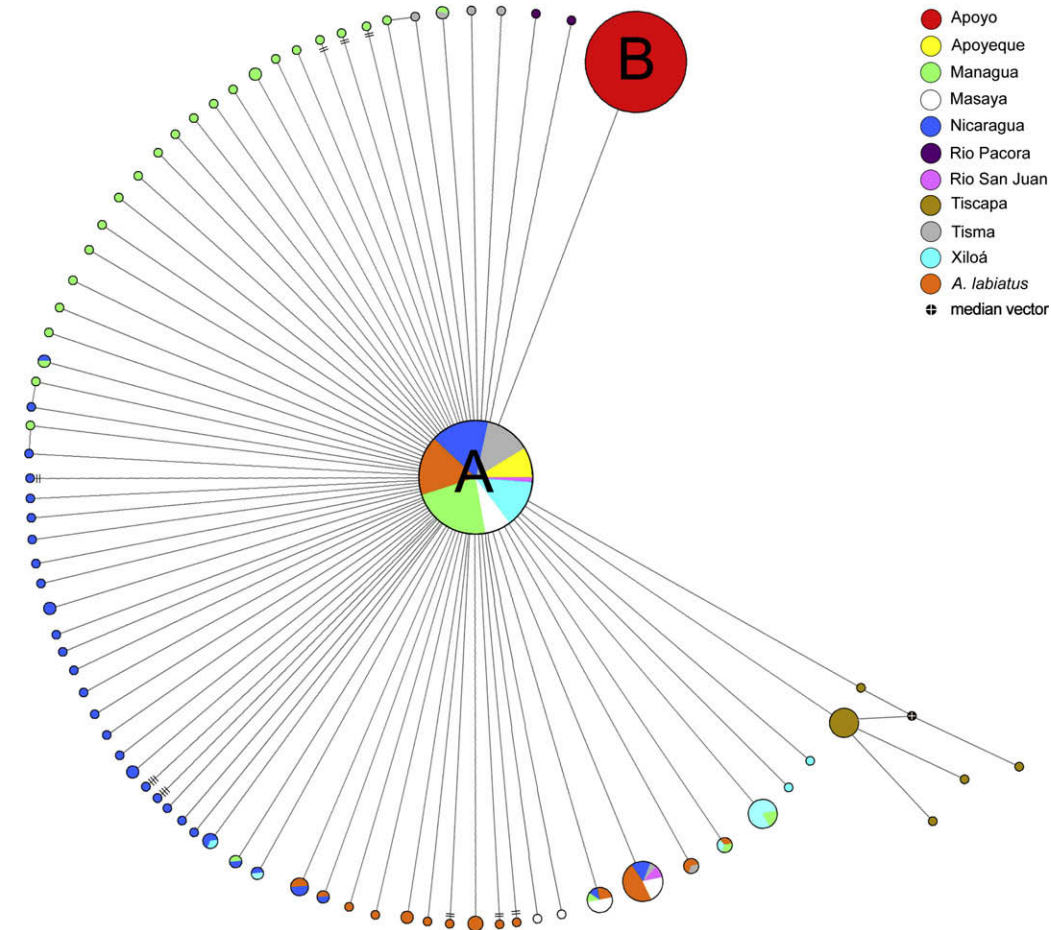


Fig. 4. Median-joining parsimony-network based on the mitochondrial control-region. Included is the central haplotype and only those directly connecting it without further complex branching. Circle size corresponds to sample size, one bar indicates one additional mutational step.

ties were detected for $K = 7$ and were confirmed with a second peak of ΔK at $K = 7$ (Fig. 7). All 20 replicates for $K = 7$ revealed the same major, cohesive genetic clusters (mean proportion of membership of each population in each of the 7 clusters in parenthesis): two for L. Apoyo (43% and 53%), one for L. Apoyeque (96%), one for A. Leon (95%), one for L. Managua (80%), two for L. Nicaragua (varying) and one for L. Xiloá (81%) with a high proportion of admixed individuals in that particular lake. As depicted in Fig. 6 Structure is highly accurate in grouping individuals from the same lake into a common cluster: for $K2$ the main groups are L. Apoyo vs. remaining individuals, for $K3$ a third cluster appears for all A. Leon samples, for $K4$ samples from L. Apoyeque and L. Xiloá are grouped together in a new cluster, for $K5$ the L. Apoyeque and L. Xiloá individuals are separated, for $K6$ a separation of *A. zaliosus* individuals from the remaining L. Apoyo samples is visible and for $K7$ a new cluster appears within the L. Nicaragua group.

Three individuals, *A. sp.* 'elongate' (ID: 72) from L. Nicaragua (9c) and two *A. sp.* 'Rio San Juan' (IDs: 76 and 77) showed admixture proportions with L. Apoyo's high-bodied species above 10%. Another three individuals from L. Nicaragua and L. Tiscapa (IDs: 57, 58, 79) showed also elevated levels of admixture, too, but are associated with the cluster containing *A. zaliosus* (25–50%, Fig. 6). Only one individual from L. Managua, *A. labiatus* (ID: 49) shows high admixture proportions from L. Managua, L. Nicaragua and L. Apoyeque clusters (Fig. 6).

Investigating further substructure for single lakes with reasonable sample size available, the whole AFLP sample was divided and separate cluster-analyses for L. Apoyo ($n = 19$), L. Nicaragua ($n = 22$) and L. Xiloá ($n = 23$) conducted. However, given the poten-

tial species level complexity of each of the lakes, such an analysis with only comparatively few individuals has to be considered preliminary. Nevertheless, there is evidence for presence of further structure partly reflecting taxonomic placement of single individuals: all *A. zaliosus* from L. Apoyo were grouped together in one cluster with only one individual showing mixed ancestry, a second cluster comprises two *A. chancho* and three morphologically not clearly assignable individuals and a third cluster contains *A. astorquii*, *A. flaveolus* and an undescribed species (*A. sp. B*). One individual unidentifiable to species level from L. Apoyo (ID: 9) showed a clear pattern of admixed origin, with alleles derived from all three clusters (Supplementary Material S3 and S4).

For the L. Nicaragua sample a model with $K = 3$ yielded the highest likelihood value (Supplementary Material S3 and S4) but did not group individuals according to current taxonomical classification nor to our *a priori* phenotypic assignment nor to geographic origin. Interestingly, individual number 77 (*A. sp.* 'Rio San Juan') whose removal had a strong effect on L. Apoyo's monophyly support (bootstrap raised from 77 to 94, Fig. 8) and which shows a clear sign of admixture in the cluster plot for the whole AFLP sample (Fig. 6) was assigned to one single cluster in the divided dataset (Supplementary Material S4).

Lake Xiloá, containing three described *Amphilophus* species, was found to contain four genetic clusters (Supplementary Material S3 and S4), partly reflecting taxonomic assignment and geographic origin: one cluster contained only *A. sagittae* from the southern shore (golds and normals), a second cluster *A. amarillo* and *A. xiloaensis* from the southern and southeastern shore, and two more containing all species except *A. xiloaensis* from northern and northeastern

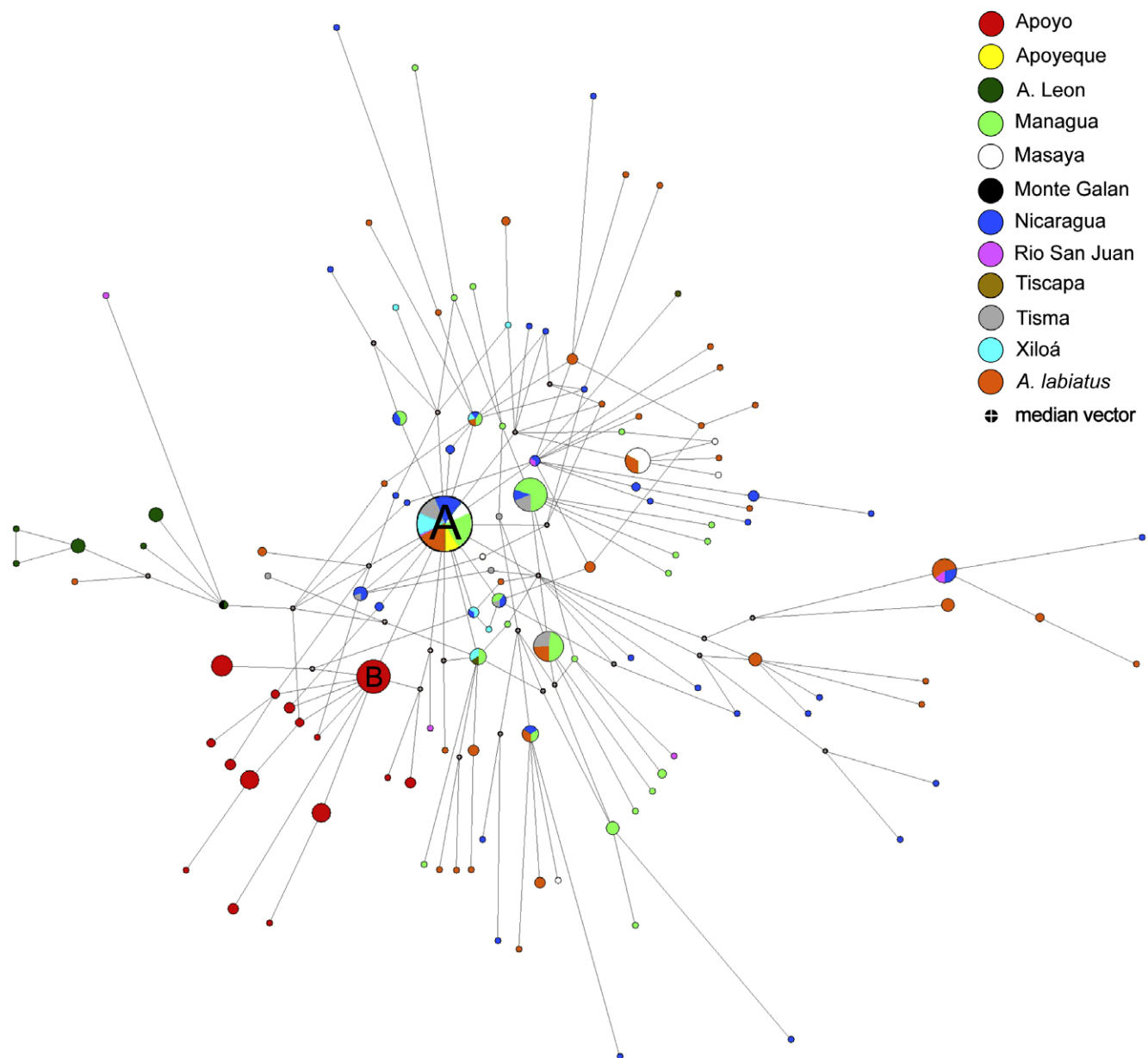


Fig. 5. Full median-joining parsimony-network based on the mitochondrial control-region. Included is the central haplotype and all remaining haplotypes connecting with further complex branching not shown in Fig. 4.

Table 1
AMOVA based on the mtDNA control region data.

Source of variation	df	Percentage of variation
Among lakes	8	27.04*
Within lakes	737	72.96*
Among crater lakes and super-lake**	1	4.62
Among lakes	7	23.69*
Within lakes	737	71.69*

* Significant (10,100 permutations).

** Super-lake: Lake Managua, Nicaragua and Tisma lagoon samples as one group.

Table 2
AMOVA based on the AFLP data.

Source of variation	df	Percentage of variation
Among lakes	5	15.96*
Within lakes	90	84.04*
Among crater lakes and super-lake**	1	0
Among lakes	4	18.10*
Within lakes	90	85.44*

* Significant (10,100 permutations).

** Super-lake: Lake Managua and Nicaragua samples as one group.

shore. Four individuals showed admixture proportions higher than 25%, interestingly all belonging to the three described species.

3.5. Homoplasly excess, leaf-stability and lineage-movement

In addition to standard bootstrapping, homoplasly excess introduced by single individuals with potential hybrid background was

tested and stability of the placement of single terminals estimated. Several specimens were identified that showed signs of admixed origin or whose position was less strongly supported than others.

While all individuals from L. A. Leon had similar LS values ($m = 0.69 \pm 0.016$ SD, [Supplementary Material S1](#)) and also the clade's bootstrap support did not vary during the Jackknife removal

Table 3Pairwise *F*_{st} values based on the mtDNA control region, min. and max. in bold.

	Apoyo	Apoyeque	A. Leon	Xiloá	Masaya	Tiscapa	Managua	Nicaragua	Tisma
Apoyo		*	*	*	*	*	*	*	*
Apoyeque	0.50640		*	*	*	*	*	*	*
A. Leon	0.76033	0.77445		*	*	*	*	*	*
Xiloá	0.49559	0.01699	0.65923		*	*	*	*	*
Masaya	0.49071	0.10179	0.52742	0.12222		*	*	*	*
Tiscapa	0.59500	0.36875	0.61739	0.29823	0.24263		*	*	*
Managua	0.46531	0.19919	0.36250	0.21524	0.24646	0.26910		*	*
Nicaragua	0.29983	0.00342	0.36535	0.01905	0.06701	0.13010	0.16447		*
Tisma	0.49252	0.12116	0.41298	0.14028	0.17491	0.22320	0.02181	0.06837	

* Significant (10,100 permutations).

Table 4

Summary genetics.

Lake	mtDNA control region			<i>F</i> _{st} Arlequin	2793 AFLP loci	
	<i>n</i> (individuals)	<i>n</i> [*] (haplotypes) total/private	<i>F</i> _{st} Arlequin		<i>n</i> (individuals)	<i>F</i> _{st} Arlequin
Apoyo	184	15/15	0.277	19	0.151	
Apoyeque	15	1/–	0.280	12	0.170	
Asososca Leon	14	6/5	0.275	12	0.173	
Managua	139	51/37	0.266	7	0.161	
Masaya	51	10/6	0.271	2	–	
Nicaragua	237	67/49	0.266	22	0.150	
Tisma	44	14/6	0.270	–	–	
Tiscapa	9	7/6	0.272	2	–	
Xiloá	45	13/5	0.276	23	0.163	

* Number of haplotypes inferred from the median-joining networks.

Table 5Pairwise *F*_{st} values based on the AFLP data, min. and max. in bold.

	Apoyo	Apoyeque	A. Leon	Xiloá	Managua	Nicaragua
Apoyo		*	*	*	*	*
Apoyeque	0.24512		*	*	*	*
A. Leon	0.24396	0.22916		*	*	*
Xiloá	0.21153	0.12478	0.20351		*	*
Managua	0.14645	0.12566	0.15541	0.10955		*
Nicaragua	0.14601	0.11397	0.13879	0.10638	0.04426	

* Significant (10,100 permutations).

experiments (Fig. 8), there were several conspicuous individuals identified from other lakes.

Exclusion of one *A. cf. labiatus* from L. Apoyeque (ID: 22) with low LS index (0.547 vs. $m = 0.68 \pm 0.0482$ SD) lead to an increase of the Apoyeque clade's bootstrap support from 72 to 83 (Fig. 8). Nevertheless, investigating its position in the NJ-tree with the LM procedure showed it to cluster within the Apoyeque clade in 100%. Removal of another L. Apoyeque individual, an *A. cf. citrinellus* (ID: 23) with an average LS index (0.70) lead to a decrease of the Apoyeque clade's bootstrap support from 72 to 59 when excluded (Fig. 8).

Removal of several individuals had a strong influence on monophyly support for the L. Apoyo clade (Fig. 8): exclusion of *A. sp.* 'Rio San Juan' (ID: 77), originally in the sister-group to the Apoyo species flock (Fig. 1) and connected to the central Apoyo-haplotype 'B' in the complete mtDNA network via two median-vectors and three single substitutions, leads to a strong increase in L. Apoyo's bootstrap support from 77 to 94 (Fig. 8). The Structure plot (Fig. 6, K7) shows that this particular individual from the San Juan River is assigned to two clusters, L. Apoyo non-*zalius* and L. Managua–Nicaragua, indicating an admixed genotype. In addition, its peculiar position is detectable in the neighbor-network (Fig. 3) and also supported by the lowest overall detected LS value (0.486, Supplementary Material S1) indicating uncertain and a weakly supported positioning in the NJ-tree. Alternative positions in the NJ-tree deduced from the LM procedure showed individual

77 to appear within the Lake Apoyo non-*zalius* group in 12% out of 1000 bootstrap replicates and in 11.5% as the basal sister taxon to Apoyo's species flock. In the remaining 25% it clusters with several different other L. Nicaragua individuals. Interestingly, exclusion of *A. sp.* (ID: 18) from L. Apoyo in the homoplasy-excess test renders L. Apoyo paraphyletic due to the aforementioned *A. sp.* 'Rio San Juan' (ID: 77) that appears as the basal sister taxon to the non-*zalius* group. This particular specimen from L. Apoyo does not show an out of average LS value (0.693, Supplementary Material S1) and its position in the NJ-tree is stable, being placed outside the L. Apoyo flock in only 3% of 1000 bootstrap replicates.

Finally, another unidentified individual from L. Apoyo (ID: 9) showed admixture ancestry, but is nested within the *A. zalius* clade in the NJ-tree (Fig. 1) with high bootstrap support (100%) and average leaf-stability value (0.736, Supplementary Material S1). The same individual was interesting in the homoplasy-excess test, too: its exclusion decreased L. Apoyo's monophyly-bootstrap support from 77 to 60 (Fig. 8).

4. Discussion

4.1. Genetic structure

The general pattern and magnitude of genetic variance discovered is largely congruent with that presented earlier (Wilson et al., 2000; Barluenga and Meyer, 2004; Bunje et al., 2007), revealing high levels of genetic differentiation between Midas cichlid populations from different lakes. This reflects (1) the geographic isolation of the crater lake populations, and (2) the close affinities between the Midas cichlid populations of the two great lakes. Genetic differentiation between color morphs from L. Apoyo presented in Wilson et al. (2000) is mirrored in our NJ-tree reconstruction as well as the Bayesian cluster analysis with the difference that here current taxonomic status was considered and not only color. It is assumed that the L. Apoyo sample in Wilson et al. (2000) was comprised of *A. zalius* (termed 'normal' in their study)

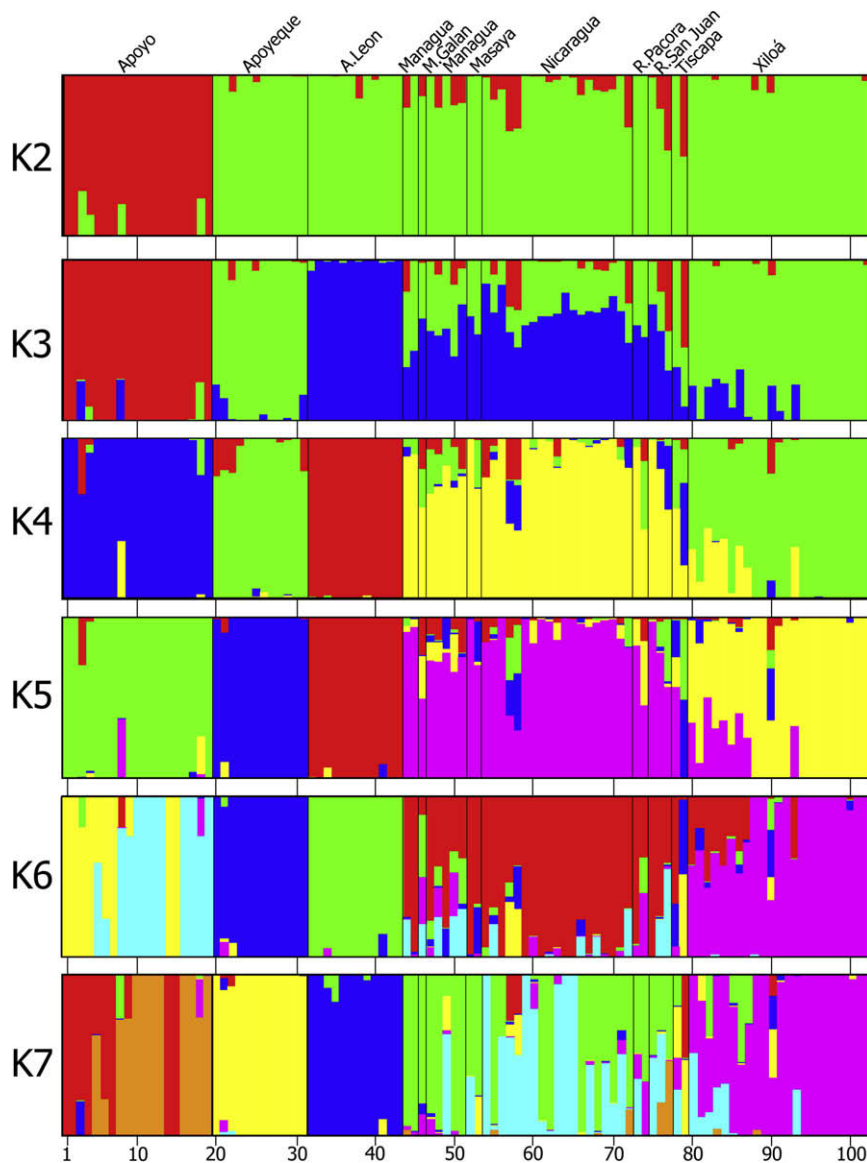


Fig. 6. Results of Structure clustering analysis for the whole AFLP dataset from K2 to K7 using Structure v2.2 without group information. K7 yielded the highest likelihood values. Lake of sample origin given above, sample ID of individuals given below.

and few individuals of the three more colorful species *A. chanco*, *A. flaveolus* and *A. sp.*, a still undescribed species (presumably referred to as ‘gold’ in their study), which are most likely not of a truly xanthoristic (‘golden’) phenotype, as these are otherwise unknown from L. Apoyo. However, our results show that there is more population subdivision within the L. Apoyo species flock than can be explained by color polymorphism or a hypothesis based on the assumption only two species to be present in L. Apoyo.

In contrast to the findings from Barluenga and Meyer (2004) but in congruence with those recently published (Elmer et al., 2009) there is support for genetic structure in L. Xiloá’s Midas cichlid population. Although both datasets support four genetic clusters in L. Xiloá, individual assignment to each cluster based on taxonomical classification in our dataset was not as clear as in Elmer et al. (2009, Fig. 2). This apparent discrepancy might be due the different segregating nature of 10 microsatellite loci used in Elmer et al. (2009) as opposed to the thousands of AFLP loci used here.

The overall similar and high levels of intralacustrine genetic differentiation (Table 4) are surprising, given the putative differences

in phenotypic diversity, species number and age of each lake. Whether this is rather due to extrinsic factors such as number, size and diversity of each founder lineage(s) or intrinsic attributes of Midas cichlids propensity to evolve differentiation, remains unclear. This pattern has to be tested critically using a substantially larger sample size from the smaller crater lakes (e.g. Apoyeque or Tiscapa) which are most likely undersampled not only with regard to the number of individuals but also with regard to the number of phenotypes identified (as compared to L. Apoyo and L. Xiloá).

4.2. Monophyletic vs. polyphyletic origin of crater lake assemblages

To establish Nicaragua’s Midas cichlids as model system for evolutionary biology especially in comparisons with African cichlid radiations, one of the most important issues is the claimed monophyly of the crater lake populations. While the monophyly of L. Apoyo’s Midas cichlids had been hypothesized before based on mtDNA, microsatellite genotyping and few AFLP loci (Wilson et al., 2000; Barluenga et al., 2006a) it remained unclear whether

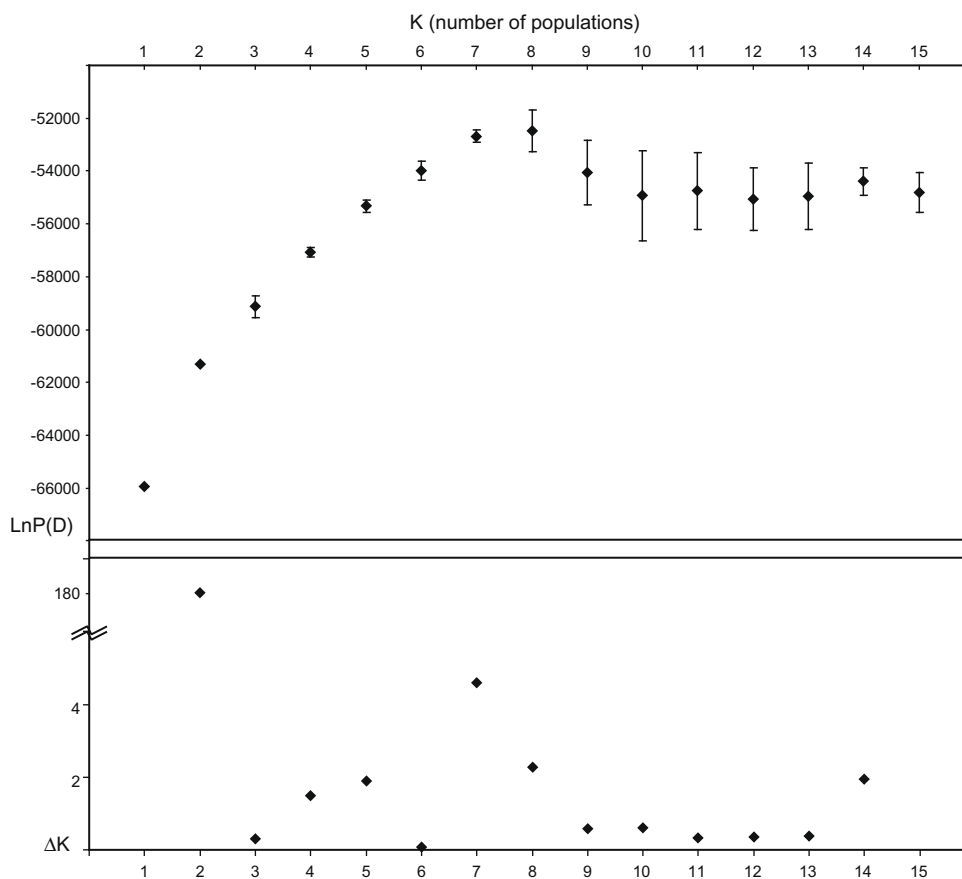


Fig. 7. Estimation criteria for the number of genetic clusters in the AFLP dataset. Above: Mean LnP(D) with SD from 20 replicates for each K , calculated without 'locprior' model (Structure v2.2). Below: Evanno's model choice criterion ' ΔK ' for the uppermost level of genetic structure.

all known species or forms of the Midas cichlid complex had been included in their studies. Consequently, doubts about the conclusion that the elongated arrow-cichlid *A. zaliosus* originated in sympatry from an ancestral *A. citrinellus* lineage in L. Apoyo persisted (Schliewen et al., 2006). The surprising finding that elongate Midas cichlids similar to *A. zaliosus* not only occur in Lakes Apoyo and Xiloá, but also in L. Nicaragua, underlined the importance of a refined individual-based taxon sampling to thoroughly test the monophyly hypothesis for the L. Apoyo Midas cichlids.

The data presented here are based on an explicit and comprehensive taxon sampling. Both, L. Apoyo and L. A. Leon had only private mtDNA-haplotypes and exhibited a predominantly monophyletic phylogenetic signal in the AFLP data. Based on geographic proximity and pairwise F_{st} values, the likely source for the L. Apoyo founder-population was L. Nicaragua. However, basal to the L. Apoyo assemblage in the AFLP NJ-tree are two individuals from the San Juan River which drains L. Nicaragua into the Caribbean. These two individuals are phenotypically drastically distinct from all other known Midas cichlids (see Fig. 1), are undoubtedly a new species and might indeed belong to an ancestral riverine population. One of these (ID: 77) shares a considerable proportion of alleles with some of the L. Apoyo 'deep-bodied' forms based on the AFLP data. One explanation could be common retained ancestral polymorphisms, although it is unclear why those alleles would have perished in the remaining L. Nicaragua and San Juan River individuals. Recent gene-flow can be ruled out as an explanation, given the geographic distance separating L. Apoyo and the San Juan River (Fig. 1). The other individual with a strong effect on monophyly support for L. Apoyo's species flock (ID:18) could phenotypically not be assigned to any described species: it's *A. flaveolus* like

coloration combined with a rather slender, *A. zaliosus* like body rendered it *a priori* plausible that it is indeed a hybrid. However, its exclusion had only little effect on the *A. zaliosus* cluster bootstrap support, lowering it from 83 to 77 not supporting its hybrid status. With a comprehensive population-genetic analysis of the L. Apoyo species flock we will be able to quantify recent and ongoing gene-flow between those incipient species and thus to identify potential hybrids (Geiger et al., in preparation).

Lake Apoyeque is the only lake where only the most common mtDNA-haplotype was recovered in 15 typed specimens. Disregarding the possibility that this is just due a sample artifact, this might imply that either the founder-population was small, that founder-population secondarily went through a genetic bottleneck and/or that colonization of L. Apoyeque occurred much more recently and only with individuals carrying the most common mtDNA-haplotype. The late colonization hypothesis gains support from the finding that the Apoyeque stratocone in its present form was created by a powerful eruption only about 1900 years ago (Freundt et al., 2006; Kutterolf et al., 2007, 2008). Despite no detectable mtDNA signal for monophyly, AFLP analysis strongly support it. Since there are at least two forms of *Amphilophus* in L. Apoyeque, a small *citrinellus*-type and a cf. *labiatus* thick-lipped form, L. Apoyeque might harbor another example of sympatric speciation, because with 2.5 km² surface area the lake is too small to allow for geographic population subdivision. This mini-flock including a thick-lipped form is also worth a more detailed examination in the light of parallel ecological adaptation as it is still unresolved whether all thick-lipped forms, occurring in lakes Apoyeque, Masaya, Managua, Nicaragua and Xiloá, are descendants from an *A. labiatus* related stock (for which we do not find support),

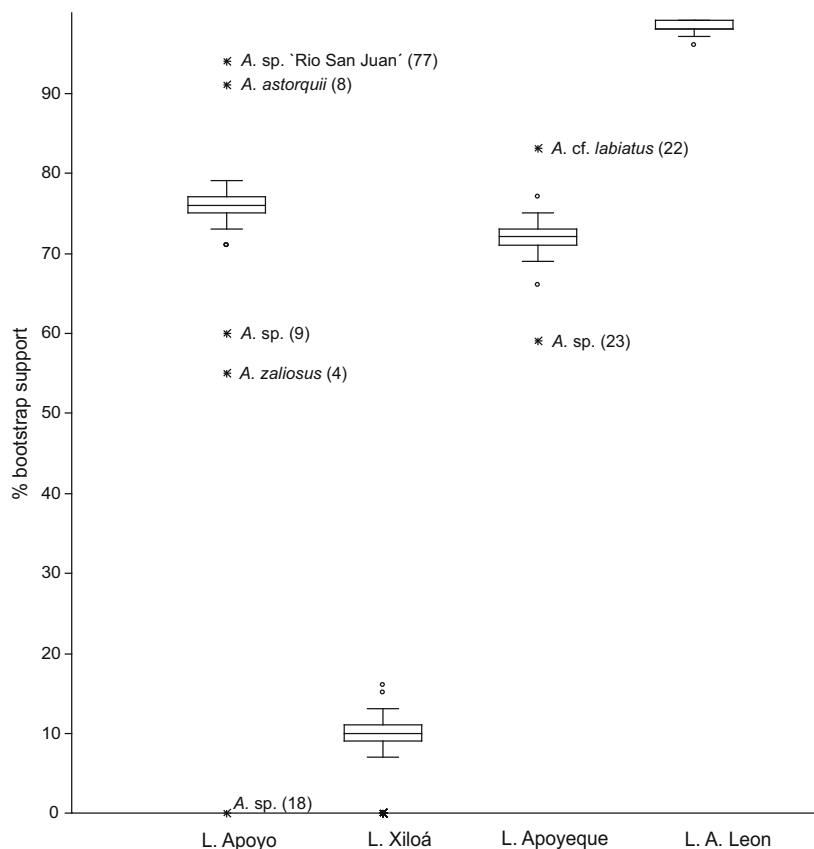


Fig. 8. Homoplasy-excess test outcome applying a modified Jackknife across taxa approach. Each single individual was removed once and bootstrap support for monophyly calculated from 1000 pseudo-replicates. Outliers indicated by asterisks. ID-numbers of individuals in brackets correspond to those used throughout.

or if there is a genetic predisposition of the proto-*Amphilophus citrinellus* stock or even cichlids in general to evolve the expression of fleshy lips under certain environmental conditions.

With already three described *Amphilophus* species and a pronounced color polymorphism in at least *A. sagittae* and *A. xiloaensis* (Stauffer and McKaye, 2002; Elmer et al., 2009), L. Xiloá's species flock has been especially attractive for evolutionary biologists. Elmer et al. (2009) have recently presented data supporting the concept of sexual selection through color assortative mating as driving factor for incipient sympatric speciation in *A. xiloaensis*. Although our dataset is limited with respect to sample size for L. Xiloá, it suggests that there is genetic structure within L. Xiloá's species flock beyond current phenotypic classification. The overall success of the Bayesian assignment test to identify *A. zaliosus* as compared to the remaining L. Apoyo species on the one hand and its "failure" to identify genotypic clusters corresponding to similar phenotypes in L. Xiloá indicates that reproductive isolation between the Xiloá species is either not complete or reflects the young age of the species flock. The Xiloá maar, created 6100 years ago (Freundt et al., 2006; Kutterolf et al., 2007, 2008) was originally part of the L. Managua basin and was probably temporarily re-connected to it until only 2000 years ago, when L. Managua's lake-level was about 3 m above today's (Cowan et al., 2002; Freundt pers. com.). In comparison to L. Apoyeque genetic variability in L. Xiloá's *Amphilophus* is significantly higher, as indicated by the higher mtDNA-haplotype richness. Given the geologic history of L. Xiloá's isolation, the founder-population, most likely once part of L. Managua's *Amphilophus* stock, was probably larger than that of neighboring L. Apoyeque, which was never connected directly with another lake and whose high crater rim does not facilitate introduction of fishes. Further, the close relatedness of L. Xiloá's species flock to

L. Managua's *Amphilophus* is expressed in low pairwise *Fst*'s. Interestingly, basal to the Xiloá species flock in the AFLP-NJ-tree are two individuals of *A. cf. labiatus*, a thick-lipped form which is rare in L. Xiloá (McKaye et al., 2002, pers. obs.). Again, there is no specific support for conspecificity with the true *A. labiatus* from L. Managua/L. Nicaragua, neither from the mtDNA-haplotype inference or NJ-tree reconstruction, nor from the Bayesian Structure analysis.

4.3. Three species vs. multi-species concept with notes on diversity

While previous and also some recent authors distinguished between a number of species based on morphological characters (e.g. Elmer et al., 2010b; Meek, 1907; McKaye et al., 2002; Stauffer et al., 2008), other authors assign numerous forms to only three species, namely *A. citrinellus*, *A. zaliosus* and *A. labiatus* (Wilson et al., 2000; Barluenga et al., 2004, 2006a; Bunje et al., 2007). The ongoing dispute about *Amphilophus* taxonomy (e.g. Villa, 1976b; Stauffer et al., 2002) is certainly based on the aforementioned high phenotypic diversity within the Midas cichlid complex which sometimes hampers ready identification in the field. To consider this issue adequately especially when testing for alternative speciation scenarios it is necessary to stick to a conservative taxonomy and only assign taxonomically valid species names to unambiguously identifiable individuals and not to each phenotype that is similar due to only one character, i.e. "elongate body form" or "thick lips". According to the most recent taxonomy, the Midas cichlid complex contains nine described species at the moment, but several more are awaiting their proper systematic treatment (McKaye et al., 2002; Geiger et al., in preparation).

Analysis of the admittedly limited sample from L. Nicaragua encompassing material from several locations and all known phe-

notypes to date but not documented before demonstrates that there is genetic structure within the L. Nicaragua Midas cichlids that is not covered by current taxonomic classification or sample location. Consequently, treating all identified phenotypes and described species as members of a highly polymorphic *A. citrinellus* does also not reflect the discovered pattern and should be discarded. The prominent basal position of four L. Nicaragua individuals belonging to four different phenotypes might be a consequence of retained alleles common to these individuals whose genotypic similarity is reflected in joint clustering in the Bayesian analysis. However, neither all individuals *a priori* identified as *A. citrinellus* nor as *A. labiatus* clustered according to species assignment or geographic origin of individuals. The result supports previous findings (e.g. Barluenga et al. (2004)) demonstrating only weak genetic differentiation between *A. citrinellus* and *A. labiatus* as reflected by multiple shared mtDNA-haplotypes.

Application of a strictly evolutionary or phylogenetic species concept will certainly lead to justified descriptions of several new species from the isolated crater lakes analogous to Midas cichlids from crater lakes Apoyo and Xiloá. A more difficult task requiring intense and dense sampling is to address the issue about the *A. citrinellus* forms from the two great Lakes Managua and Nicaragua. At the moment it is not possible to decide between alternative hypothesis, i.e. extreme intraspecific and interlacustrine polymorphism vs. a concept including multiple reproductively isolated species, which are separated ecologically, sexual and spatially and/or based on purely sexually selected characters. At the moment, it seems odd that there is a much higher diversity in several small crater lakes of relative recent origin than in the two large and ancient lakes (~500.000 mya old, Bussing, 1976; including the San Juan River system) that served as reservoirs from which the crater lake founders had been recruited. However, compared to the shallow Lakes Nicaragua (mean ~ 13 m) and Managua (mean ~ 9 m) some crater lakes offer a new and probably more stable ecological opportunity because they are very deep (e.g. Apoyo mean ~ 142 m, Xiloá mean ~ 60 m).

Future research on Nicaraguan Midas cichlids focusing on speciation mechanisms should therefore include the understudied populations from e.g. Lakes A. Leon, Apoyeque and Tiscapa as comparisons to the better examined Lakes Apoyo and Xiloá.

5. Outlook

The phylogenetic reconstruction of the Midas cichlid species flock is not a simple case, because phylogenetic patterns are not always unambiguous and complex and taxon sampling remains incomplete. Even the seemingly monophyletic species assemblages as of crater L. Apoyo retains phylogenetic signal that appears incompatible with a monophyly of the flock in the strict sense. The few individuals that had an obvious effect on the monophyly probability for e.g. L. Apoyo demonstrate the importance of an individual-based approach for the inference of phylogenetic relationships within this complex. It is highly likely, that the inclusion of new populations both from the great lakes as well as from the San Juan River system into the phylogenetic analysis might still produce alternative phylogenetic scenarios and consequently alternative hypotheses about the evolutionary factors that have influenced speciation the Midas species complex. The complexity of this geologically very young system nevertheless offers a unique possibility to study different speciation scenarios in situ, because of multiple parallel cases of spatially, ecologically and historically diversification within a single meta-system. For example, all three investigated crater lake assemblages (L. Apoyo, L. Xiloá, L. A. Leon) qualify as a natural triplicate experiment for parallel adaptive speciation, because similar phenotypes apparently have evolved inde-

pendently and their small size both in terms of species numbers and area render a total taxon sampling approach possible. Undoubtedly however, the analyses presented here show that the diversity in the Midas cichlid complex is not compatible with the traditional three-species taxonomy (*A. citrinellus*, *A. labiatus* and *A. zaliosus*) and that further taxonomic work is mandatory before refined hypotheses are possible.

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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.ympbev.2010.05.015](https://doi.org/10.1016/j.ympbev.2010.05.015).

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