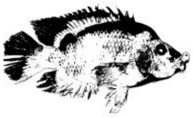
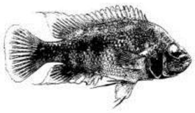
# PHYLOGENETIC RELATIONSHIPS AND EVOLUTIONARY ECOLOGY OF NICARAGUAN MIDAS-CICHLIDS (*AMPHILOPHUS* SPP.)

Z·S·M Zoologische Staatssammlung München





DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER FAKULTÄT FÜR BIOLOGIE

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THE THEORY OF EVOLUTION BY CUMULATIVE NATURAL SELECTION IS THE ONLY THEORY WE KNOW OF THAT IS IN PRINCIPLE CAPABLE OF EXPLAINING THE EXISTENCE OF ORGANIZED COMPLEXITY.

RICHARD DAWKINS

BRITISH ETHOLOGIST, GENETICIST, & POPULARIZER OF GENETICS (1941 - )

## EHRENWÖRTLICHE VERSICHERUNG

ICH VERSICHERE HIERMIT EHRENWÖRTLICH, DASS DIE VORGELEGTE DISSERTATION VON MIR SELBSTÄNDIG UND OHNE UNERLAUBTE BEIHILFE ANGEFERTIGT IST.

## ERKLÄRUNG

HIERMIT ERKLÄRE ICH, DASS ICH MICH ANDERWEITIG EINER DOKTORPRÜFUNG OHNE ERFOLG **NICHT** UNTERZOGEN HABE UND DASS DIE DISSERTATION NICHT GANZ ODER IN WESENTLICHEN TEILEN EINER ANDEREN PRÜFUNGSKOMMISSION VORGELEGT WORDEN IST.

## Publications from the work presented in this dissertation

**Geiger MF**, McCrary JK, Schliewen UK (2010)
Not a simple case - A first comprehensive phylogenetic hypothesis for the Midas cichlid complex in Nicaragua (Teleostei: Cichlidae: Amphilophus).
Molecular Phylogenetics and Evolution, 56, 1011–1124.

**Geiger MF**, McCrary JK, Stauffer, JR (2010)
Description of two new species of the Midas cichlid complex (Teleostei: Cichlidae) from Lake Apoyo, Nicaragua.
Proceedings of the Biological Society of Washington, 123, 159–173.

**Geiger MF**, Mayr C, McCrary JK, Schliewen UK
Molecular Ecology of the Lake Apoyo Midas Cichlid Species Flock.
(submitted to Molecular Ecology)

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*CD including online supporting information of Articles I & III*
*+ full specimen list*
*+ pdf - files of publications*

## GENERAL INTRODUCTION PART I – STUDY SYSTEM

Cichlid fish species flocks (Teleostei: Perciformes: Cichlidae) of the African lakes have served for decades as prime model systems for evolutionary biology in general and speciation research in particular (Kocher 2004; Seehausen 2006). Alone 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 sheer size of these mega-diverse 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*. Flocks with fewer species that are endemic to small lakes, e.g. crater lakes, thus represent an interesting alternative model system to study adaptive radiations, if the analysis of complete species richness is also within the focus. Quite well known are the monophyletic cichlid flocks of Cameroonian crater lakes with five to eleven species per lake (Schliewen et al. 2001; Schliewen & Klee 2004; Stiassny et al. 1992; Trewavas 1972). More recently, crater lake species flocks of the Nicaraguan Midas cichlid complex (genus *Amphilophus*) attracted the attention of evolutionary biologists (Barlow 1976; Barluenga & Meyer 2004; Barluenga et al. 2006a; Elmer et al. 2009 & 2010a; McKaye et al. 2002; Stauffer & McKaye 2002; Stauffer et al. 2008; Wilson et al. 2000). Both, Nicaraguan *Amphilophus* cichlids as well as Cameroonian crater lake cichlids belong to phylogenetic lineages only distantly related to the great 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 has been no established phylogenetic species-level framework for the Midas cichlid species complex prior to this work.

Nicaraguan Midas cichlids (*Amphilophus* spp.) are named after the famous King Midas of the Greek mythology who was given the fatal ability to turn everything he touched into gold – until he starved as wealthy man. The name Midas cichlid derives from the fact that, in some Nicaraguan lakes, brightly colored *Amphilophus* individuals can be found that have lost the melanophores that usually build patterns of black bars, yielding individuals ranging in color from white to orange or red, and collectively termed 'gold'. They are found in varying abundances up to 10% in more turbid lakes (Barlow 1976) and are known from at least four of the to date described eleven species in this species complex.

Fig. 1 The Pacific Versant of Nicaragua, including the Great Lakes and crater lakes of this study (modified from Barluenga et al. 2006).

Midas cichlids occur in all major freshwater-bodies within the Rio San Juan drainage in Nicaragua: they are very abundant in the two large and old rift lakes Nicaragua and Managua but also inhabit rivers and the majority of the crater lakes west of the two great lakes (Table 1 & Fig. 1).

Almost 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, 1978; Elmer et al. 2010b; McKaye 1977, 1980; McKaye et al. 2002; Wilson et al. 2000; Stauffer et al. 2008).

Only until recently, one group of researchers considered 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 & Munsey 1976; Barluenga & Meyer 2004; Barluenga et al. 2006a&b; Bunje et al. 2007; Wilson et al. 2000). The opposite view claims that the phenotypic diversity of Midas cichlid species complex falls into multiple species per lake including the crater lakes (McKaye et al. 2002; Stauffer & McKaye 2002; Stauffer et al. 2008). It was also this discrepancy between observed and described diversity that has contributed to the initiation of this PhD study. 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 eleven species at the moment, but may contain 30 or more species (McKaye et al. 2002; pers. obs.).

| taxon | author | type locality | estimated min. age |
|---|---|---|---|
| *A. citrinellus* | Günther 1864 | L. Nicaragua | > 500.000 y$^+$ |
| *A. labiatus* | Günther 1864 | L. Managua | > 500.000 y$^+$ |
| *A. amarillo* | Stauffer & McKaye 2002 | L. Xiloá | < 2.000 – 6.000 y* |
| *A. sagittae* | Stauffer & McKaye 2002 | L. Xiloá | |
| *A. xiloaensis* | Stauffer & McKaye 2002 | L. Xiloá | |
| *A. astorquii* | Stauffer, McCrary & Black 2008 | L. Apoyo | < 21.000 – 25.000 y*° |
| *A. chancho* | Stauffer, McCrary & Black 2008 | L. Apoyo | |
| *A. flaveolus* | Stauffer, McCrary & Black 2008 | L. Apoyo | |
| *A. globosus* | Geiger, McCrary & Stauffer 2010 | L. Apoyo | |
| *A. supercilius* | Geiger, McCrary & Stauffer 2010 | L. Apoyo | |
| *A. zaliosus* | Barlow & Munsey 1976 | L. Apoyo | |

**Table 1 Current taxonomy of valid Midas cichlid species. $^+$Bussing 1976; *Freundt et al 2006; ° Sussmann 1982.**

# GENERAL INTRODUCTION PART II – SPECIATION THEORY

Although more than 150 years have now passed since Darwin's famous book "On the origin of species" has been published (Darwin 1859), one of the central and fundamental issues in evolutionary biology remains the question of **how** species evolve (e.g. Coyne & Orr 2004). The origin of new species has puzzled evolutionary biologists for decades, and while early studies focused on how species evolved under Darwin's introduced **natural selection** within a single habitat (e.g. Woltereck 1931; Brooks 1950), subsequent work sought for alternatives to natural selection (e.g. Mayr 1942; Futuyama & Mayer 1980). Later on, with the advent of molecular biology, underlying genetic aspects of speciation have gained widespread interest (e.g. Coyne & Orr 1998; Via 2002). Today, in the era of genomics where unprecedented amounts of genetic information are readily available (given sufficient resources) and are combined with ecological and morphological data, we will likely see greater advance in our understanding of evolution and speciation than ever before (Butlin 2010).

Given the complexity of interactions that individuals and populations experience between conspecifics, with heterospecifics and with the environment, different **modes of speciation** have been classified to facilitate the study of speciation. The most widely accepted framework is a classification based on geographic conditions and on the underlying mechanisms of speciation (Coyne & Orr 2004). Generally, the process of speciation can be finalized when a subpopulation has either achieved **prezygotic** isolation, i.e. does not interbreed anymore with its antecessors through either geographic or behavioral barriers or a subpopulation has reached **postzygotic** isolation, i.e. when the genomes have changed to a point of incompatibility. While in the former case viable hybrid offspring can result from interbreeding, no viable offspring will be produced once postzygotic isolation has been established between populations.

## Modes of speciation – geographic view

**Allopatric speciation** describes the origin of a new species as the consequence of fragmentation of an initially panmictic distributed population into subpopulations, usually by a geological event, such as the uplift of mountains. Its reality is quite undisputed (e.g. Mayr 1942; Kawata & Yoshimura 2000; Mallet et al. 2009) and good examples for its occurrence include island populations, with Darwin's finches of the Galapagos Islands being the most well-known example. Once geographically separate, gene flow is greatly reduced and subpopulations potentially diverge independently in different directions. The process is accompanied by mutation accumulation and genetic drift and can be driven by diverging adaptation to a particular environment which can then result in a morphologically distinct and reproductively isolated cluster of individuals. Alternatively, as mutation and drift are mainly stochastic and random processes, morphology does not necessarily change, which would lead to cryptic species that form genetically distinct clusters, but are morphologically often indistinguishable (e.g. Hebert et al. 2004; Elmer et al. 2007). Even the opposite scenario, morphologically distinct clusters of individuals that are not reproductively isolated from each other can result from allopatric speciation (Stelkens et al. 2009). Intrinsic pre- or postzygotic barriers to reproduction have not evolved between those species as they cannot interbreed due to geographic isolation. Especially when the geography of a particular region allows for direct correlation with a reconstructed phylogeny, evidence for an allopatric speciation scenario can reasonably easy be documented (e.g. Mayr 1942).

**Parapatric speciation** describes a situation where populations with diverging ecological adaptations have either overlapping or adjacent distributions, and migration between those populations occurs and results in accidental hybridization. Because either parental species is better adapted to its own habitat, crosses between those populations will usually show below-average fitness in both habitats and are thus selected against. The process from initial differentiation until complete speciation with strong reproductive isolation is often aided by a process called **reinforcement**. It describes the increase of reproductive isolation by natural selection when less fit hybrid individuals are selected against and thus assortative mating in both parental populations provides a fitness advantage. Often cited examples include so called ‘ring species’, like the circumpolar living *Larus* gulls species ring, a complex of adjacent gull populations or sub-species distributed around the North Pole that largely hybridize among each other except for the two ‘end’ species in the series, which are too distantly related to interbreed. However, more recent findings have shown that this example is much more complicated than thought before (Liebers et al. 2004) and it was already put forward that it is almost impossible to demonstrate parapatric speciation in nature since alternative explanations are almost always possible. Those include secondary contact of in allopatry diverged populations or competition between related species that prevent them from occurring in the same area (e.g. Coyne & Orr 2004).

**Sympatric speciation** refers to a scenario where a new species emerges within a freely breeding population without geographic isolation, which is however only one of about a dozen published definitions (Fitzpatrick et al. 2008). Although it is theoretically possible under a variety of more or less stringent conditions (e.g. Maynard Smith 1966; Turner & Burrows 1995; Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Kawata & Yoshimura 2000; Almeida & de Abreu 2003; Ripa 2009; van Doorn et al. 2009), it is considered uncommon in nature and still only few unequivocal examples are generally accepted (Bolnick & Fitzpatrick 2007). Sympatric speciation can be a relatively fast process if adaptation to distinct ecological niches is coupled with assortative mating – a fact that inspired some to use the term ‘magic trait’ for a trait under natural selection that also serves as a cue for assortative mating. As outlined above for parapatric speciation, the process can be accelerated by reinforcement through selection against intermediate hybrid individuals, thus favoring the evolution of prezygotic reproductive isolation. *Rhagoletis* apple maggot flies are among the most famous examples (Bush 1968), but promising candidates for the study of underlying processes of sympatric speciation are fish species flocks endemic to small lakes. They usually offer the possibility to examine speciation processes without confounding effects of allopatric population differentiation if a single colonization event as inferred from molecular genetics implies their monophyly (e.g. Schliewen et al. 2006; Mallet et al. 2009; Losos & Mahler 2010). Beside West African crater lake cichlids (e.g. Schliewen et al. 2001; Schliewen & Klee 2004) as study system, Nicaraguan Midas cichlids from crater lakes have recently come into the focus of evolutionary biologists

(Barluenga & Meyer 2004; Barluenga et al. 2006a; Schliewen et al. 2006; Elmer et al. 2009; Elmer et al. 2010a; Elmer et al. 2010b; Wilson et al. 2000).

## Modes of speciation – Mechanisms

**Natural selection** is the almost all-embracing key process of evolution which leads to the establishment **or** disappearance of fitness relevant biological traits in a population. It works through effects on survival and reproduction of the trait-bearer and is based on four components: variation, inheritance, population growth and differential survival (e.g. Endler 1986). **Variation - i**ndividuals within a population exhibit variation in appearance, behavior or any other trait. **Inheritance -** some traits are passed on from parent to offspring, i.e. are heritable. **Population growth -** usually, populations produce more offspring than local resources could support, which leads to competition for resources and substantial mortality in each generation. **Differential survival -** individuals with traits well suited for exploiting local resources will reproduce more successfully and produce more offspring, which ultimately changes the frequency of a particular, likely beneficial trait in the population. Selection acts on the frequency of a particular trait and can take the form of **stabilizing**, **directional**, or **diversifying** selection (e.g. Coyne & Orr 2004). Under stabilizing selection, extreme varieties are eliminated and the frequency distribution is the same as in the generation before, which is probably the most common form of natural selection, but often mistaken for no selection. Under directional selection, individuals with traits from one end of the distribution do especially well, and so the frequency of the trait in subsequent generations is shifted from where it was in the parental generation into one direction. Under diversifying or disruptive selection, both extreme varieties of a trait are favored at the expense of intermediates. Although it is rather uncommon, it is of theoretical interest because it provides a mechanism for speciation without geographic isolation.

In a species with allopatrically occurring populations, natural selection may accidentally lead to reproductive isolation between those populations as a consequence of adaptation to differing habitats. These cases, when speciation has mainly been driven by diverging ecological adaptations, such as resource utilization, are regarded a particular case of natural selection (Schluter 1998). In scenarios where there is gene flow between populations, i.e. under para- or sympatric conditions, the hypothesis is that because intermediates between two evolving forms suffer reduced fitness divergence of those forms is facilitated through the establishment of some reproductive isolation. Once weak reproductive isolation has been established through e.g. assortative mating, reinforcement can lead to complete sexual isolation and help completing the speciation process.

**Sexual selection** arises most often from competition between males for attractive females or from the preferences females exhibit for certain characters males display (Futuyama 1998). This form of intraspecific competition can be generalized as the variability in reproductive fitness as a consequence of differential mating success. The outcomes are characters which provide either an advantage in male-male competition (e.g. body size) or enhance the attractiveness of one sex (e.g. ornaments). Those characters are usually over-expressed relative to the same feature in the other sex. Sexual selection had a reputation as major cause of speciation since it directly affects reproductive isolation and also because theoretical models supported its plausibility. However, the case for sexual selection might not be as strongly supported as allopatric speciation for instance (Ritchie 2007).

**Hybridization** can generate and increase standing genetic variation which forms the substrate for new, beneficial adaptive trait combinations suitable for exploiting resources not utilized previously (Dowling & Secor 1997, Stelkens et al. 2009). The same mechanisms concurrently prevent the loss of genetic variation due to strong selection prevailing in sympatric speciation scenarios and may even induce speciation (Mallet, 2007). The product is not necessarily the formation of a hybrid species, but rather an increase in genetic variance with the introgressing form disappearing in a hybrid swarm. Theory indicates that adaptation can be rapid when evolution acts on standing genetic variation instead of relying on the rather rare occurrence of beneficial mutations (Barrett & Schluter 2008; Wolf et al. 2010).

## SUMMARY - AIMS OF THIS THESIS

The first major aim of this study was to compile a sound phylogenetic hypothesis for the whole Midas cichlid complex encompassing all described species and several potential new species based on an exhaustive and individualized sampling from so far unsampled lakes. All published phylogenetic hypotheses (Barluenga & Meyer 2004; Barluenga et al. 2006a; Wilson et al. 2000) were based on the three species concept (*A. citrinellus*, *A. labiatus* and *A. zaliosus*) and built upon mitochondrial haplotype data or few nuclear loci only. This has resulted in a debate about 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 that with the given unclear taxon sampling including only ''*A. citrinellus* Apoyo" and *A. zaliosus* and with evidence for mitochondrial monophyly alone it cannot be argued that the whole assemblage is monophyletic, because mitochondrial monophyly does not necessarily imply flock monophyly. The null hypothesis of multiple invasions or introgression into L. Apoyo across species borders remained a valid alternative, which needed to be tested with a representative taxon sampling across the whole Midas cichlid flock and the Nicaraguan lakes system with individualized nuclear multilocus data.

The second major aim was a systematic-taxonomic reevaluation of the L. Apoyo species assemblage as basis for a more detailed study of the evolutionary processes underlying the diversification within this particular Midas cichlid species flock. According to Barluenga et al. (2004 & 2006) *A. zaliosus* had its origin in a sympatric speciation process in L. Apoyo within the last 10.000 years. However, the authors ignored the fact that McKaye et al. documented the existence of three or more species already in 2002 which had subsequently been described later on (Stauffer et al. 2008). Recognition of the species-level diversity in this system is a necessary prerequisite for reconstructing the evolutionary history of speciation. That the authors failed to mention that five, rather than two forms are known from L. Apoyo and that several more fully described species of the *A. citrinellus* species complex exist in the area additionally casted doubts on the validity of the drawn conclusions (Schliewen et al 2006).

The third major aim was to infer the significance of hybridization, sexual selection and natural selection for the formation of a complete and complex species flock *in statu nascendi*, and thus to re-evaluate previous findings concerning the mode of speciation of the L. Apoyo Midas cichlid species flock. Studies examining the factors that maintain species integrity and cohesiveness despite ongoing gene flow between incipient species rarely included combined analyses of eco-morphological with molecular genetic data (but see e.g. Herder et al. 2008, Tobler & Carson 2010). Specifically, we hypothesized to find increased levels of genetic differentiation between species that are not strongly ecologically divergent, but which differ in breeding coloration if sexual selection dominates divergent selection in this system. In contrast, higher levels of genetic differentiation of ecologically divergent rather than ecologically similar pairs are to be expected if divergent natural selection was the dominant force shaping diversity, hereby assuming that ecological divergence is a proxy for divergent natural selection (Nosil 2009). Together with the comprehensive framework of the *Amphilophus* system of the Nicaraguan lakes and the clarification of the taxonomic background, the last study was designed to finalize the provision of baseline analyses necessary for the establishment of the L. Apoyo system as a comparative model to the African crater lake cichlids.

## MATERIALS EXAMINED

All included *Amphilophus* individuals were caught in Nicaragua during three field trips in the dry seasons (November-April) of 2006/07 (Geiger), 2007/08 (Geiger) and 2008/09 (McCrary). Fishes from crater lakes Apoyo, Apoyeque, Asososca Leon and Xiloá were caught SCUBA diving or snorkelling with harpoon while material from lakes Managua, Masaya, Monte Galan, Nicaragua and Tiscapa was caught with gill nets (40mm mesh-width). Fishes were anesthetised, preserved in 7-10% formalin, individually tagged, and fin-samples taken for molecular genetic studies. Photos of live individuals were taken and notes on coloration made in the field. Fishes are preserved in 75% ethanol and whole body and tissue vouchers stored permanently at Bavarian State Collection of Zoology, Munich (ZSM) with their respective collection numbers. Raw extracted DNA aliquots are also stored permanently at ZSM within the DNA Bank Network and are available to the scientific community upon request.

While basically all collected material was used to characterise the complete morpho-space occupied by the Midas cichlid complex for article I (Geiger et al. 2010a), due to limited resources only a representative set of individuals was used for the phylogenetic reconstruction covering the phenotypic and geographic range. The focus of articles II (Geiger et al. 2010b) and III (Geiger et al. submitted) were the Midas cichlids occurring in L. Apoyo, and thus only few ‘out-group’ individuals were included for the phylogenetic reconstruction.

## METHODS

### Taxon Sampling

One individual of each, *Amphilophus lyonsi* (aquarium stock, articles I & III) and *Astatheros rostratus* (L. Nicaragua, article I) were chosen as outgroup taxa, their adequacy as a valid outgroup supported by e.g. Concheiro et al. (2007) or Říčan et al. (2008).

Where possible, described species were identified according to the most recent species descriptions, or in other cases, where labelled 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 labelled *A.* cf. *labiatus*. The addition 'gold' to individual names describes fishes that have lost their melanophores as adults and thus their pattern of black bars and spots.

### 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. To this end we applied an already established geometric-morphometric landmark approach (Rohlf & Marcus 1993; Rohlf 2006a&b; Sheets 2003), which provides an efficient way to characterise body shape variation (Klingenberg et al. 2003; Parsons et al. 2003). In Geiger et al. (2010a) we used this approach to choose a subset of individuals for the molecular genetic study that represent the complete phenotypic variability of the Midas cichlid complex. Focussing on the small scale radiation in L. Apoyo in Geiger et al. (submitted) we used the method to capture information on body shape variation and to compare the assignment to morphological similar groups with the assignment to genetic entities. Since seven individuals could not be assigned to any of the six species *a priori* - possibly due to hybrid origin - a canonical variate analysis (CVA) with assignment test (Nolte & Sheets 2005) was applied to explore those individuals' phenotypic affinities. For details on data acquisition and analysis see Geiger et al. (2010a), Geiger at al. (submitted) and the literature aforementioned.

The systematic-taxonomic evaluation of the small species flock endemic to L. Apoyo in article II (Geiger et al. 2010b) required to apply traditional morphometric methods, e.g. directly measuring a set of exactly defined distances on preserved fish-bodies. Counts and measurements follow Barel et al. (1977) and Stauffer (1991, 1994), with exceptions and technical details given in Geiger et al. (2010b). Additionally to traditional measurements, we examined the caudal skeletons of ten individuals of each of the six L. Apoyo *Amphilophus* species using high-resolution x-ray pictures that have been produced at University Hospital (KUM) in Grosshadern, Munich. Statistical analyses of the data include principal component analysis (PCA) and sheared principal component analysis (SPCA) and are explained in Geiger et al. (2010b).

### DNA Extraction, PCR and mtDNA- and AFLP Genotyping

Genomic DNA was extracted using the QIAGEN® DNeasy® 96 Tissue Kit for animal tissues according to the protocol provided by the manufacturer. For a total of 275 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 (cf. Geiger et al. 2010a). Sequencing of the ca. 790 bp long fragment was done at the sequencing service of the Department of Biology of the Ludwig Maximilian University (Munich). Additional 575 sequences were included for the phylogenetic analysis from published control-region sequence data (Barluenga & Meyer 2004; Barluenga et al. 2006a). 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 (Geiger et al. 2010a) and 126 (Geiger et al. submitted) individuals was genotyped with twenty selective amplifications using enzymes EcoRI and MseI with three selective bases. Details for sizing, bin-calling, quality checks and AFLP matrix construction are given in the aforementioned articles. The combination of the rather slowly evolving mitochondrial marker with fast evolving nuclear markers such as AFLP should allow for better estimates of the colonization history and past and current gene flow between populations than a single marker system alone.

**Phylogeny reconstruction**

The pruned final alignment contained 752 sequences and 720 bp including gaps (for details of alignment construction see Geiger et al. 2010a), and was reduced to 201 unique haplotypes to construct median-joining haplotype networks containing all shortest least complex phylogenetic trees (all maximum parsimony or MP trees). Since it was known from previous studies that the mtDNA control region alone does not provide enough evolutionary resolution for untangling the complete phylogenetic relationships between single Midas cichlid species within a single tree (Barluenga & Meyer 2004; Wilson et al. 2000), we only considered network reconstructions for our purposes. 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). A similar approach was used in Geiger et al. (submitted), although there was less complexity in the networks simply due to the smaller number of haplotypes present in L. Apoyo.

For the tree- and network reconstruction based on AFLP data a simple distance matrix was calculated since so far no general accepted, model based approach for AFLP data analysis exists. We chose the Link et al. (1995) distance-measure to compute a pairwise genetic distance matrix. Link's formula is based on Jaccard's similarity measure (Jaccard 1908) 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. Technical details for the reconstruction methods are given in Geiger et al. (2010a).

As an assessment of 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. The LS index measures the consistency of each taxon'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.

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 Geiger et al. (2010a), additionally 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 et al. (2006). 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 & Moulton 2004) as implemented in SplitsTree (Huson 1998).

**Outlier locus detection (only article III)**

In order to discern between effects from neutral processes and selection which might influence divergence in L. Apoyo Midas cichlids (cf. Geiger et al. submitted), a Bayesian method was applied to identify potential candidate loci under selection, implemented in Bayescan 1.0 using default settings (Foll & Gaggiotto 2008). Performance of Bayescan was evaluated with two alternative software programs to identify loci under directional selection: Dfdist (www.rubic.rdg.ac.uk/~mab/stuff/), most commonly used for AFLP markers (Pérez-Figueroa et al. 2010), and SAM (Joost et al. 2007), which performs multiple univariate logistic regressions to test for associations between allelic frequencies and environmental variables (see CCA details below for coding of environmental variables).

**Inference of genetic structure**

We used the individual-based Bayesian algorithm implemented in Structure 2.2 (Pritchard et al. 2000; Falush et al. 2007) to identify the number of genetically differentiated clusters without *a priori* group designation based on the AFLP data. Complementary, the approach of Evanno et al. (2005) was applied to detect the uppermost hierarchical level of genetic structure. Structure 2.2 was also used to test for immigrant or misclassified individuals and to visualize population genetic structure. For details of parameter configuration see Geiger et al. (2010a) and Geiger et al. (submitted).

The structure of genetic diversity was investigated using hierarchical analyses of molecular variance (AMOVA) as implemented in Arlequin 3.11 for both, the mtDNA and AFLP data independently. Molecular variance in Geiger et al. (2010a) 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). In the L. Apoyo study (Geiger et al. submitted) molecular variance was estimated among and within 1) the six species, 2) the three sampling locations within L. Apoyo, and 3) three breeding-coloration groups. According to their prevalent ground coloration when breeding, individuals were pooled into three groups: 1) *A. astorquii*, *A. zaliosus*, and *A. supercilius* (black), 2) *A. chancho* and *A. globosus* (yellow) and 3) *A. flaveolus* (green).

Genetic differentiation between geographically isolated populations (lakes) or species was estimated using F-statistics on uncorrected p-distances (Weir & Cockerham 1984) as implemented in Arlequin 3.11, and their significance tested by permutating haplotypes among populations as well as generating bootstrap confidence intervals. Loci identified to be under directional selection were removed from the AFLP matrix and AMOVAs as well as F-statistics recalculated as a measure of presumably neutral differentiation. Additionally, we performed Structure runs and PCA as graphical representation of population differentiation on the AFLP matrix containing outlier loci only, and on the presumably neutral AFLP matrix without outlier loci (Geiger et al. submitted).

### Stable isotope analysis (SIA, only article III)

For an estimation of ecological differentiation with the null hypothesis of no dietary differentiation between the six sympatric L. Apoyo *Amphilophus* species, we analyzed a total of 132 samples presumably covering all major components of the lacustrine food-web. Muscle samples for stable isotope analysis were taken from a total of 84 *Amphilophus* individuals from all six species and from 16 individuals belonging to all other known fish species from L. Apoyo, except introduced *Oreochromis* spp. (McCrary et al. 2007). Various fish species with different ecological characteristics such as the poeciliid *Poecilia* sp., the atherinid silverside *Atherinella* cf. *sardina*, the predatorious bigmouth sleeper goby *Gobiomorus dormitor*, and the largest cichlid in L. Apoyo, *Parachromis managuensis* were included to estimate the relative position of the *Amphilophus* in L. Apoyo's food-web. Additionally, we drew plankton samples at two different depths (5-7m and 15m) and collected aufwuchs from rocks close to hydrothermal springs (mainly consisting of filamentous green algae, cyanobacteria and attached diatoms) and benthic flocculent material from littoral zones (mainly *Terpsinoë americana*, *Campylodiscus clypeus* and other diatoms). Finally, *Pyrgophorus coronatus*, an abundant snail and important dietary component of certain *Amphilophus* species (McCrary et al. 2008) was included (without shell) as well as *Chara* sp., the only macrophyte and preferred habitat for *P. coronatus* in that lake. Details for sample preparation and analysis are given in Geiger et al. (submitted). As a proxy for realized niche-breadth we obtained the convex-hull area in the $\delta^{13}C$ / $\delta^{15}N$ bi-plot for all included *Amphilophus* individuals and calculated the ratio between this and each single species convex-hull areas as a measure of degree of specialization (Layman et al. 2007).

### Correlating genetic, morphometric and ecologic differentiation (only article III)

Testing for patterns of correlation between species-pairs genetic, morphometric and ecologic differentiation (Fig. 2) we used Mantel tests and used as input matrices the pairwise $F_{ST}$'s from the complete AFLP data, the Euclidean distances between species pairs mean $\delta^{13}C$ and $\delta^{15}N$ and the Euclidean distances between species pairs mean for each of the first four principal components based on the geometric morphometric data. As graphical representation of the relationships between those matrices, linear regressions based on ordinary least squares were generated in PAST (Hammer et al. 2001) including a 95% confidence band for the fitted line.

Additionally, a canonical correspondence analysis (CCA) was conducted to test for associations between the distribution of AFLP loci and predictor variables, treating the genetic data as dependent variables (Fig. 2). The statistical framework of CCA provides an efficient way to relate patterns of genetic variation to descriptive variables (Angers et al. 1999). The underlying eigenvalue ordination technique allows direct analysis of the relationships between multivariate 'ecological' data tables (ter Braak 1986; ter Braak & Verdonschot 1995), integrating regression and ordination without assuming linearity in the response of 'species' to environmental variables. Using this approach, we aimed to identify single AFLP loci via CCA that are explicitly related to 'environmental' variables.

Each individual in the CCA was characterised by following variables: species (decomposed into six variables), trophic position (see below), breeding coloration (categorized as black, yellow or green) and principal component scores one to four from the geometric morphometrics. Coding of variables was the same in SAM to detect loci under selection. Since we did not obtain stable isotope ratios for all individuals included in the molecular genetic study, different CCA data sets were created including 1) all genotyped individuals with raw $\delta^{15}$N and $\delta^{13}$C values and species mean $\delta^{15}$N and $\delta^{13}$C for individuals not included in the SIA, 2) all genotyped individuals with each species' trophic position derived from $\delta^{15}$N and categorized as three trophic levels, and 3) only those individuals for which both, stable isotope and AFLP data were available. Additional CCAs were conducted with and without species assignment coded as variables and independently for the AFLP matrix containing the presumably neutral loci only and a matrix containing outlier loci only. See Geiger et al. (submitted) for details on CCA settings.

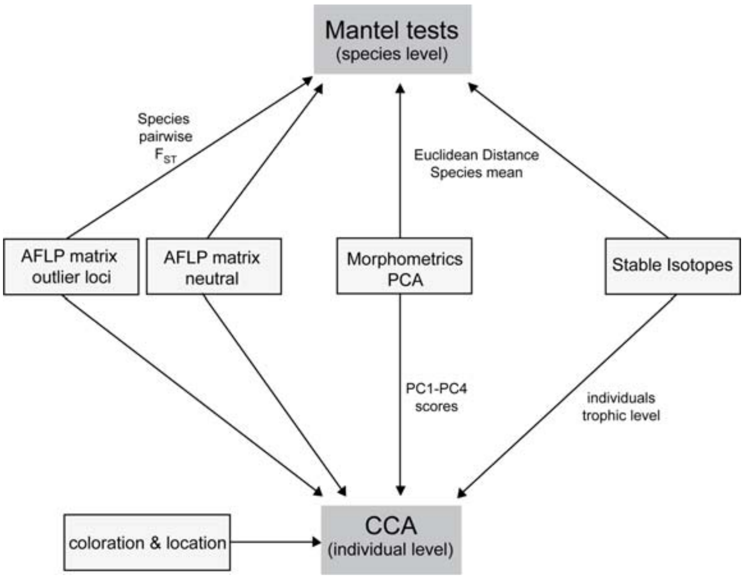

Fig. 2. Flow chart diagram showing the general scheme of the joint analysis of genetic, morphometric and ecologic data.

## RESULTS & DISCUSSION

All major aims of the PhD thesis had been achieved while M. Geiger was doing his doctorate at the Bavarian State Collection of Zoology in Munich and resulted in the publication of three articles in international, peer-reviewed journals which are ISI-indexed. A summary of the most significant results with discussion follows for each article, followed by a combined synopsis in German of all articles.

### *Molecular phylogeny of Nicaraguan Midas Cichlids*

Geiger MF, McCrary JK, Schliewen UK (2010) Not a simple case - A first comprehensive phylogenetic hypothesis for the Midas cichlid complex in Nicaragua (Teleostei: Cichlidae: Amphilophus). Molecular Phylogenetics and Evolution, 56, 1011–1124.

Until recently no attempt has been made to comprehensively reconstruct phylogenetic relationships of the highly diverse and recently evolved Nicaraguan Midas cichlid species complex. We presented a first multi-locus 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 molecular genetic 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.

The general pattern and magnitude of genetic variance discovered reflects the geographic isolation of the crater lake populations, and 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. Additionally, we detected 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. Contrasting the findings from Barluenga and Meyer (2004) but in line with those recently published (Elmer et al. 2009) four genetic clusters in L. Xiloá were detected, although individual assignment to each cluster based on taxonomical classification in our dataset was not as clear as in Elmer et al. (2009). The apparent discrepancy might be attributed to the different segregating nature of microsatellite markers as opposed to the thousands of AFLP loci used in our study. The overall similar levels of intralacustrine genetic differentiation are surprising, given the putative differences in phenotypic diversity, species number and age of each lake. Whether this is due to extrinsic factors as number, size and diversity of each founder lineage(s) or intrinsic attributes remains unclear. This pattern has to be tested critically using a substantially larger sample size.

Two crater lakes, Apoyo and Asososca Leon had only private mtDNA-haplotypes and exhibited a predominantly monophyletic phylogenetic signal in the AFLP data (Fig. 3). 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 phylogeny are individuals from the San Juan River which drains L. Nicaragua into the Caribbean. They are phenotypically distinct from all other Midas cichlids, represent most likely a new species and might indeed belong to an ancestral riverine population of Midas cichlids.

Despite no detectable mtDNA signal for monophyly, AFLP analysis supports the monophyly of the crater lake Apoyeque Midas cichlid population. 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 harbour another example of sympatric speciation, because with 2.5 km$^2$ surface area the lake is too small to allow for geographic subdivision (Elmer et al. 2010b). This mini-flock is also worth a more detailed examination in the light of parallel ecological adaptation as it is still unresolved whether all thick-lipped forms in the Midas cichlid complex are descendants from an *A. labiatus* related stock, for which we do not find support, or if there is a genetic predisposition of the proto-*Amphilophus citrinellus* stock to evolve the expression of fleshy lips under certain environmental conditions.

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. It has been especially attractive for evolutionary biologists with three described *Amphilophus* species and a pronounced color-polymorphism in *A. sagittae* and *A. xiloaensis* (Stauffer & McKaye 2002; Elmer et al. 2009). The observed 'failure' of our Bayesian assignment test to identify genotypic clusters corresponding to similar phenotypes in L. Xiloá indicates that reproductive isolation between the Xiloá species is not complete, likely reflecting the young age of this small species flock.

Analysis of the sample from L. Nicaragua encompassing material from several locations and various previously unknown phenotypes 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 phenotypes and described species as members of a single, highly polymorphic species does not reflect the discovered pattern. The basal position of four individuals belonging to four different phenotypes could be a consequence of common retained alleles whose genotypic similarity is reflected in joint clustering in the Bayesian analysis. Neither all individuals identified *a priori* as *A. citrinellus* nor as *A. labiatus* clustered according to their species assignment or geographic origin of individuals (Fig. 3). This supports previous findings (e.g. Barluenga & Meyer 2004) demonstrating only weak genetic differentiation between the two species as reflected by multiple shared mtDNA haplotypes. Surely, application of a strictly evolutionary or phylogenetic species-concept will certainly lead to justified descriptions of several new species from the isolated crater lakes but most likely also from L. Nicaragua, analogous to Midas cichlids from crater lakes Apoyo and Xiloá.

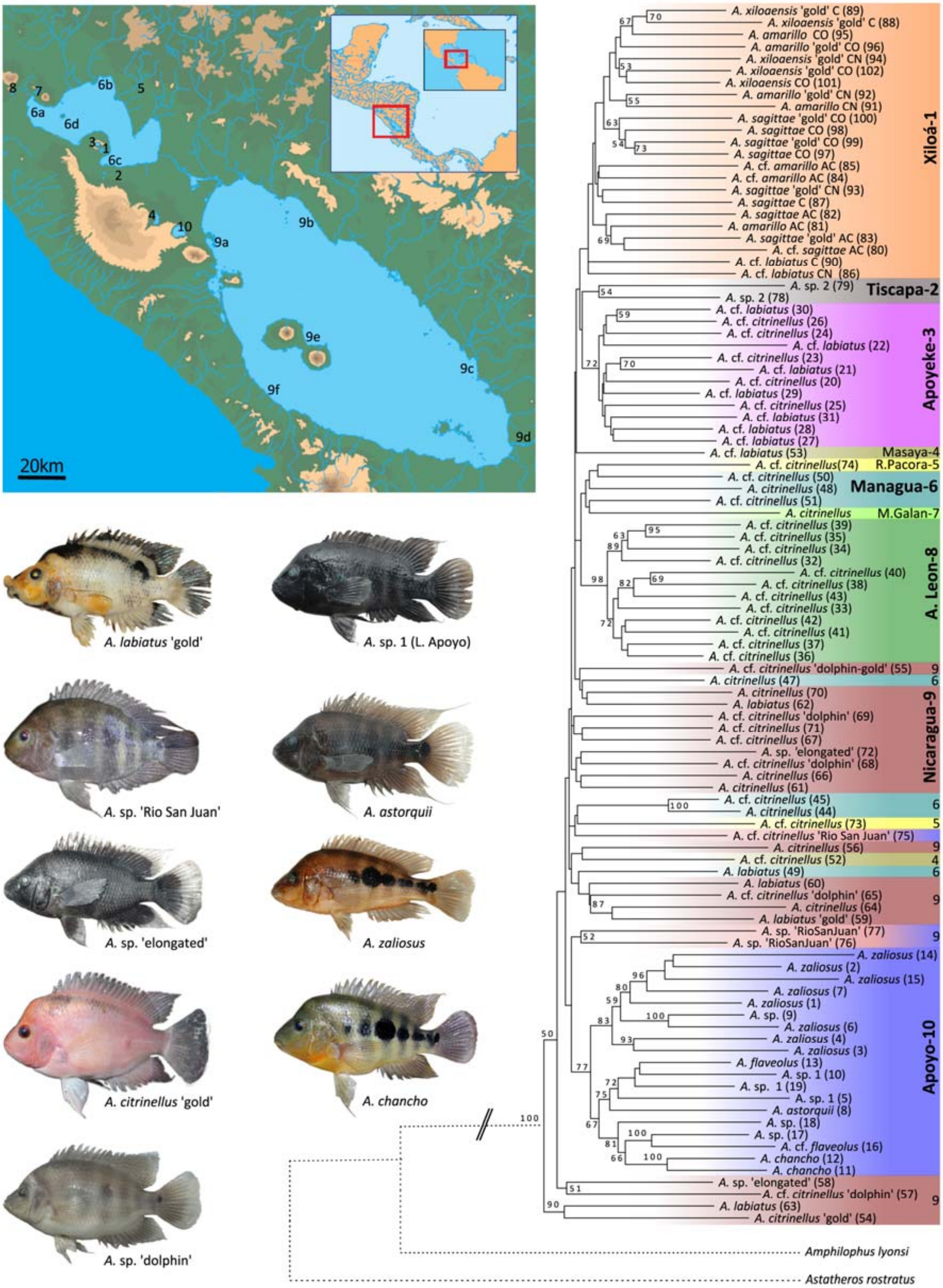

Fig. 3. AFLP NJ-tree with bootstrap supports and sample ID given in brackets, map indicating sample locations and some representative species of the Midas cichlid complex (from Geiger et al. 2010a).

For now, it seems odd that there is higher diversity in several small crater lakes of relative recent origin than in the two large and ancient lakes (ca. 500.000 mya old, Bussing 1976) that served as reservoirs from which the crater lake founders had been recruited. However, compared to the shallow Lakes Nicaragua - mean depth ca. 13m - and Managua - mean depth ca. 9m - some crater lakes offer a new and probably more stable ecological opportunity because they are very deep (e.g. Apoyo mean depth ca. 142m, Xiloá mean depth ca. 60m). 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.

## *Taxonomic evaluation of Lake Apoyo Amphilophus*

Geiger MF, McCrary JK, Stauffer, JR (2010) Description of two new species of the Midas cichlid complex (Teleostei: Cichlidae) from Lake Apoyo, Nicaragua. Proceedings of the Biological Society of Washington, 123, 159–173.

The systematic-taxonomic reevaluation of the L. Apoyo species assemblage was intended to deliver the basis for a more detailed study of the evolutionary processes underlying diversification within this particular Midas cichlid species flock. Clearly, recognition of the species-level diversity in this system is a necessary prerequisite for reconstructing the evolutionary history of speciation. But, although the mode of speciation in Apoyo Midas cichlids has already been in the focus of several studies (Wilson 2000; Barluenga & Meyer 2004; Barluenga et al. 2006), these authors always either failed to mention that five, rather than two forms were known from L. Apoyo or did not explicitly specify what their sampling was based on and the material was not stored at an accessible research collection. After intensively having collected in L. Apoyo, it became soon clear that the already before formulated doubts about the quality of evidence for a sympatric speciation scenario (Schliewen et al. 2006) were justified, as the observed diversity of Midas cichlid phenotypes clearly fell into several distinct clusters.

As a result of detailed morphometric comparisons with type material of *Amphilophus zaliosus* (Barlow 1976), *A. astorquii*, *A. chancho*, and *A. flaveolus* (all Stauffer, McCrary & Black 2008), two species belonging to the *A. citrinellus* (Günther, 1864) species complex endemic to L. Apoyo were described. Both species exhibit unique phenotypic characters that have not been found in other members of the L. Apoyo *Amphilophus*. Furthermore, they breed assortatively in L. Apoyo and can readily be distinguished in the field from all other described species found in that lake. Including the two described species, six species that form a monophyletic species assemblage within the Midas cichlid complex inhabit L. Apoyo, Nicaragua.

*Amphilophus supercilius* can be distinguished from all other described *Amphilophus* by its rounded oval-shaped caudal fin supported by strong and short fin rays. In all other species of the complex, the caudal fins are subtruncate, more triangular shaped and do not exhibit such short and robust outer fin rays. In *A. supercilius* the two outermost principal caudal-fin rays are shorter than in other species of *Amphilophus* from L. Apoyo and do not reach to the posterior fourth of the caudal fin, thus giving the fin its oval shape. Additionally, it differs from other species by various standard measurements and coloration features that will not be presented in detail. The specific epithet is an adjective from the Latin word meaning eyebrow or 'frowning being', referring to the strongly developed portion of the neurocranium that borders the eye cavity and gives the species the appearance of possessing eyebrows or a frowning appearance. The new species was earlier reported as *A.* 'barlow' in a report on breeding ecology (McCrary & López 2008). It breeds between September and March during the dry season with peaks in October and February. Breeding pairs were encountered in depths ranging between 1.5–20 m where rocks form suitable burrows or crevices. It was often observed to breed in direct vicinity to *A. zaliosus*, and it is noteworthy to mention that all observed pairs (n>30, MFG pers. obs.) were between conspecifics. Of all *Amphilophus* from L. Apoyo, *A. supercilius* had the most generalist diet and stomachs contained fish remains, fish-eggs, molluscs, and the macrophyte *Chara* (McCrary et al. pers. obs.).

*Amphilophus globosus* can be readily distinguished from all other L. Apoyo *Amphilophus* by its distinctive high-bodied shape in lateral view, conspicuous bright yellow coloration, and the absence of clear and continuous dark or black lateral bars in non-breeding coloration. Additionally, it differs from other species by various standard measurements that will not be presented in detail. The specific epithet is an adjective from the Latin word meaning round or globated, which refers to the general appearance of this high-bodied species. This new species was previously not identified as a distinct species and thus its breeding ecology was not catalogued separately. It has been observed breeding with conspecifics over *Chara* vegetation at 4–8 m depth (n=5; JKM pers. obs.). Interestingly, while at least some individuals of all other examined L. Apoyo species of *Amphilophus* had molluscs in their stomachs, in ten radio-graphed *A. globosus* individuals, we did not observe any molluscs or shell remains.

Multivariate analysis of the morphometric and meristic data support the heterospecificity of all six described species, and all morphometric, ecological, and genetic results to date strongly suggest that the original founder stock has evolved into multi-species lineages under sympatric conditions. It seems more parsimonious to assume independent parallel sympatric speciation in the isolated Nicaraguan crater lakes than to accept the idea that the endemic species of *Amphilophus* in each respective water body would have had wider distributions and undergone subsequent local extinctions.

## *Molecular Ecology of the L. Apoyo species flock*

Geiger MF, Mayr C, McCrary JK, Schliewen UK. Molecular Ecology of the Lake Apoyo Midas Cichlid Species Flock. (submitted to Molecular Ecology on April, 14 2011)

The aim of this part of the PhD thesis was to infer the significance of hybridization, sexual selection and natural selection for the formation of a complete and complex species flock, and thus to re-evaluate previous published findings concerning the mode of speciation of the L. Apoyo Midas cichlid species flock. It directly builds upon the systematic-taxonomic examination of the L. Apoyo flock and seeks to explore factors that maintain species integrity and cohesiveness despite ongoing gene flow. To this end, a comprehensive data set of population genetic (mtDNA, AFLP), morphological (body shape) and ecological (isotopic signatures) proxies of species level differentiation for a representative set of 126 individuals of all six L. Apoyo *Amphilophus* species and several potential hybrid individuals was established. Signatures of divergence were extracted from each data set to identify system specific factors potentially contributing to the sympatric speciation process.

The geometric morphometric analyses support morphological differentiation between the six described species and a CVA assignment test correctly classified 95.7% of the individuals. Only *A. chancho* showed considerable overlap in morphospace with *A. flaveolus* and two of the seven included potential hybrid individuals were unambiguously assigned to one species, the remaining were not significantly or ambiguously assigned.

In terms of a phylogenetic species concept the recovered phylogeny delivered different but overall low levels of monophyly support for the six *Amphilophus* species. The tree topology based on the Link et al. (1995) distance measure on 2297 AFLP loci contains taxonomically homogeneous and heterogeneous clades, and only *A. globosus* forms a monophylum. As consequence of the recovered topology with heterogeneous clades, the phylogeny based tests for homoplasy-excess and lineage-movement where only performed for selected clades and not for species specific groups only. For the homoplasy-excess test we conducted 36 removal experiments and excluded once at a time either each clade with bootstrap support (BS) >25 %, all individuals of a single species or each of the potential hybrid individuals. Conspicuous results were the increase of BS for the monophyly of *A. globosus* from m=30.2% to 50% upon removal of *A. chancho*, and the monophyletic grouping of the otherwise non-monophyletic *A. zaliosus* upon removal of either *A. chancho*, *A. astorquii* or *A. supercilius*. This hints to a possible hybridization component in the speciation event of the latter three species. The lineage-movement procedure showed that seven conspicuous *A. zaliosus* individuals appeared in 38.8% of 2000 bootstrap replicates outside the whole Midas cichlid complex as basal sister to *A. lyonsi*, indicating a potential introgression event by an unknown allochthonous contributor.

The stable isotope analysis (SIA) supports the notion of dietary differentiation among the six *Amphilophus* species, and the detected range of $\delta^{15}N$ from 3.3 to 8.9‰ suggests the existence of at least two trophic levels. *Amphilophus globosus* exhibited the lowest $\delta^{15}N$ values (m=3.9‰), *A. astorquii* showed intermediate $\delta^{15}N$ levels (m=5.5‰) and *A. supercilius* had the highest mean $\delta^{15}N$ of all examined species (m=7.8‰). Pairwise comparisons revealed significant differences in $\delta^{15}N$ between all *Amphilophus* species, except pairs *A. astorquii* / *A. flaveolus* and *A. zaliosus* / *A. supercilius*.

Using Bayescan 1.0 to identify potential candidate loci under directional selection from the AFLP matrix, 49 loci (ca. 2%) were detected to be influenced by directional selection applying the strictest criteria 'decisive'. Further analyses of the AFLP data were conducted on both, the complete matrix and on the reduced matrix (without the 49 loci), which we refer to as the neutral matrix. All pairwise $F_{ST}$'s obtained from the complete and the reduced, neutral AFLP matrix were significant and ranged between 0.052 - 0.223 based on pairwise differences for the complete matrix and between 0.036 - 0.141 for the neutral matrix. Exclusion of the 49 loci detected to be under selection lead to a $F_{ST}$ decrease by 30.8 ± 6.8%. Pairwise $F_{ST}$'s were differently strong influenced by the exclusion of the 49 loci, and a weak correlation between $F_{ST}$ and decrease was observed ($r=0.63$, $p<0.05$). The effect ranged from a decrease by 42.8% (*A. chancho* / *A. zaliosus*) to 20.9% (*A. astorquii* / *A. flaveolus*). Excluding the 49 outlier loci had a clear effect on AMOVA outcomes: among species variation decreased by one third while within species variation increased by ca. 5%. While AMOVA revealed ca. 6.5% of variation between coloration groups using all loci, removal of the 49 loci lead to a complete loss of genetic variation explained by this coloration-based grouping and a slight reduction in variance explained by the trophic level.

Findings from the Bayesian cluster analysis with Structure v2.2 without *a priori* species designation suggest different levels of population integrity of the six species and point to the presence of hierarchical genetic structure with six clusters to be most likely, corresponding to the six species. The Structure runs on the reduced AFLP matrix and on the 49 outlier loci matrix without *a priori* species assignation delivered extremely different results. No differentiated, coherent clusters were detected in the neutral AFLP matrix, irrespective of K. Only the seven conspicuous *A. zaliosus* individuals showed assignment probabilities around 50% to a non-Apoyo cluster. To the contrary, including only the 49 outlier loci in the Structure runs was highly informative: 1) six was the most likely number of genetically differentiated groups, identical to the outcome with the complete AFLP matrix; 2) all A. zaliosus individuals grouped together, while they were split based on the complete matrix; 3) a much higher percentage of individuals showed elevated admixture proportions as compared to the complete AFLP matrix and 4) only 8.9 % (compared to 80.2% based on the complete matrix) of the *A. astorquii* individuals showed assignment probabilities above 95 % to a unique cluster.

We found a moderate correlation between pairwise distances from the morphometric data with the pairwise AFLP $F_{ST}$ values. Congruently, the Mantel statistic was significant for a correlation between Euclidean distances based on PC3 and pairwise AFLP $F_{ST}$ only ($r=0.58$, $p<0.05$). The strongest correlation was detected between the ecologic descriptor based on the SIA and, again, the Euclidean distances based on PC3 from the morphometric data (Mantel $r=0.89$, $p<0.001$).

We tried to extract factors that are related to AFLP loci distribution using canonical correspondence analysis (CCA), and emerging patterns can be summarized as follows: 'coloration' and 'trophic position' almost always significantly improved the model of the CCA on the outlier AFLP matrix while this was not the case for the neutral matrix; the opposite was found for variables 'PC3' of the geometric morphometrics and 'location', which were almost always significantly associated with the neutral AFLP matrix and not with the outlier loci distribution. Species assignment included as a variable differed in importance between the CCAs, being always significantly related to the outlier loci distribution for *A. chancho*, *A. globosus* and *A. zaliosus*, but not for the remaining species, and to a smaller extent to the neutral AFLP matrix.

In sum we identified eco-phenotypic covariance structure with neutral and non-neutral genetic differentiation that differentially support a role of both, divergent natural, and sexual selection for shaping current species divergence in L. Apoyo. Body coloration, likely involved in sexual selection, was identified as potential key character for maintaining species cohesion, while ecological selection judged from the proxies applied yielded a comparatively weak signature. Five out of the six species were found to be polyphyletic and showed substantial levels of gene flow between species. We conclude that the shared alleles partially result from gene flow and not only from ancestral polymorphisms, since otherwise we would anticipate stronger incongruence between genotypic and phenotypic cohesiveness. Within *A. zaliosus* several individuals showed putative allochthonous phylogenetic affinities and levels of neutral differentiation not related to divergent selection within L. Apoyo indicating a past introgression event from outside. We find no strong indication that any of the six species has originated from 'homoploid hybrid speciation' sensu Mallet (2007), i.e. that hybridization has had a primary role in the origin of one of the species. In that case we expected to find a similar signature of introgression in any of the six species as has been described for the seven 'aberrant' *A. zaliosus* individuals above. However, we demonstrate the existence of various levels of gene flow between species pairs, underlining their incipient status and a potentially non-detrimental or even beneficial role of hybridization. The observed pronounced genetic, phenotypic and ecological differentiation between *A. globosus* and *A. zaliosus* supports a scenario of primary divergence in those species as opposed to a secondary divergence including a hybridization component for *A. astorquii*, *A. chancho*, *A. flaveolus* and *A. supercilius* as deduced from the homoplasy-excess tests and cluster analyses. Our findings demonstrate the possibility that introgression of allochthonous alleles into seemingly fully isolated crater lake species flocks can take place, and should not be dismissed as potentially confounding factors.

## SUMMARY (GERMAN)

Mit der vorliegenden Arbeit wurde ein Beitrag zur evolutionsbiologischen Grundlagenforschung geleistet welcher letztlich darauf abzielt, mit dem nicaraguanischen *Amphilophus* cf. *citrinellus* Komplex ein geeignetes Alternativmodell zu den bekannten afrikanischen Artenschwärmen zu etablieren. Nach eingehender Literaturrecherche wurde während zwei dreimonatiger Sammelreisen nach Nicaragua die nötige Materialbasis in Form einer Aufsammlung von ca. 800 adulten, individualisiert beprobten *Amphilophus* Buntbarschen aus sieben Kraterseen (Apoyo, Apoyeque, Asososca Leon, Masaya, Monte Galan, Tiscapa, Xiloá), den beiden großen, alten tektonischen Seen Managua und Nicaragua sowie dem San Juan Fluss geschaffen.

Basierend auf dem gesammelten Material wurde zum ersten Mal eine umfassende phylogenetische Hypothese für den gesamten Arten-Komplex mittels molekulargenetischen Methoden (mtDNS & AFLP) erarbeitet und dabei die Besiedelungsgeschichte der einzelnen Seen untersucht. Hierbei zeigte sich deutlich die bisher scheinbar übersehene Komplexität des Systems welche sich in unterschiedlichen Monophylie-Wahrscheinlichkeiten und cytonukleärer Diskordanz bei einigen Populationen äußert. Dies führt dazu, dass für einige Seen die Plausibilität sympatrischer Artentstehung größer ist als für andere. Zudem demonstriert die Arbeit, dass das bisher angewendete Drei-Arten Konzept für den Gesamtkomplex in keiner Weise der tatsächlichen Diversität gerecht wird und ein individualisierter Ansatz nötig ist, um verlässliche Aussagen über evolutionäre Prozesse zu treffen.

Eine eingehende, morphologisch-vergleichende Untersuchung der *Amphilophus* Population aus dem Apoyo See, einem etwa 21.000 Jahre alten Kratersee, führte im Rahmen dieser Arbeit zur Beschreibung zweier neuer Arten, *A. globosus* und *A. supercilius*. Dies erhöht die Anzahl der im Apoyo See endemisch vorkommenden Arten auf sechs, welche dort innerhalb den letzten ca. 20.000 Jahren entstanden sein müssen. Noch in einem Artikel von 2006 in der renommierten Zeitschrift *Nature* berichten die Autoren nur von zwei Arten in diesem See und stützen ihre Artentstehungshypothese auf einer eindeutig unvollständigen Materialbasis.

Im letzten Teil der Arbeit wurden anhand des Apoyo Sees exemplarisch mögliche Artbildungsmechanismen untersucht, die zu der zuvor charakterisierten Diversität geführt haben. Hierzu wurde die genetische Differenzierung (mtDNS & AFLP) mit morphometrischer und ökologischer (stabile Isotopen $^{12}C/^{13}C$ und $^{14}N/^{15}N$) Differenzierung verglichen und auf Korrelationen hin untersucht. Durch Aufteilung der genetischen Varianz in eine neutrale und eine nicht-neutrale, d.h. vermutlich von Selektion beeinflusste Komponente fanden sich Hinweise darauf, dass für die momentane Kohärenz der sechs Arten im Apoyo See wahrscheinlich sexuelle Selektion wichtiger ist als natürliche (ökologische) Selektion. Wir schließen auf sexuelle Selektion während des sympatrischen Artbildungsprozesses, da in unseren Daten der Erklärungswert der Brutpflegefärbung für die genetische Varianz von ca. 6.5 auf 0% fällt, wenn die unter Selektion stehenden Loci entfernt werden. Ein solcher Effekt auf die genetische Varianz wurde für die Trophiestufe oder den Fundort nicht nachgewiesen. Schließlich konnten wir bei *A. zaliosus* Introgression von einer allochthonen Quelle nachweisen und den Genfluss zwischen den einzelnen Arten abschätzen. Demnach findet Hybridisierung statt, die einzelnen Arten evolvieren aber trotz unvollständiger reproduktiver Isolation als morphologisch und genetisch kohärente Einheiten.

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## CURRICULUM VITAE

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**Academic Education:**

1999 - 2005:
Ludwig Maximilian University Munich, Germany: Biology (Zoology, Palaeontology, Ecology and Fishery-Biology)

2005 - 2006:
Diploma Thesis, “A morphometric and molecular study of selected populations of *Gymnocephalus cernuus* (Linnaeus, 1758) (Teleostei:Percidae) from central Europe” (Advisor: Dr. U. Schliewen, Bavarian State Collection of Zoology, Munich (ZSM))
Diploma Biology (“Diplom Biologe Univ.”)

Since 2007:
PhD, “Phylogenetic relationships and evolutionary ecology of Nicaraguan Midas cichlids (*Amphilophus* spp.)” (Advisor: Prof.Dr. G. Haszprunar & Dr. U. Schliewen, ZSM)

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**Research Experience:**

2000 - 2004: Student Assistant at Max-Planck Institute for Behavioral Physiology in Seewiesen (Aquaria management and realization of habitat-choice and clonal-competition experiments with *Poecilia formosa* and other viviparous fish; Flow Cytometry)

2002 - 2008: Student Assistant at ZSM, Section Ichthyology (EDP acquisition of the ichthyological collection, assistance in the project “Diversity of Bavarian Fishes” including field work and sample processing)

VI. 2010 - IV. 2011: Project Co-leader, DFG funded “DNA Bank Network” at ZSM

Since V. 2011: Project Coordinator FREDIE (Freshwater Identification for Europe) at ZFMK (Bonn)

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**Presentations:**

III.2007: Presentation at the conference “Aportes a la taxonomía de la mojarra común (*Amphilophus* c.f. *citrinellus*) y su conservación” in Managua, Nicaragua.

IX.2007: Poster presentation at the ECI XII (XII. European Congress of Ichthyology) in Dubrovnik, Croatia: “A morphometric and molecular study of selected populations of *Gymnocephalus cernuus* (Linnaeus, 1758) (Teleostei:Percidae) from central Europe”.

XI.2007: Presentation at ZSM for EESlmu Master students: “An introduction into morphometrics” in Munich, Germany.

XI.2007: Presentation at ZSM “Science days”: “Midas cichlids in Nicaragua” in Munich, Germany.

I.2008: Presentation at National Park Masaya Volcano: “Species Concepts and Midas Cichlids in Nicaragua” in Masaya, Nicaragua.

IX.2008: Presentation at DATZ-Forum „Buntbarsche“: “Vielfalt und Taxonomie des Midas-Cichliden Komplexes in Nicaragua (*Amphilophus* c.f. *citrinellus*)**”** in Stuttgart, Germany.

IV.2009: Presentation at DCG/IAG: “Nicaragua, der Zitronenbuntbarsch und seine Geheimnisse…” in Germaringen, Germany.

XI.2009: Presentation at ZSM “Open-house”: “Auf Buntbarschjagd im Kratersee – Abenteuer Nicaragua” in Munich, Germany.

XI.2009: Presentation at the International Conference on Evolutionary Ecology of Fishes – Diversification, Adaptation and Speciation: “Not a simple case. The Midas cichlid complex – a first comprehensive phylogenetic hypothesis” in Berlin, Germany.

IX.2010: Poster presentation at conference “Tools for Identifying Biodiversity: Progress and Problems” in Paris: “DNA Bank Network – connecting biological collections and sequence databases by means of long-term DNA storage with online accession”.

**Peer-reviewed publications:**

Lamatsch, D.K., Lampert, K.P., Fischer, P., **Geiger, M.**, Schlupp, I., Schartl, M. Diploid Amazon mollies (*Poecilia formosa*) show a higher fitness than triploids in clonal competition experiments. Evolutionary Ecology **23**, 687-697 (2009).

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**Geiger, M.F.**, McCrary, J.K., Stauffer, J.R., Jr. Description of two new species of the Midas cichlid complex (Teleostei: Cichlidae) from Lake Apoyo, Nicaragua. Proceedings of the Biological Society of Washington, **132**(2), 159-173 (2010).

**Geiger, M.F.**, McCrary, J.K., Schliewen, U.K. Not a simple case – a first comprehensive phylogenetic hypothesis for the Midas cichlid complex in Nicaragua (Teleostei: Cichlidae: *Amphilophus*). Molecular Phylogenetics and Evolution **56**(3), 1011-1024 (2010).

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Straube, N., **Geiger M.F.**, Wedekind, J. & Kirchhauser, J. X.: Second generation parthenogenesis in the Whitespotted Bambooshark *Chiloscyllium plagiosum*. Intended to be submitted to Journal of Heredity.

**Non peer-reviewed publications:**

Lechner, W., **Geiger, M.**, Werner, A. Neues aus der Gattung *Hypancistrus.* DATZ **11**, 6-13 (2005).

Lechner, W., **Geiger, M.**, Werner, A. Neues aus der Gattung *Hypancistrus.* (Schluss) DATZ **12**, 10-17 (2005).

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- Spanish advanced knowledge
- Latin qualification
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- Experience with ABI 3130 genetic analyser
- Phylogenetic and population genetic analyses
- EDP (Soft- & Hardware installation, Office, Photoshop, Illustrator etc.)
- Diving license (AOWD – Advanced Open Water Diver, PADI)
- Fishing & electrofishing license
- International driving license

## *Article I*

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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.

## 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). Alone 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 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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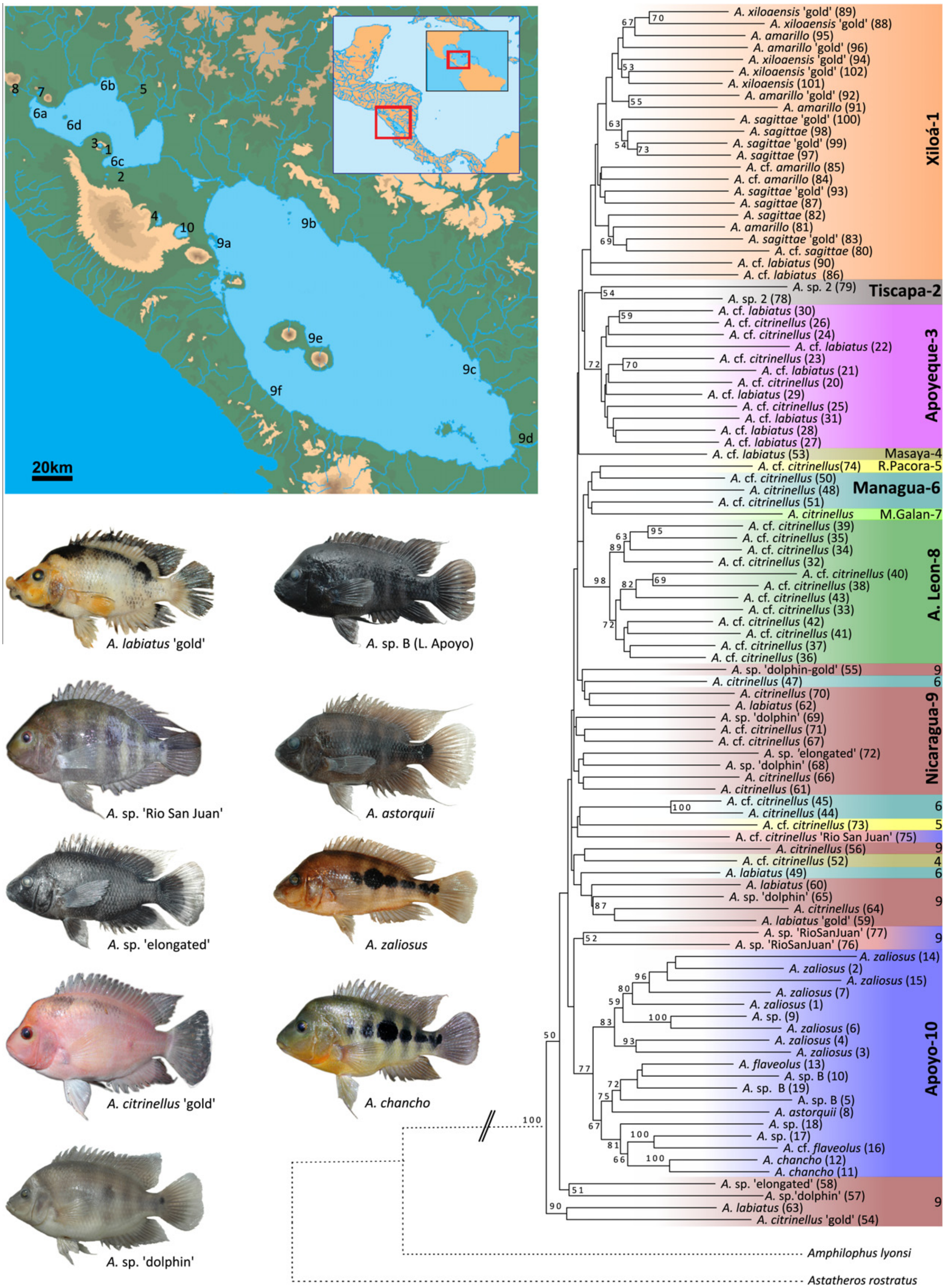

**Fig. 1.** AFLP NJ-tree with bootstrap supports and sample ID given in brackets, map indicating sample locations and some representative species of the Midas cichlid complex.

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 crater 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. zaliosus*) 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. zaliosus* 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 Říčan 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′-ATATACACATGTCACGTAAG-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

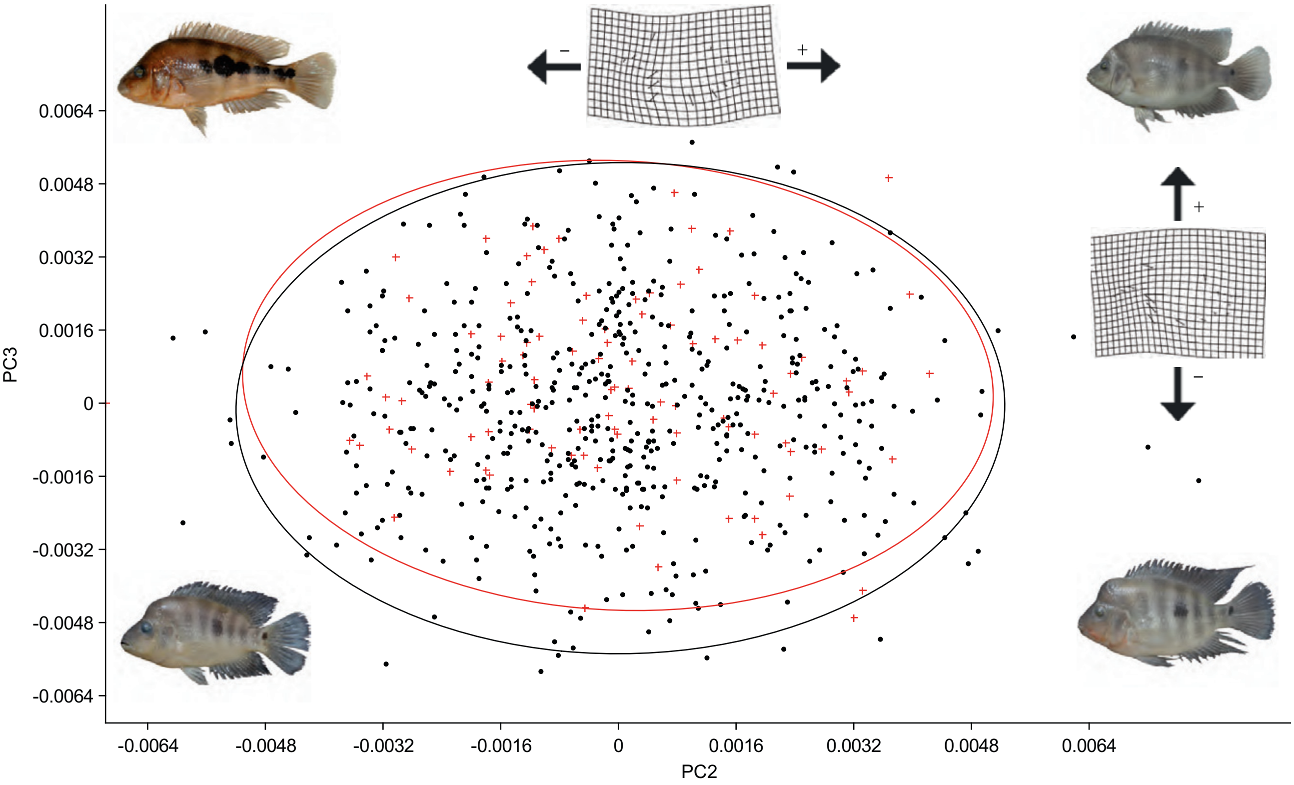

**Fig. 2.** Plot of the second vs. the third principal component of the partial-warp scores from the geometric morphometric landmark analysis (647 individuals) with deformation grids for each axes and representative individuals. Black ellipse: 95% confidence interval of the whole sample. Red ellipse: 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 taxon'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 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 $\alpha$ (Dirichlet parameter for the degree of admixture) and $\lambda$ (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 $\Delta K$ 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. zaliosus* 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. xiloaensis*) 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 (∼73%) variation is attributable to within lake differentiation, whereas only ∼27% can be explained by between-lake differences and only ∼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 partitioned as ∼16% among and ∼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 Fst values derived from the AFLPs reflects the geographic history of the two once permanently connected lakes. Pairwise Fst 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 Fst 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 Fst 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 Fst of 0.270. All pairwise Fst values from the AFLP data were significant (Table 5) and range from 0.04 to 0.25. Lake-specific Fst values were again very homogeneous (0.151–0.173) and show that all groups do equally support the global Fst 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 $\Delta K$, 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 LnP(D) (Fig. 7), the highest log-likelihood probabili-

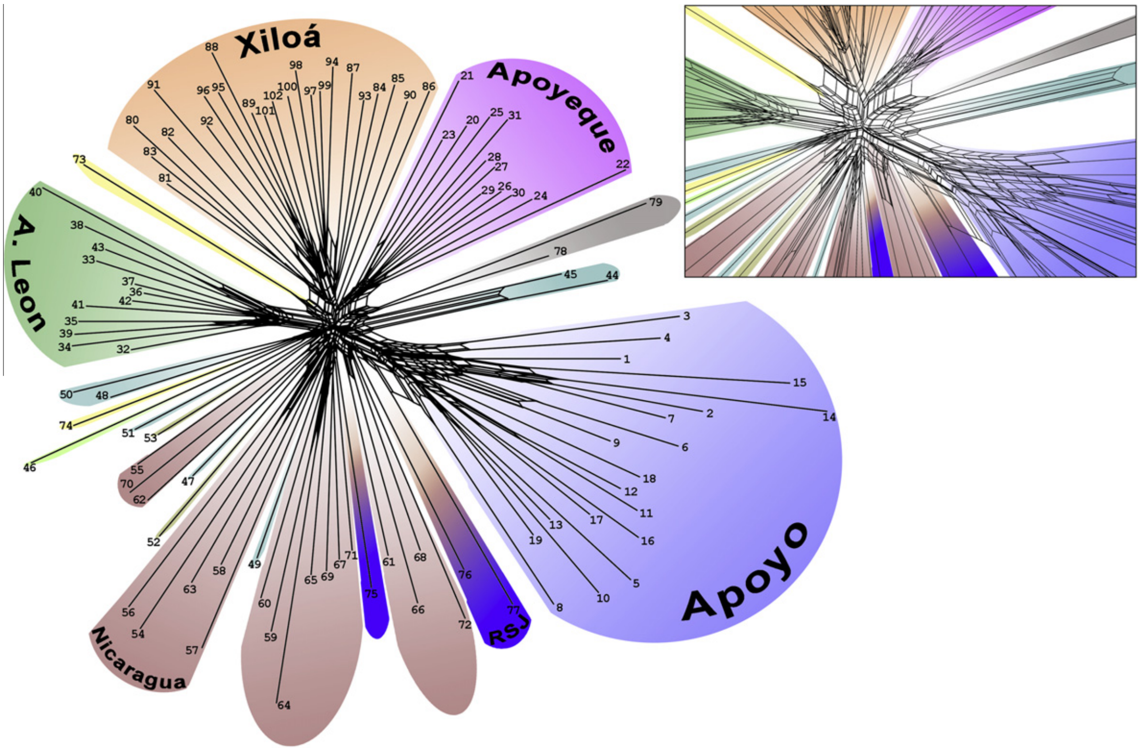

**Fig. 3.** AFLP neighbor-network based on Link’s genetic distances. Sample IDs are those used throughout. Conflicting phylogenetic signal in the center magnified top right.

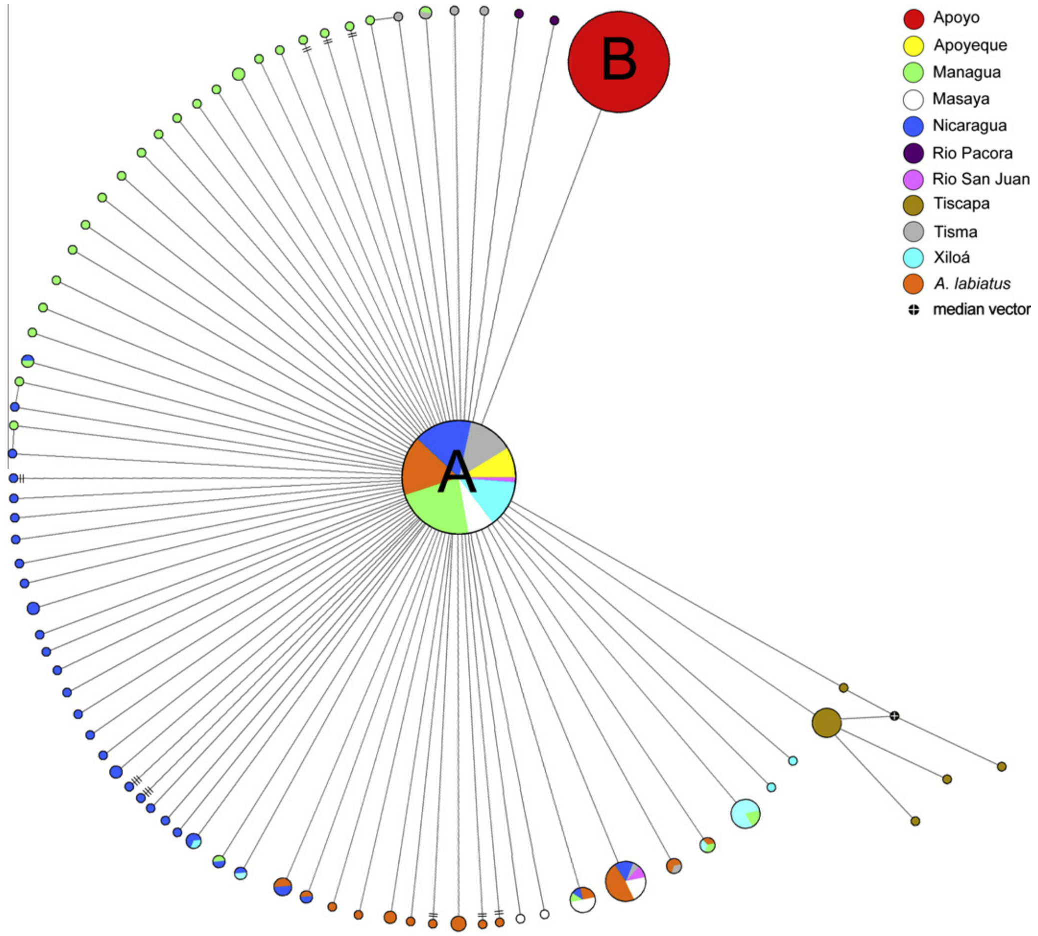

**Fig. 4.** Median-joining parsimony-network based on the mitochondrial control-region. Included is the central haplotype and only those directly connecting it without further complex branching. Circle size corresponds to sample size, one bar indicates one additional mutational step.

ties were detected for $K = 7$ and were confirmed with a second peak of $\Delta K$ 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 *K*2 the main groups are L. Apoyo vs. remaining individuals, for *K*3 a third cluster appears for all A. Leon samples, for *K*4 samples from L. Apoyeque and L. Xiloá are grouped together in a new cluster, for *K*5 the L. Apoyeque and L. Xiloá individuals are separated, for *K*6 a separation of *A. zaliosus* individuals from the remaining L. Apoyo samples is visible and for *K*7 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 potential 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

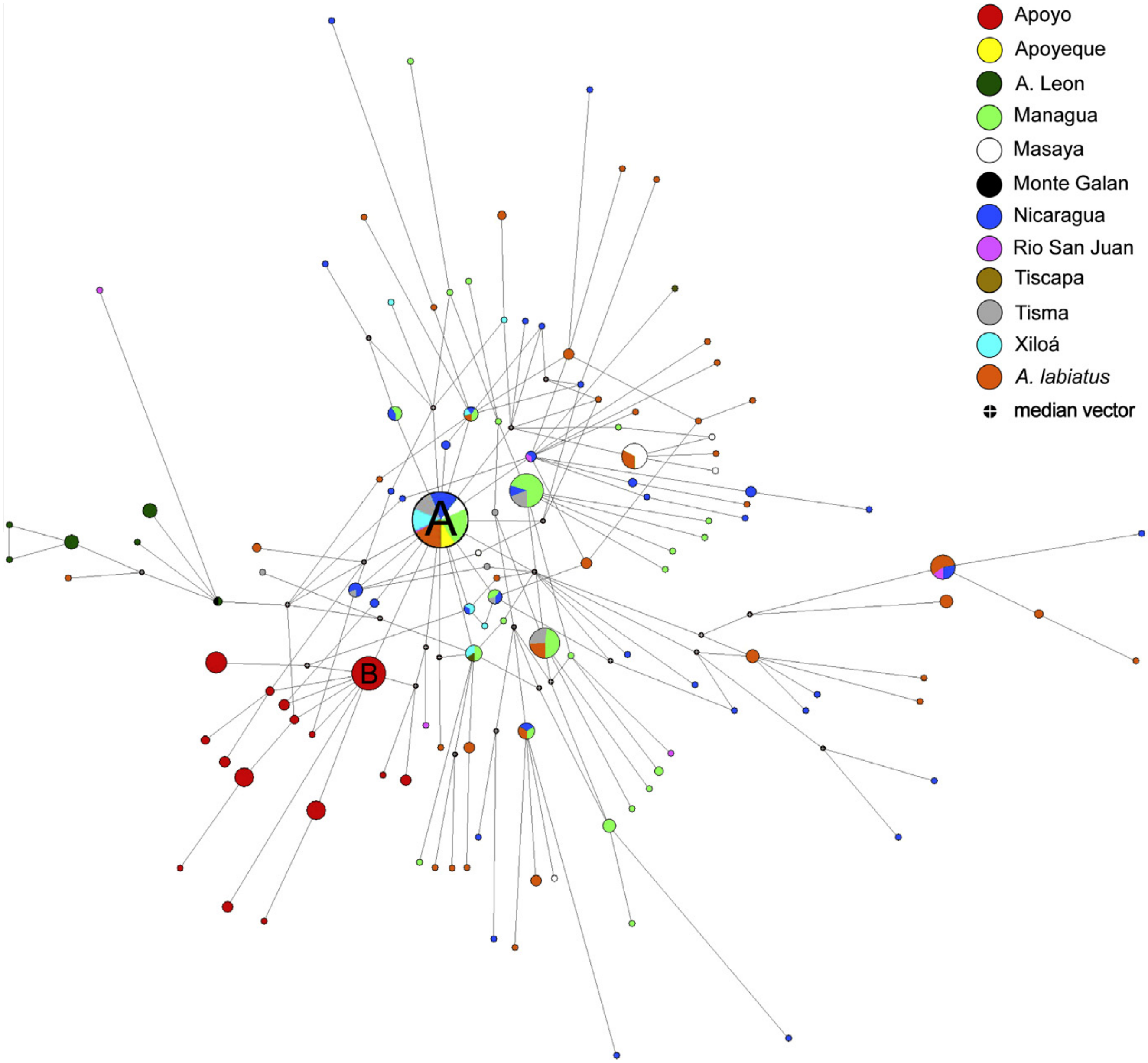

**Fig. 5.** Full median-joining parsimony-network based on the mitochondrial control-region. Included is the central haplotype and all remaining haplotypes connecting with further complex branching not shown in Fig. 4.

**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. Homoplasy excess, leaf-stability and lineage-movement*

In addition to standard bootstrapping, homoplasy 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 sings 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 3**
Pairwise Fst 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.

| | mtDNA control region | | | 2793 AFLP loci | |
|---|---|---|---|---|---|
| Lake | *n* (individuals) | $n^*$ (haplotypes) total/private | Fst Arlequin | *n* (individuals) | Fst 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 5**
Pairwise Fst 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, *K*7) shows that this particular individual from the San Juan River is assigned to two clusters, L. Apoyo non-*zaliosus* 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-zaliosus 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-*zaliosus* 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. zaliosus* 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. zaliosus* (termed 'normal' in their study)

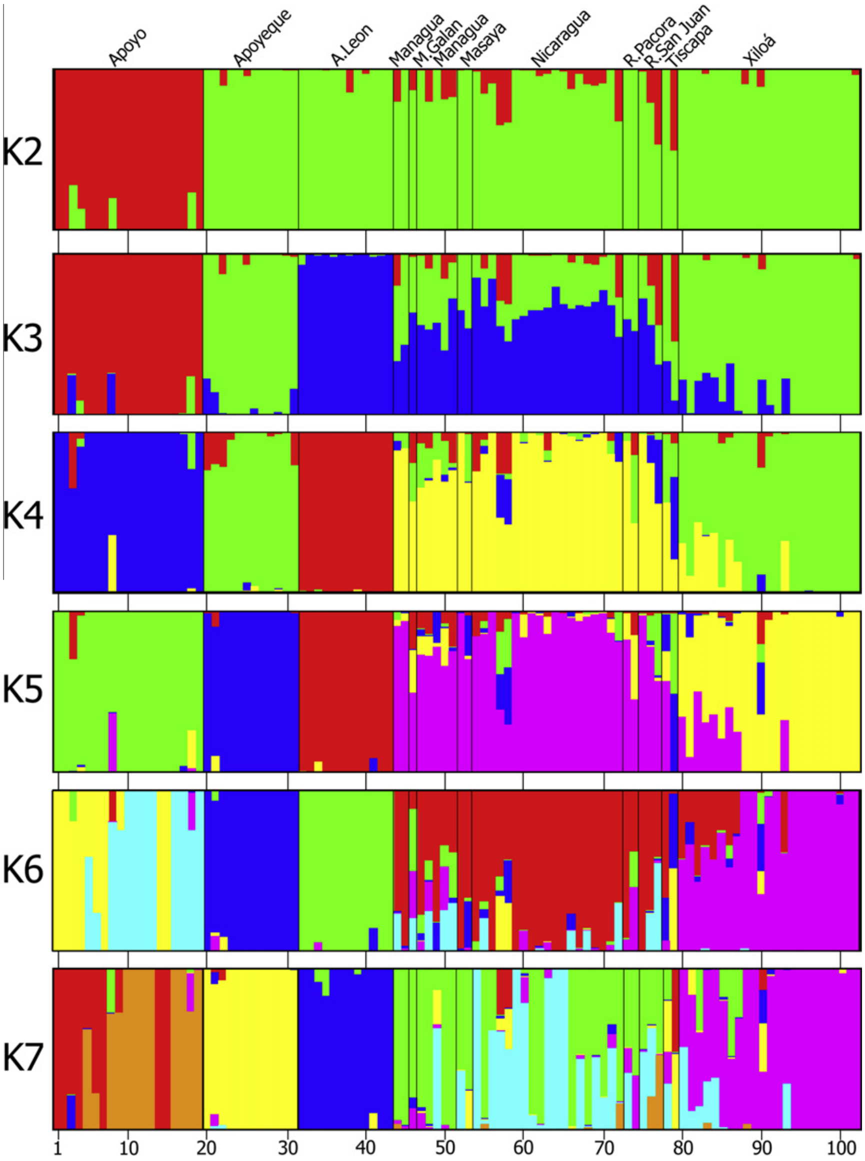

**Fig. 6.** Results of Structure clustering analysis for the whole AFLP dataset from $K2$ to $K7$ using Structure v2.2 without group information. $K7$ yielded the highest likelihood values. Lake of sample origin given above, sample ID of individuals given below.

and few individuals of the three more colorful species *A. chancho*, *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. Xiloa).

### 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

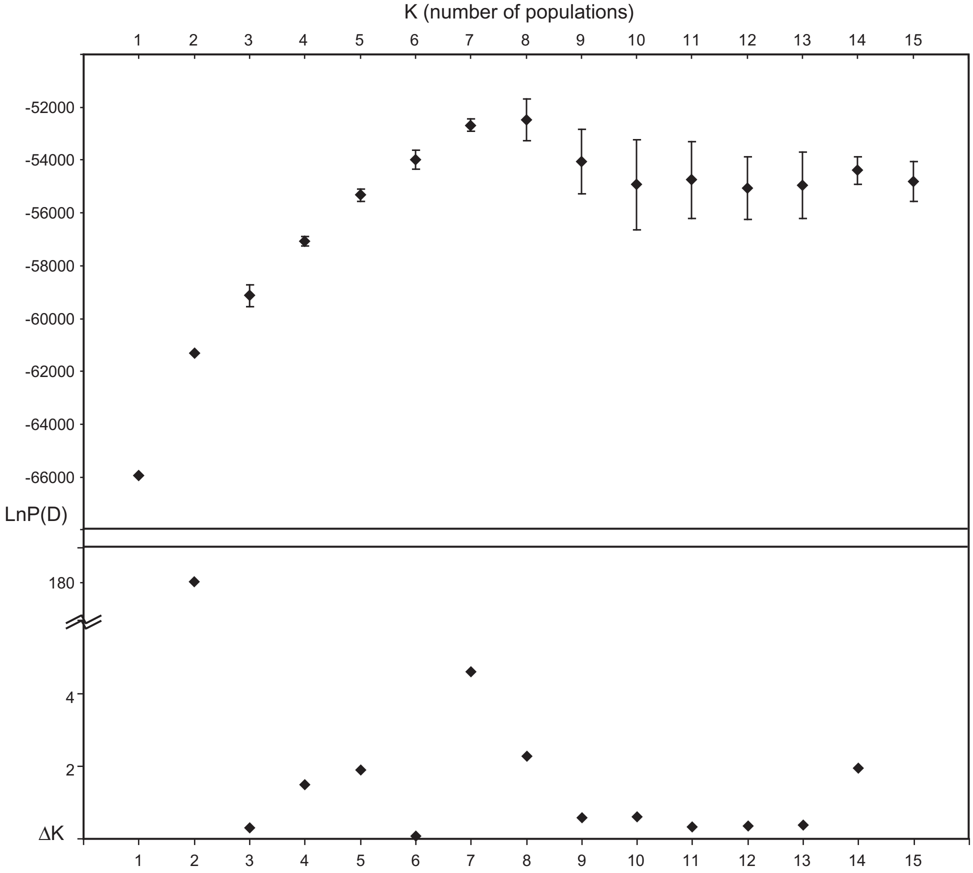

**Fig. 7.** Estimation criteria for the number of genetic clusters in the AFLP dataset. Above: Mean LnP(D) with SD from 20 replicates for each *K*, calculated without 'locprior' model (Structure v2.2). Below: Evanno's model choice criterion 'Δ*K*' for the uppermost level of genetic structure.

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 Fst 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 km$^2$ 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),

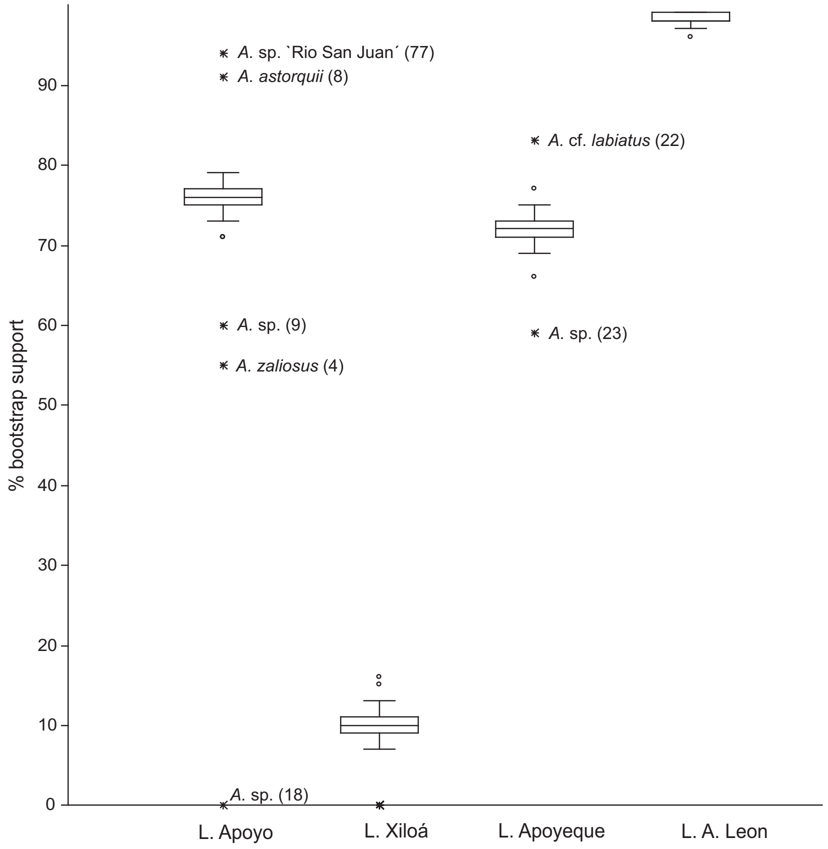

**Fig. 8.** Homoplasy-excess test outcome applying a modified Jackknife across taxa approach. Each single individual was removed once and bootstrap support for monophyly calculated from 1000 pseudo-replicates. Outliers indicated by asterisks. ID-numbers of individuals in brackets correspond to those 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 conspecifity 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 phenotypes 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 independently 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.ympev.2010.05.015.

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## *Article II*

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# Description of two new species of the Midas cichlid complex (Teleostei: Cichlidae) from Lake Apoyo, Nicaragua

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*Abstract.*—Two species belonging to the *Amphilophus citrinellus* (Günther, 1864) species complex endemic to Lake Apoyo, Nicaragua are described. Both species exhibit unique phenotypic characters that have not been found in other members of the species complex. Furthermore, they breed assortatively in Lake Apoyo and can readily be distinguished in the field from all other described species found in that lake. Including the two herein described species, six species that form a monophyletic species assemblage within the Midas cichlid complex inhabit Lake Apoyo, Nicaragua.

Midas cichlids are monogamous substrate spawners that form pairs only during breeding season, when they aggressively defend their territory and fry. The name Midas cichlid derives from the fact that, in some Nicaraguan lakes, brightly coloured *Amphilophus* individuals can be found that have lost the melanophores that build patterns of black bars, yielding individuals ranging in color from white to orange or red, and collectively termed 'gold.' They are found in varying abundance in more turbid lakes but not in Lake Apoyo (Barlow 1976).

The Midas cichlid complex makes an excellent study subject for evolutionary biology and taxonomy. Historically, Barlow & Munsey (1976) recognized only three species in the Midas cichlid complex; however, we are now able to differentiate several species. Field observations using SCUBA, extensive collecting efforts, and proper treatment of collected material for museum collections allowed the description of three endemic species from Lake Xiloá in 2002 and three endemic species from Lake Apoyo in 2008 (Stauffer & McKaye 2002, Stauffer et al. 2008). Findings from molecular genetic studies suggested that both Lake Xiloá and Lake Apoyo contained other undescribed *Amphilophus* and that all examined individuals in one lake are closely related to each other based on microsatellite genotyping of three loci (McKaye et al. 2002). This conclusion was supported by mitochondrial DNA sequence data, which demonstrated that fishes from Lake Apoyo were more closely related to each other than to fishes from other lakes (Barluenga & Meyer 2004, Barluenga et al. 2006).

The recent origins of Lakes Xiloá (<10,000 bp, BANIC 1977) and Apoyo (<23,000 bp; BANIC 1977, Sussman 1985) coupled with their endemic Midas cichlids has inspired biologists to speculate and investigate the possibility of sympatric speciation (Barlow 1976, McKaye et al. 2002, Barluenga et al. 2006,

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but see Schliewen et al. 2006). The small size of Lake Apoyo (20.92 km$^2$ surface area) and its rather homogeneous habitat with syntopic breeding of its endemic *Amphilophus* species (McCrary & López 2008), support the theory that sympatric speciation has occurred (Wilson et al. 2000, Barluenga et al. 2006).

A complete taxonomic inventory of the region is essential to discover the biologically most meaningful explanations of the phylogenetic history within the Midas cichlid complex. We provide here a description of the fifth and sixth members of Lake Apoyo's endemic species of *Amphilophus*.

## Site Description

Lake Apoyo is situated within an almost circular volcanic caldera of 36.32 km$^2$, about 4 km west of Lake Nicaragua. The lake's water surface occupies 20.92 km$^2$; its diameter measuring more than 4 km with a maximum depth 178 m (CIRA 2008). The caldera was created by a series of volcanic eruptions, the last one occurred about 23,000 years ago (Sussman 1985). The water level of Lake Apoyo is 70 m above sea level (masl), with effluents limited to subterranean filtration through highly permeable geologic layers toward neighboring Lake Nicaragua (31 masl; 5.4 hm$^3$ pa). The lake has undergone a 15 m decrease in water level since 1950 (CIRA 2008). The water of oligotrophic Lake Apoyo is warm (27–29.5°C), alkaline (pH = 8.1), and rather saline with a conductivity of 3310 µS and Na$^+$ of 640 mg/l (Parello et al. 2008).

Compared to some of the smaller volcanic crater lakes in Nicaragua, the fish fauna of Lake Apoyo is depauperate (Waid et al. 1999) and the native forms include: one atherinid *Atherinella sardina*, one poeciliid *Poecilia* cf. *sphenops*, and several cichlids: *Parachromis managuensis* and at least six (four of which have been described) endemic species of the genus *Amphilophus*. *Gobiomorus dormitor* and at least three species of tilapias have been introduced in the past two decades (Waid et al. 1999, McCrary et al. 2007). There are no open-water connections to any other water body; thus, it is speculated that these fishes might have entered the lake via humans, piscivorous birds, and/or climatic events (Stauffer et al. 2008).

## Materials and Methods

We collected fishes in three field trips during the dry seasons (Nov–Apr) of 2006/2007, 2007/2008, and 2008/2009. Individuals were caught by SCUBA divers using harpoons following field identification. Fishes were anesthetised, preserved in 10% formalin, individually tagged, and fin-samples taken for molecular genetic studies. Photos of live individuals were taken and notes on coloration made in the field. Fins were pinned, fishes preserved in 10% formalin, and permanently preserved in 70% ethanol. Counts and measurements follow Barel et al. (1977) and Stauffer (1991, 1994), except that head depth was measured from the hyoid symphysis to the top of the head at 90° angle to the horizontal body axis. Measurements are point-to-point using a dial calliper to the nearest 0.01 mm, and taken from the left side of specimens. Following abbreviations are used in the tables:

ADAA—anterior insertion of dorsal fin to anterior insertion of anal fin;
PDPA—posterior insertion of dorsal fin to posterior insertion of anal fin;
ADPA—anterior insertion of dorsal fin to posterior insertion of anal fin;
PDAA—posterior insertion of dorsal fin to anterior insertion of anal fin;
PDVC—posterior insertion of dorsal fin to ventral portion of caudal fin;
PADC—posterior insertion of anal fin to dorsal portion of caudal fin;
ADPV—anterior insertion of dorsal fin to insertion of pelvic fin;
PDPV—posterior insertion of dorsal fin to insertion of pelvic fin.

Additionally, we examined the caudal skeletons of ten individuals of each of the six Lake Apoyo species of *Amphilophus* using high-resolution x-ray pictures. We only used specimens with complete and straight caudal-fin rays and counted the number of: principal (segmented) rays, dorsal and ventral procurrent rays (sensu Chakrabarty 2007), procurrent and principal rays inserting at the haemal spine of the preural centrum (sensu De Schepper et al. 2004), principal rays of the parhypural (sensu Chakrabarty 2007), and principal rays inserting in each hypural. We also measured the length of the first ventral principal caudal-fin ray and the length of the lower central caudal-fin ray.

Since sex of Midas cichlids can only be determined by invasive methods or direct observation of breeding pairs, we did not group individuals according to sex and did not test for sexual dimorphism in morphometrics. Institutional abbreviations follow Leviton et al. (1985). Morphometric data were analyzed using sheared principal component analysis (SPCA), which factors the covariance matrix and restricts size variation to the first principal component (Humphries et al. 1981, Bookstein et al. 1985). Meristic data were analyzed using principal component analysis (PCA) in which the correlation matrix was factored. Comparisons among species were illustrated by plotting the sheared second and third principal components (2SPCA, 3SPCA) of the morphometric data or the 2SPCA and first principal components (PCA) of the meristic data. The Statistical Analysis System (SAS) software was used to calculate both PCA and SPCA.

As the monophyly of Lake Apoyo's species of *Amphilophus* flock has been hypothesized previously (Barluenga & Meyer 2004, Barluenga et al. 2006) and tested thoroughly including all known species and the two new species (Geiger et al. pers. obs.), data of the new species were compared to only those species of *Amphilophus* native to Lake Apoyo. To that purpose, we obtained meristic and morphometric data for the holotypes and seven paratypes each of, *A. astorquii*, *A. chancho*, and *A. flaveolus* as well as eight paratypes of *A. zaliosus* (see comparison material).

## Results

### ***Amphilophus supercilius***, new species

Fig. 1, Table 1

*Holotype.*—ZSM 38821, adult male, 166.6 mm SL, 27 Feb 2007, Spanish Coast, Lake Apoyo NE shore, Nicaragua, 11°56′5.43″N, 86°00′46.88″W, DNA tag: 247.

*Paratypes.*—All paratypes were collected from Lake Apoyo, Nicaragua. ZSM 37347, 163.6 mm SL, 11 Feb 2008, rock formation called "los hongos," S shore, 11°54′20.79″N, 86°01′45.40″W, DNA tag: 735. ZSM 37348, 142.4 mm SL, 11 Feb 2008, rock formation called "los hongos," S shore, 11°54′20.79″N, 86°01′45.40″W, DNA tag: 723. ZSM 37351, 2, 157.8–166.7 mm SL, 11 Feb 2008, rock formation called "los hongos," S shore, 11°54′20.79″N, 86°01′45.40″W, DNA tags: 728 & 736. ZSM 38751, 143.2–146.3 mm SL, 22 Jan 2007, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tags: 50 & 164. ZSM 38752, 159.9 mm SL, 17 Apr 2009, Fte Cruz de Mayo, 11°55′29.14″N, 86°03′22.04″W, DNA tag: 629. ZSM 38753, 3, 129.8–148.9 mm SL, 19 Jan 2007, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tags: 39, 42, & 59. ZSM 38754, 150.8 mm SL, same data as for holotype, DNA tag: 250. PSU 4768, 132.5–167.8 mm SL, same data as for holotype, DNA tags: 251 & 252.

*Non-type material.*—ZSM 38776, 2 (out of 3), 24 Jan 2007, NE shore, Spanish coast, 11°56′04.43″N, 86°00′46.88″W. ZSM 38777, 1, 5 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W. ZSM 38778, 1, 8 Jan 2007, W shore, along

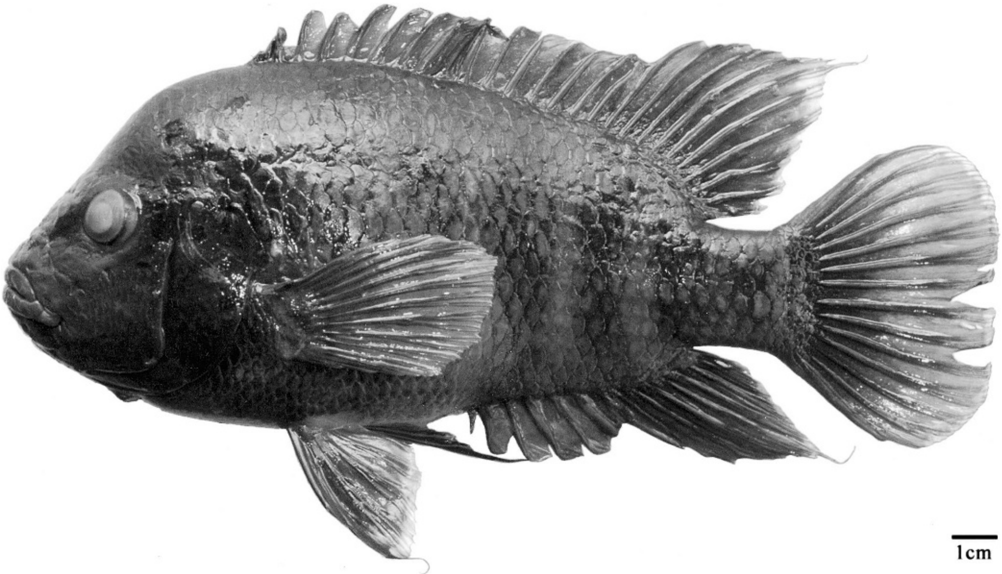

Fig. 1. *Amphilophus supercilius*, holotype, ZSM 38821, adult male, 166.6 mm SL, 27 Feb 2007, Spanish Coast, Lake Apoyo NE shore, Nicaragua 11°56′5.43″N, 86°00′46.88″W, DNA-tag: 247.

public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W. ZSM 38780, 1, 8 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W.

*Diagnosis*.—*Amphilophus supercilius* can be distinguished from all other described *Amphilophus* by its rounded oval-shaped caudal fin supported by strong and short fin rays; in other species of the complex, caudal fins are subtruncate and more triangular shaped. This is due to the fact that in *A. supercilius* the two outermost principal caudal-fin rays are shorter than in other species of *Amphilophus* from Lake Apoyo (see Figs. 1, 2) and do not reach to the posterior fourth of the caudal fin, thus giving the fin its oval shape.

Additionally, it differs from *A. zaliosus* by a greater body depth (44.4–49.4% SL vs. 34.9–41.1% SL), a greater PDAA (36.1–40.1% SL vs. 32.5–35.2% SL), and a greater ADPV (43.4–49.4% SL vs. 34.6–42.8% SL). The dark green/black head of *A. supercilius* and dark violet breast and throat distinguish it from *A. chancho* and *A. flaveolus*, which have a yellow/green head, and in *A. chancho* a bright lemon yellow throat. Pectoral-fin rays in *A. supercilius* are yellow and in *A. astorquii* they are usually gray.

*Description*.—Jaws isognathus (Fig. 1), scale rows on cheek 4 in holotype, 4–6 in paratypes; scales along lateral line ctenoid; holotype with 33 lateral-line scales, paratypes with 27–33; pored scales posterior to hypural plate 0–2. Head deep (91.1–117.2% HL); head length 33.8–36.7% SL. Eye small; horizontal eye diameter 22.6–25.5% HL; vertical eye diameter 22–26.5% HL. Mouth small (lower jaw length 37.1–43.1% HL) and not extending to anterior edge of orbit. Morphometric ratios and meristics in Table 1.

Live males and females with similar color. Breeding individuals with nearly black to dark green ground color dorsally, black to dark gray laterally with five strong, black, vertical bars under dorsal fin. One additional vertical bar extending pre-dorsal to pectoral-fin origin, another strong black bar on the caudal peduncle and one black blotch on caudal-fin base. Belly and breast dark with violet shine.

Table 1.—Morphometric and meristic values of *Amphilophus supercilius* collected from Lake Apoyo, Nicaragua. Means, standard deviation, and range include holotype ($n = 14$). See Materials and Methods for explanation of abbreviations. Holotype ZSM catalog number: 38821. Paratype catalog numbers: ZSM 37347, ZSM 37348, ZSM 37351–38754; PSU 4768.

| Variable | Holotype | $\bar{X}$ | *SD* | Range |
|---|---|---|---|---|
| Standard length | 166.6 | 150.1 | 12.5 | 129.8–167.8 |
| Head length | 58.5 | 53 | 5 | 46.2–60.9 |
| Percent standard length | | | | |
| Head length | 35.1 | 35.4 | 0.8 | 33.8–36.7 |
| Body depth | 47.5 | 46.9 | 1.3 | 44.4–49.4 |
| Snout to dorsal-fin origin | 41.2 | 42.2 | 1.3 | 40.2–44.4 |
| Snout to pelvic-fin origin | 41.6 | 42.6 | 1 | 40.7–44.5 |
| Caudal peduncal length | 12.5 | 12 | 0.7 | 11.1–13.1 |
| Least caudal peduncle depth | 15.7 | 15.4 | 0.5 | 14.3–16.2 |
| Dorsal-fin base length | 61.5 | 58.3 | 1.7 | 55.9–61.5 |
| ADAA | 52.6 | 53.2 | 1.2 | 51–55.2 |
| PDPA | 17.2 | 17.1 | 0.4 | 16.2–17.6 |
| ADPA | 67.4 | 64.8 | 1.4 | 62.9–67.4 |
| PDAA | 39.2 | 37.8 | 1.1 | 36.1–40.1 |
| PDVC | 20.2 | 19.5 | 0.7 | 18.2–20.6 |
| PADC | 19.7 | 20 | 0.8 | 18.6–21.6 |
| ADPV | 47.2 | 46.5 | 1.6 | 43.4–49.4 |
| PDPV | 59.6 | 59.1 | 1.1 | 57.2–61.1 |
| Percent head length | | | | |
| Horizontal eye diameter | 24.7 | 24.4 | 1 | 22.6–25.5 |
| Vertical eye diameter | 22.7 | 24 | 1.6 | 22–26.5 |
| Snout length | 36.5 | 38.8 | 1.9 | 36.3–42.5 |
| Postorbital head length | 40.5 | 38.8 | 1.4 | 36.4–40.5 |
| Preorbital depth | 28.6 | 25.5 | 1.9 | 22.5–28.6 |
| Lower-jaw length | 41.9 | 40.7 | 2 | 37.1–43.1 |
| Cheek depth | 34.1 | 29.6 | 2.2 | 26.7–34.1 |
| Head depth | 110.2 | 104.1 | 8.2 | 91.1–117.2 |
| Counts | | Mode | Frequency | Range |
| Dorsal-fin spines | 17 | 17 | 50 | 16–17 |
| Dorsal-fin rays | 10 | 11 | 57.1 | 10–11 |
| Anal-fin spines | 7 | 7 | 92.9 | 6–7 |
| Anal-fin rays | 8 | 8 | 50 | 7–8 |
| Pelvic-fin rays | 5 | 5 | | |
| Pectoral-fin rays | 14 | 15 | 50 | 14–15 |
| Lateral-line scales | 33 | 31 | 35.7 | 27–33 |
| Pored scales post. lat line | 2 | 1 | 57.1 | 0–2 |
| Scale rows cheek | 4 | 4 | 50 | 4–6 |

Pectoral fin with yellow/golden rays and transparent membrane. Dorsal, anal, and pelvic-fin membranes sooty transparent with greenish shine. Caudal-fin membrane with dark sooty anterior half fading into lighter reddish/violet transparent posterior half. Iris with golden rim. Preserved specimens almost uniformly black. Dorsal one-third of lateral side black or dark gray, fading to dark violet/gray ventrally; black lateral bars very faint or absent. Black caudal spot on post hypural onto caudal fin. Breast and belly black or dark brown.

*Etymology*.—Specific epithet is an adjective from the Latin word meaning eyebrow or "frowning being," referring to the strongly developed portion of the

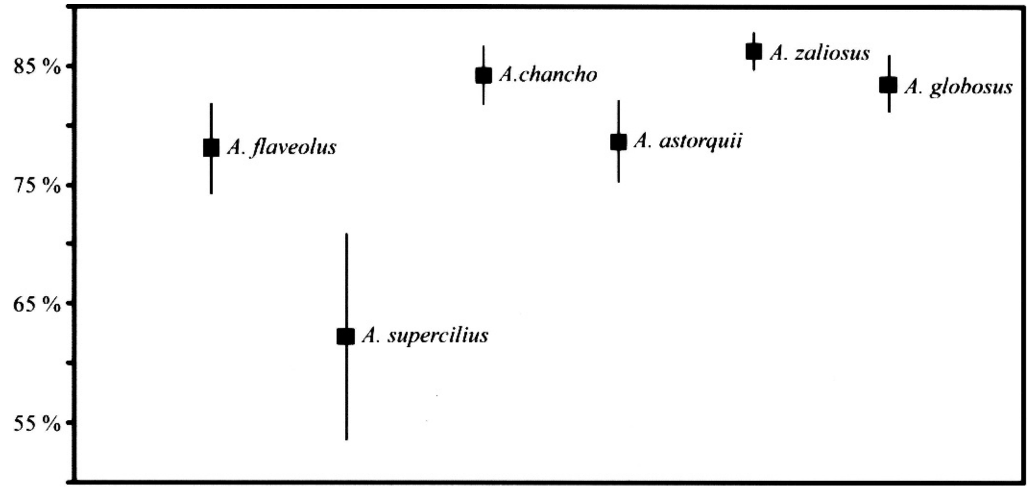

Fig. 2. Mean with 95% confidence interval of length of first principal ventral caudal-fin ray in caudal-fin length ratio of ten individuals of each of the six Lake Apoyo *Amphilophus* species.

neurocranium that borders the eye cavity and gives the species the appearance of possessing eyebrows and a frowning appearance.

*Notes on biology*.—*Amphilophus supercilius* was earlier reported as *A.* 'barlow' in a report on breeding ecology (McCrary & López 2008). This species breeds between September and March during the dry season with peaks in October and February. Breeding pairs can be encountered in depths ranging between 1.5–20 m where rocks form suitable burrows or crevices. It was often found to breed in direct vicinity to *A. zaliosus*, and it is noteworthy to mention that all observed pairs ($n > 30$, MFG pers. obs.) were between conspecifics. Of all the species of *Amphilophus* in Lake Apoyo, *A. supercilius* had the most generalist diet; stomachs contained fish-remains, fish-eggs, molluscs, and the macrophyte *Chara* (McCrary et al. pers. obs.).

### ***Amphilophus globosus***, new species
Fig. 3, Table 2

*Holotype*.—ZSM 38822, adult male, 158.8 mm SL, 17 Apr 2009, Fte Ranchos, Lake Apoyo NW shore, Nicaragua, 11°55′54.00″N, 86°03′10.80″W, DNA tag: 630.

*Paratypes*.—All paratypes were collected from Lake Apoyo, Nicaragua. ZSM 38755, 106.2 mm SL, 11 Apr 2009, Fte Lorenzo Guerrero, 11°55′13.07″N, 86°03′24.10″W, DNA tag: 618. ZSM 38756, 95.7 mm SL, 17 Apr 2009, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tag: 627. ZSM 38757, 3, 108.7–136 mm SL, 13 Apr 2009, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tags: 621, 622, & 625. ZSM 38758, 3, 110.2–111.2 mm SL, 17 Apr 2009, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tags: 631 & 636. ZSM 38759, 3, 130–135.8 mm SL, 17 Apr 2009, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tags: 640–642. PSU 4742, 6, 87.7–164.6 mm SL, 17 Apr 2009, Fte Cruz de mayo, 11°55′29.14″N, 86°03′22.04″W, DNA tags: 632–635, 638, 639. PSU 4743, 106 mm SL, 17 Apr 2009, Fte Lorenzo Guerrero, 11°55′13.07″N, 86°03′24.10″W, DNA tag: 617. PSU 4744, 2, 97.5–124.7 mm SL, 13 Apr 2009, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W, DNA tags: 623, 624. PSU 4745, 135.4 mm SL, 17 Apr 2009, Fte Escuela, 11°56′14.59″N, 86°03′10″W, DNA tag: 625.

*Diagnosis*.—*Amphilophus globosus* can be distinguished from all other Lake

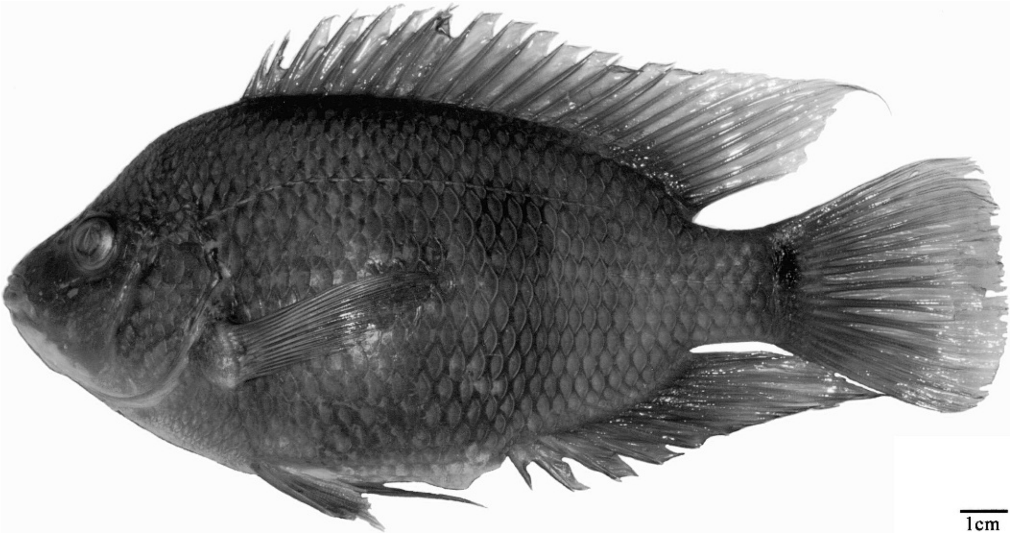

Fig. 3. *Amphilophus globosus*, holotype, ZSM 38822, adult male, 158.8 mm SL, 17 Apr 2009, Fte Ranchos, Lake Apoyo NW shore, Nicaragua, 11°55′54.00″N, 86°03′10.80″W, DNA tag: 630.

Apoyo species *Amphilophus* by its distinctive shape in lateral view, conspicuous bright yellow/greenish coloration, and the absence of clear and continuous dark or black lateral bars in non-breeding coloration.

*Amphilophus globosus* differs in a number of morphometric measurements from the remaining species of *Amphilophus* in Lake Apoyo: it differs from *A. zaliosus* by a smaller caudal-peduncle length (9.6–12.3% SL vs. 12.7–14.5% SL), a greater dorsal-fin base length (59–63.4% SL vs. 55.2–58.2% SL), greater PDAA (36.9–40.5% SL vs. 32.5–35.2% SL), greater ADPV (44–47.9% SL vs. 34.6–42.8% SL), greater PDPV (61–67.4% SL vs. 53.3–58.6% SL) and a smaller lower-jaw length (35.3–40.1% HL vs. 40.8–43.3% HL). *Amphilophus globosus* has a smaller least caudal-peduncle depth than *A. supercilius* (12.6–14.3% SL vs. 14.3–16.2% SL) and a greater PDPV (61–67.4% SL vs. 57.2–61.1% SL). *Amphilophus globosus* can be distinguished from *A. chancho* by a greater dorsal-fin base length (59–63.4% SL vs. 54.7–58.1% SL), and shorter head length (31.1–34.3% SL vs. 35.7–38.1% SL). It differs from *A. flaveolus* by a greater PDPV (61–67.4% SL vs. 56.7–59% SL. Compared to *A. astorquii* the new species has a shorter head length (31.1–34.3% SL vs. 34.5–38.9% SL).

*Description.*—Jaws isognathus (Fig. 3), scale rows on cheek 5 in holotype, 4–6 in paratypes; scales along lateral line ctenoid; holotype with 30 lateral-line scales, paratypes with 28–35; pored scales posterior to hypural plate 1–2. Head depth 75.1–98.8% HL; head length 31.1–34.3% SL. Eye small; horizontal eye diameter 22.1–28% HL; vertical eye diameter 22.5–28.5% HL. Mouth small (lower jaw length 35.3–40.1% HL) and not to anterior edge of orbit. Morphometric ratios and meristics in Table 2.

Live males and females with similar color. Breeding adults greenish yellow background color in the dorsal region and head, yellow in the abdomen and ventral region, broken by seven dark vertical bars plus dark caudal spot. Pectoral fin transparent and yellowish, other fins dark. Non-breeding adults with very faint vertical bars, usually imperceptible in the field, over yellow/greenish ground color dorsally, yellow laterally with one characteristic, strong, black

Table 2.—Morphometric and meristic values of *Amphilophus globosus* collected from Lake Apoyo, Nicaragua. Means, standard deviation, and range include holotype ($n = 22$). See Materials and Methods for explanation of abbreviations. Holotype ZSM catalog number: 38822. Paratype catalog numbers: ZSM 38755–38759; PSU 4742–4745.

| Variable | Holotype | $\bar{X}$ | *SD* | Range |
|---|---|---|---|---|
| Standard length | 158.8 | 120.6 | 19.8 | 88.5–166.7 |
| Head length | 50.9 | 39.3 | 5.8 | 30.2–51.9 |
| Percent standard length | | | | |
| Head length | 32.1 | 32.7 | 0.9 | 31.1–34.3 |
| Body depth | 49 | 48.5 | 1.4 | 45.4–51.6 |
| Snout to dorsal-fin origin | 38.5 | 38.6 | 0.9 | 37.1–40.4 |
| Snout to pelvic-fin origin | 41.9 | 41.5 | 1.1 | 39.6–43.6 |
| Caudal peduncal length | 10.5 | 10.7 | 0.7 | 9.5–12.3 |
| Least caudal peduncle depth | 14.3 | 13.5 | 0.5 | 12.6–14.3 |
| Dorsal-fin base length | 59.9 | 60.9 | 1.1 | 59–63.4 |
| ADAA | 56.2 | 55.2 | 1.3 | 52.8–57.2 |
| PDPA | 17.5 | 17 | 0.5 | 16.1–17.7 |
| ADPA | 66.3 | 66.4 | 1.1 | 64.7–69.2 |
| PDAA | 37.8 | 38.5 | 1 | 36.9–40.5 |
| PDVC | 19.1 | 18.4 | 0.7 | 16.9–19.7 |
| PADC | 19.2 | 18.3 | 0.8 | 16.7–19.4 |
| ADPV | 47.4 | 46.1 | 1.2 | 44–47.9 |
| PDPV | 64.7 | 63 | 1.5 | 61–67.4 |
| Percent head length | | | | |
| Horizontal eye diameter | 22.9 | 24.8 | 1.4 | 22.1–28 |
| Vertical eye diameter | 23.2 | 25.2 | 1.7 | 22.5–28.5 |
| Snout length | 38.9 | 36.2 | 1.6 | 33.4–38.9 |
| Postorbital head length | 42.2 | 40.1 | 1.5 | 36.5–42.2 |
| Preorbital depth | 25.1 | 22.7 | 1.7 | 20–26.7 |
| Lower-jaw length | 37.7 | 37.6 | 1.2 | 35.3–40.1 |
| Cheek depth | 29.8 | 26.7 | 2.6 | 22.1–32.6 |
| Head depth | 98.8 | 85 | 6.1 | 75.1–98.8 |
| Counts | | Mode | Frequency | Range |
| Dorsal-fin spines | 17 | 17 | 68.2 | 17–18 |
| Dorsal-fin rays | 10 | 10 | 81.8 | 10–11 |
| Anal-fin spines | 6 | 7 | 90.9 | 6–8 |
| Anal-fin rays | 7 | 8 | 54.5 | 7–8 |
| Pelvic-fin rays | 5 | 5 | | |
| Pectoral-fin rays | 14 | 14 | 68.2 | 13–15 |
| Lateral-line scales | 30 | 32 | 40.9 | 28–35 |
| Pored scales post. lat line | 2 | 2 | 63.6 | 1–2 |
| Scale rows cheek | 5 | 4 | 72.7 | 4–6 |

blotch usually on third bar under dorsal fin (“spotted,” sensu Barlow 1976). One black blotch on caudal-fin base. Head yellow/green. Belly and breast bright yellow/olive-green, gular cream white. Pectoral fin with transparent rays and membrane. Dorsal, anal, and pelvic-fin membranes transparent with yellow/orange shine. Caudal-fin membrane transparent or yellowish. Iris with golden/orange rim and dark vertical bar.

Preserved specimens dark brown dorsally, lighter ventrally. Gular white; belly light brown. Lateral bars very faint or absent. Black spot laterally instead of third bar, caudal spot on post-hypural onto caudal fin usually in area above lateral line.

*Etymology*.—Specific epithet is an adjective from the Latin word meaning round or globated, referred to the general appearance of this high-bodied species.

*Notes on biology*.—*Amphilophus globosus* was previously not identified as a distinct species and thus its breeding ecology was not catalogued separately. It has been observed breeding with conspecifics over *Chara* vegetation at 4–8 m depth, in March 2009 and in January 2010 ($n$ = 5; JKM pers. obs.). Interestingly, while at least some individuals of the other Lake Apoyo species of *Amphilophus* had molluscs in their stomachs, in the ten radio-graphed *A. globosus* individuals, we did not observe any molluscs or shell remains.

## Discussion

George Barlow († 14 Jul 2007) first noted the higher variability in certain characters in *Amphilophus* from Lake Apoyo compared to those from other lakes in Nicaragua (Barlow 1976). We agree with him that Lake Apoyo's depauperate fish fauna and the lack of potential competitors probably have lead to character release which might have favored the observed diversification. Nicaragua's crater lakes offer a unique possibility to study such processes. Several isolated crater lakes with differing species assemblages have most probably been seeded by independent source populations of *Amphilophus* given the geographic conditions (Waid et al. 1999). All morphometric, ecological, and genetic results to date in Lake Apoyo and in Lake Xiloá strongly suggest that the original founder stocks have evolved into multi-species lineages under sympatric conditions. In our view, it is more parsimonious to assume independent parallel sympatric speciation in those isolated crater lakes than to accept the idea that the now endemic species of *Amphilophus* in each respective water body would have had wider distributions and undergone subsequent local extinctions.

*Amphilophus supercilius* and *A. globosus* are morphologically distinct from each other and from the four other species of *Amphilophus* inhabiting Lake Apoyo (Figs. 1, 3, 7). We conducted multivariate analysis of the morphometric and meristic data to support the heterospecificity of all six described species.

The plot of the sheared second principal component of the morphometric data (SPC2) versus the sheared third principal component of the morphometric data (SPC3, Fig. 4) clearly demonstrates that the minimum polygon cluster of *A. globosus* is distinct from those of the remaining species of *Amphilophus* from Lake Apoyo and that the minimum polygon cluster of *A. supercilius* is distinct from that of *A. zaliosus* and *A. chancho*. Size accounts for 93.3%, the sheared second principal component for 2.7%, and the sheared third principal component for 1.4% of variation. Variables with the highest loadings on the sheared second principal component in decreasing order of importance are head depth (−0.37), body depth (0.33), PDPV (0.32) and lower-jaw length (−0.28). Variables with the highest standardized scoring coefficients on the sheared third principal component are caudal-peduncle length (0.64), head depth (−0.38) and cheek depth (−0.28).

The plot of the first principal component of the meristic data (PC1) versus the SPC2 (Fig. 5) shows that the minimum polygon clusters of *A. astorquii* and *A. supercilius* do not overlap. Size accounts for 93.1% and the second principal component for 1.9% of total variation. Variables with the highest loadings on the sheared second principal component in decreasing order of importance are cheek depth (−0.52), preorbital depth (−0.42) and head depth (−0.35). The first principal component of the meristic data accounts for 21.6% of the total variation.

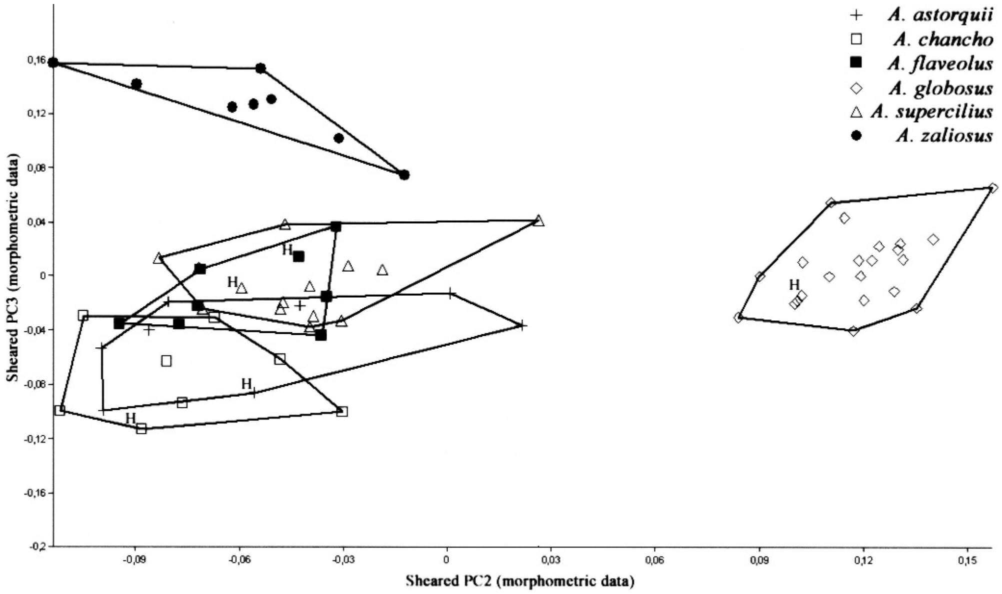

Fig. 4. Plot of the sheared second and third principal component of the morphometric data of *Amphilophus astorquii*, *A. chancho*, *A. flaveolus*, *A. globosus*, *A. supercilius*, and *A. zaliosus*. H demarks the position of the holotype of each species, except for *A. zaliosus*.

Variables with the highest standardized scoring coefficients on the first principal component of the meristic data in decreasing order are dorsal-fin rays (0.61), pored scales posterior to lateral line (−0.50) and anal-fin rays (0.44).

When the data for *A. flaveolus* and *A. supercilius* are analyzed separately, the

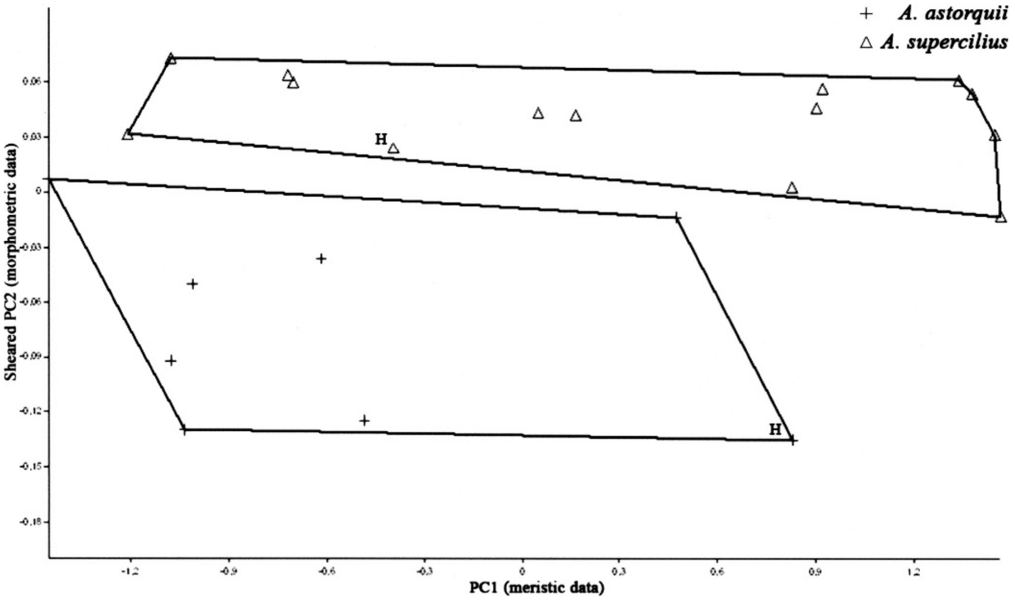

Fig. 5. Plot of the first principal component of the meristic data against the sheared second principal component of the morphometric data of *Amphilophus astorquii* and *A. supercilius*. H demarks the position of the holotype of each species.

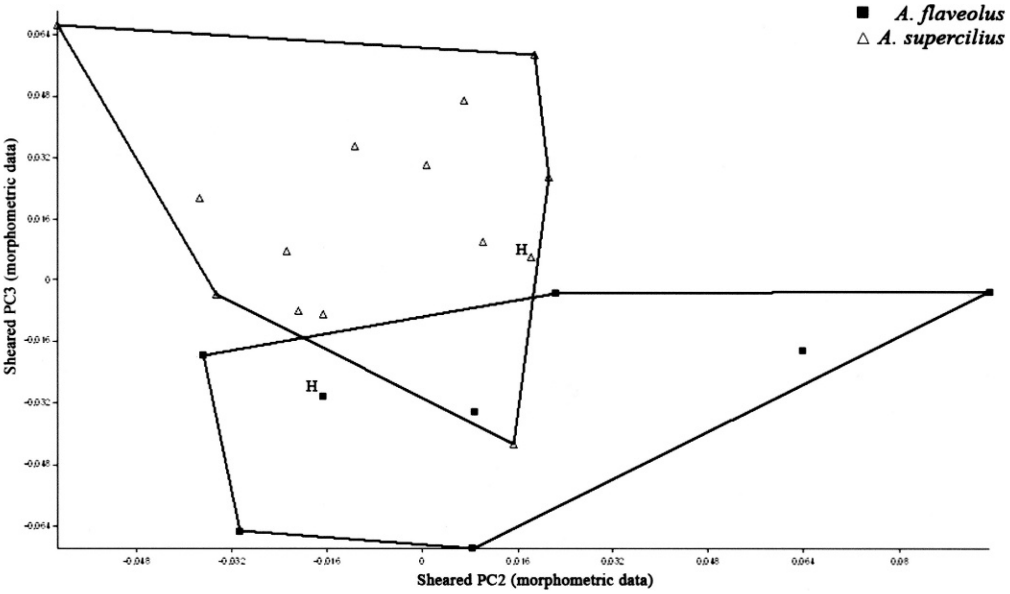

Fig. 6. Plot of the sheared second and third principal component of the morphometric data of *Amphilophus flaveolus* and *A. supercilius*. H demarks the position of the holotype of each species.

minimum polygon clusters between the species show almost no overlap (Fig. 6), only one individual of *A. supercilius* clusters within the polygon of *A. flaveolus*. Size accounts for 94.6%, the sheared second principal component for 1.1% and the sheared third principal component for 1.0% of total variation. Parameters with the highest loadings on the second principal component are cheek depth (0.56), horizontal eye diameter (0.39) and vertical eye diameter (0.31). Those variables that loaded highest on the third principal component are head depth (−0.61) and vertical eye diameter (0.40).

Comparison of the caudal-fin skeleton between the six species endemic to Lake Apoyo revealed only very little structural variation (Table 3) most likely reflecting the recent (<20,000 yr) origin of this species assemblage. In all examined individuals, the caudal-fin base consists of

Table 3.—Meristic values of the caudal-fin skeleton for ten radio-graphed individuals of each of the six Lake Apoyo species of *Amphilophus*. Princ.: principal (segmented); proc.: procurrent (non-segmented); HSP: haemal spine of the preural centrum.

| | *A. astorquii* | | *A. chancho* | | *A. flaveolus* | | *A. globosus* | | *A. supercilius* | | *A. zaliosus* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Character | Min. | Max. | Min. | Max. | Min. | Max. | Min. | Max. | Min. | Max. | Min. | Max. |
| Princ. caudal-fin rays | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| Ventral proc. rays | 3 | 3 | 3 | 3 | 3 | 4 | 3 | 4 | 3 | 4 | 2 | 3 |
| Dorsal proc. rays | 3 | 4 | 3 | 4 | 3 | 4 | 4 | 4 | 3 | 5 | 3 | 4 |
| Proc. rays HSP | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Princ. rays HSP | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Parhypural princ. rays | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 2 |
| Hypural 1, princ. rays | 3 | 3 | 3 | 4 | 3 | 4 | 3 | 3 | 3 | 4 | 3 | 4 |
| Hypural 2, princ. rays | 2 | 2 | 1 | 2 | 1 | 2 | 2 | 2 | 1 | 2 | 1 | 2 |
| Hypural 3, princ. rays | 2 | 3 | 2 | 2 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 |
| Hypural 4, princ. rays | 3 | 4 | 4 | 4 | 3 | 5 | 3 | 4 | 3 | 4 | 3 | 4 |
| Hypural 5, princ. rays | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 3 | 2 | 3 |

Fig. 7. Upper left: *Amphilophus astorquii*; Upper right: *A. chancho*; Middle left: *A. flaveolus*; Middle right: *A. globosus*; Lower left: *A. supercilius*; Lower right: *A. zaliosus*.

five hypuralia, only one individual of *A. supercilius* had the two dorsal hypuralia fused into one plate. It is thus remarkable that in the newly described *A. supercilius* such an 'aberrant' caudal fin with significantly (two-tailed *t*-test, $P < 0.05$) shorter outer principal caudal-fin rays has evolved, despite the observed conservative nature of the caudal skeleton.

Combining the findings from our morphometric analysis with the observed differences in coloration among taxa (Fig. 7), together with different preferences for time and site of breeding of *A. supercilius* (McCrary & López 2008) strongly support the validity of the newly described species. More basic taxonomic research in Nicaragua is necessary to build the basis for a comprehensive phylogenetic hypothesis for the Midas cichlid complex. To render this species complex a sustainable model system for evolutionary biology and biodiversity, a sound systematic analysis of the whole species assemblage is a necessary prerequisite.

Unfortunately, released exotic tilapias, e.g., *Oreochromis aureus* and *O. niloticus*, in Lake Apoyo (McCrary et al. 2007) threaten the endemic fish fauna. The range of negative consequences is manifold, and it was even suggested that introduced tilapias in Lake Apoyo may have been responsible for the outbreak of blindness in native cichlids (McCrary et al. 2007). The disappearance of submerged vegetation *Chara* sp. (Characeae) for more than five years was clearly correlated with the introduction of tilapias in Lake Apoyo (McCrary et al. 2007). This is especially dramatic since *Chara* beds serve as important nesting sites and refugia for juveniles of certain cichlid species, as well as the preferred habitat for the snail *Pyrgophorus coronatus* which is an essential dietary component of certain species of *Amphilophus* (McCrary et al. 2008). The addition of two new members of the endemic fish fauna of Lake Apoyo underscores the importance of efforts that have been undertaken to protect Laguna de Apoyo Nature Reserve, as a reservoir of endemic species and a study site for sympatric speciation processes.

## Comparison Material

*Amphilophus astorquii.*—Holotype: PSU 4518, 107.7 mm SL, 10 Nov 2002, Casa Rosal, Lake Apoyo, Nicaragua 11°55.74′N, 86°03′10.80″W. Paratypes: PSU 4519, 3 (out of 4), 107.1–114.8 mm SL, 10 Nov 2002, Casa Rosal 11°55.74′N, 86°03′10.80″W; PSU 4520, 1, 108.4 mm SL, 20 Dec 2002, Bajadero Granada, 11°55.41′N, 86°00.72′W; PSU 4523, 1, 118.6 mm SL, 21 Dec 2002, Bajadero Granada, 11°55.41′N, 86°00.72′W; PSU 4525, 1, 109.7 mm SL; 22 Dec 2002, Casa Rosal, 11°55.74′N, 86°03′10.80″W; PSU 4528, 1, 129.7 mm SL, 24 Dec 2002, Lado Este OL, 11°54.54′N, 86°00.50′W. Non-types (x-rays): ZSM 39110, 1 (out of 2), 18 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39111, 2, 19 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39112, 1 (out of 3), 16 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39114, 1 (out of 5), 23 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39153, 2 (out of 3), 23 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39159, 3, 16 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39166, 2, 24 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39167, 1 (out of 4), 16 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W.

*Amphilophus chancho.*—Holotype: PSU 4500, 1, 207 mm SL, 10 Nov 2002, Granada Bajadero, Lake Apoyo, Nicaragua, 11°55.41′N, 86°00.72′W. Paratypes: PSU 4508, 1, 175.2 mm SL, 11 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W; PSU 4509, 1, 165.4 mm SL, 12 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W; PSU 4511, 1, 147.7 mm SL, 14 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W; PSU 4512, 1, 107.6 mm SL, 15 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W; PSU 4513, 1, 107.6 mm SL, 16 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W; PSU 4423, 1, 228.8 mm SL, 9 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W; PSU 4419, 1, 242 mm SL, 9 Dec 2003, Fte Ranchos, 11°55′54.00″N, 86°03′10.80″W.

Non-types: ZSM 39109, 1 (out of 2), 17 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39128, 1 (out of 2), 17 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39130, 2, 23 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39132, 1, 2 Feb 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39168, 1 (out of 2), 16 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39169, 2, 17 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39175, 1, 17 Feb 2007, S shore, "Diria Bajadero," 11°54′08″N, 86°02′38″W; ZSM 39176, 1, 16 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39184, 1, 15 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39186, 1, 23 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39187, 1, 12 Feb 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W.

*Amphilophus flaveolus*.—Holotype: PSU 4515, 1, 126.9 mm SL, 10 Dec 2003, Otro Lado, Lake Apoyo, Nicaragua 11°54.22′N, 86°01.72′W. Paratypes: PSU 4515, 2, 92.2–130.3 mm SL, 10 Dec 2003, Otro Lado, 11°54.22′N, 86°01.72′W; PSU 4517, 5 (out of 8), 112.7–136.4 mm SL, 28 Nov 2003, Otro Lado, 11°54.22′N, 86°01.72′W. Non-types: ZSM 39116, 1, 24 Jan 2007, NE shore, "Granada Bajadero," 11°55.41′N, 86°00.72′W; ZSM 39117, 4, 26 Jan 2007, S shore, "Diria Bajadero," 11°54′08″N, 86°02′38″W; ZSM 39133, 1 (out of 2), 24 Jan 2007, NE shore, "Granada Bajadero," 11°55.41′N, 86°00.72′W; ZSM 39134, 2, 27 Feb 2007, NE shore, "Granada Bajadero," 11°55.41′N, 86°00.72′W; ZSM 39135, 1, 14 Feb 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39136, 2, 1 Feb 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39171, 1, 24 Feb 2007, S shore, "Diria Bajadero," 11°54′08″N, 86°02′38″W.

*Amphilophus zaliosus*.—Paratypes: CAS 29105, 5 (out of 10), 112.4–126.2 mm SL, 2 Aug 1969, E shore of Lake Apoyo, Nicaragua; USNM 212181, 3 (out of 10), 125.4–141mm SL, 2 Aug 1969, E shore of Lake Apoyo, Nicaragua. Non-types: ZSM 39107, 1, 15 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39108, 1, 6 Feb 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39119, 2, 17 Feb 2007, S shore, "Diria Bajadero," 11°54′08″N, 86°02′38″W; ZSM 39125, 1, 16 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39140, 1 (out of 3), 22 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39141, 2, 19 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39142, 1, 24 Jan 2007, NE shore, "Granada Bajadero," 11°55.41′N, 86°00.72′W; ZSM 39172, 1, 1 Feb 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W; ZSM 39173, 1, 23 Jan 2007, W shore, along public beach between Spanish school and bars, 11°56′14.59″N, 86°03′10.00″W.

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## *Manuscript III*

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# Evolutionary ecology of the Midas cichlid flock endemic to Crater Lake Apoyo, Nicaragua

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# Evolutionary ecology of the Midas cichlid flock endemic to Crater Lake Apoyo, Nicaragua

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## Abstract

A comprehensive data set of population genetic (mtDNA, AFLPs), morphological (body shape) and ecological (isotopic signatures) proxies of species level differentiation for a representative set of individuals of all six endemic *Amphilophus* species occurring in Crater Lake Apoyo (Nicaragua) was established. Signatures of divergence were extracted from each data set to identify system specific factors potentially contributing to the sympatric speciation process. AFLP genetic differentiation was partitioned into a neutral and non-neutral component applying outlier-loci detection approaches and patterns of correlation between divergence proxies tested using Mantel tests and Canonical Correspondence Analysis. Significant eco-phenotypic covariance with AFLP data suggests a role of both, divergent natural, and sexual selection for shaping species divergence in L. Apoyo. Breeding coloration was identified as potential key character for maintaining species cohesion, while ecological selection deduced from isotopic niche segregation displayed a much weaker signature. Bayesian clustering methods revealed substantial levels of admixture between species, but despite incomplete reproductive isolation, all six species apparently evolve as morphologically and genetically coherent units. Analysis of neutral genetic variation revealed several *A. zaliosus* as being introgressed by an unknown allochthonous contributor, hereby rendering the sympatrically evolving L. Apoyo flock polyphyletic.

## Introduction

Speciation as the cessation of gene flow between closely related populations can be classified into three types: allopatric, parapatric, sympatric. While allopatric speciation occurs by complete geographic isolation and its reality is undisputed (e.g. Kawata & Yoshimura 2000; Mallet et al. 2009), parapatric speciation occurs under a limited extent of gene flow (e.g. Gavrilets et al. 2000), as can be the case of species distributed as populations along an environmental gradient (cline) with varying local adaptations. Most species with limited gene flow between adjacent populations thus have the potential for parapatric speciation. We refer to sympatric speciation as a scenario where a new species emerges within a freely breeding population without geographic isolation, which is however only one of about a dozen published definitions (Fitzpatrick et al. 2008). Although it is theoretically possible under a variety of more or less stringent conditions (e.g. Maynard Smith 1966; Turner & Burrows 1995; Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Kawata & Yoshimura 2000; Almeida & de Abreu 2003; Ripa 2009; van Doorn et al. 2009), it is considered uncommon in nature and only few unequivocal examples are generally accepted (Bolnick & Fitzpatrick 2007).

Among the most promising candidates for sympatric speciation are cichlid fish species flocks endemic to small crater lakes. They usually offer the possibility to examine speciation processes without confounding effects of allopatric population differentiation if a single colonization event as inferred from molecular genetics implies their monophyly (e.g. Schliewen et al. 2006; Mallet et al. 2009; Losos & Mahler 2010). But even without full monophyly, sympatric speciation is considered possible, if amalgamation of different lineages (hybridization, introgression) renders a member of - or the flock itself - polyphyletic (Schliewen & Klee 2004). Under the assumption that a species flock is evolving as a genetically interconnected entity, a polyphyletic flock may nevertheless generate species diversity by sympatric speciation. With or without monophyly, a sympatric speciation scenario is then possible, albeit with different onset conditions with regard to standing genetic variation introduced by multiple versus a single source of genetic variation. What has remained comparatively unstudied in species flocks are patterns of species cohesion and phenotypic divergence in relation to their ecology and patterns of gene flow among evolving species (but see e.g. Herder et al. 2008, Tobler & Carson 2010).

Among Crater Lake cichlid species flocks, Midas cichlids endemic to L. Apoyo (Nicaragua) constitute a promising model to study patterns and processes of speciation due to its accessibility and feasibility to sample all species, i. e. *Amphilophus astorquii*, *A. chancho* and *A. flaveolus*, *A. globosus*, *A. supercilius* and *A. zaliosus* (Barlow & Munsey

1976, Stauffer et al. 2008, Geiger et al. 2010a). While most recent studies dealt either with taxonomic questions or tried to shed light on issues addressing the mode of speciation in L. Apoyo's *Amphilophus*, a comprehensive picture completely representing the genomic diversity of all six species and divergence between them has not been presented to date.

The putative monophyly of the flock has been documented based on mitochondrial DNA and nuclear markers (Barluenga & Meyer 2004; Barluenga et al. 2006; Geiger et al. 2010b). However, data left room for a secondary introgression scenario from outside L. Apoyo (Schliewen et al. 2006; Geiger et al. 2010b). Undoubtedly, rapid speciation has occurred - most probably in sympatry (Wilson et al. 2000; Barluenga et al. 2006), but the relative importance of sexual and natural selection as well as the role of hybridization and introgression had not been investigated. Ecological disruptive selection has been proposed as the main factor leading to speciation of *A. zaliosus* out of the original L. Apoyo *Amphilophus* stock (Barluenga et al. 2006). A comparative transcriptome study between *A. astorquii* and *A. zaliosus* identified six ESTs with signals of strong diversifying selection with BLAST assigned functions in biosynthetic - metabolic processes, brain development and cognition, response to hormone stimuli and in the nervous system (Elmer et al. 2010a). Whether the discovered sequence differences are a product of adaptive molecular evolution and/or selection or directly play a role in speciation remains to be tested.

Studies on another Midas cichlid species flock from Lake Xiloá (Nicaragua) indicate that colour assortative mating contributes to sympatric divergence between 'gold' (brightly colored) and 'normal' (black striped) morphs of Midas cichlids (Elmer et al. 2009) while no support for reproductive isolation based on trophic polymorphisms was found (Wilson et al. 2000). Both studies revealed significant intralake population genetic structure supporting a scenario of divergence in sympatry. Yet another recent study on Midas cichlids examined the genetic and eco - morphologic differentiation between two probably very young (< 100 years old) forms in Lake Apoyeque (Nicaragua) that differ in lip morphology, one with normal (thin) and the other with thick lips (Elmer et al. 2010b). Interestingly, bimodal distributions of eco-morphological features were documented - compatible with expectations of an initial stage of ecological speciation under disruptive selection - but no (significant) genetic differentiation was detected based on mitochondrial DNA and 11 microsatellites (Elmer et al. 2010b).

Divergent sexual selection may ultimately lead to assortative mating with various levels of reproductive isolation among incipient species pairs, largely dependent on the strength of disruptive selection. Deduced from a number of behavioural studies (mostly focused on ‘gold’ vs. ‘normal’ colored *Amphilophus,* i.e. not yet applying recent taxonomy, sexual selection in Midas cichlids is working on a basis of female choosiness and male-male competition for breeding sites with a role of body size and coloration (e.g. Barlow 1973 & 1976; Barlow & Rogers 1978; Barlow et al. 1977; McKaye 1986).

Divergent selection acts both on standing genetic variation and mutation as source for increased differentiation during the speciation process. Seehausen (2004) developed two related hypotheses for the role of increased levels of standing genetic variation based on hybridization in adaptive radiations: the ‘hybrid swarm origin’ hypothesis posits that out of several unrelated lineages hybrid swarms form after the invasion of a new habitat. Hereby increased levels of heritable phenotypic variation evolve quickly based on reshuffled genetic variation. The ‘syngameon’ hypothesis suggests a prominent role for evolution of increased species assemblage complexity, if a group of genetically weakly but ecologically distinctive species exchange genetic material after they had speciated. In both cases, adaptive divergence is facilitated by initial hybridization generating new adaptive trait combinations whereas ongoing hybridization helps maintaining high levels of genetic variation that would otherwise be reduced by directional selection. Speciation may be affected in different ways, either by being provided with genetic variation for the evolution of novel adaptive traits, or directly through hybrid speciation. Hybrid swarm theories of evolutionary radiation combine these two ideas (Seehausen 2004; Mallet 2007).

A common generalization from various studies and simulations is that divergence with gene flow in sympatry is easy and rapid if the naturally selected ‘magic’ traits diverge and pleiotropically lead to reproductive isolation (e.g. Gavrilets 2004; Bolnick & Fitzpatrick 2007; Feulner et al. 2009). Given the very recent origin of the L. Apoyo radiation (< 20.000 B.P.) it is not unlikely that divergently selected ‘magic’ traits integrating ecological performance and mate choice, i.e. body shape and coloration, lead to reproductive isolation.

In a multidisciplinary and comprehensive approach we present results from a study of L. Apoyo’s Midas cichlids including all six described species and several potential hybrid individuals. Combined findings from molecular population genetics, phylogenetics, geometric morphometrics and stable isotope analysis (SIA) are used to infer the significance of hybridization, sexual selection and natural selection for the formation of a complete and complex species flock *in statu nascendi*. We (1) use genetic differentiation as measured with F-statistics, hierarchical AMOVAs and Bayescan clustering as a proxy for the strength of divergent selection and reduced gene flow. If sexual selection dominates divergent selection in L. Apoyo Midas cichlids we expect to detect increased levels of differentiation between species that are not strongly ecologically divergent, but which differ in breeding coloration. In contrast, we expect higher levels of genetic differentiation of ecologically divergent rather than ecologically similar pairs if divergent natural selection was the dominant force, hereby assuming that ecological divergence is a proxy for divergent natural selection (Nosil 2009a). We use SIA to quantify niche segregation, and hence ecological differentiation between species pairs, and mine for ecological correlations with genetic and morphologic differentiation. Both, natural and sexual selection potentially contribute to overall morphology in cichlids (Salzburger 2009), but genetic drift in localized populations remains a valid alternative for morphological divergence to occur (e.g. for sticklebacks: Leinonen et al. 2006). Therefore, we (2) compare body shape variation among all species and individuals and correlate it to neutral and presumably non-neutral genetic and ecological differentiation to estimate the relative importance of the two factors. Finally (3) we assess the role of introgression and hybrid speciation among sympatric L. Apoyo *Amphilophus* in the face of apparent species cohesiveness by evaluating signatures of introgression. Together with the comprehensive framework of the *Amphilophus* system of the Nicaraguan lakes (Geiger et al. 2010b) and the clarification of the taxonomic background (Geiger et al. 2010a), this study is designed to finalize the provision of baseline analyses necessary for more detailed and focussed studies of sympatric speciation processes in L. Apoyo.

## Materials and Methods

All included individuals were caught during three field seasons from January to April in 2007, December to March in 2007/2008 and April 2009 in L. Apoyo. With an estimated age of less than 23,000 years (BANIC 1977; Sussman 1982) L. Apoyo is one of the younger volcanic Crater lakes in Nicaragua, situated within an almost circular caldera about 4 km west of L. Nicaragua. Its surface occupies 20.92 km$^2$, its diameter measuring more than 4 km and its maximum depth 178 m (CIRA 2008). Fishes were caught SCUBA diving with harpoon following field identification and anesthetized and killed using an overdose of clove oil. Each specimen was photographed to document live-coloration and preserved with pinned fins in 4-10% formalin. Individual whole body and tissue vouchers are stored permanently at Bavarian State Collection Munich (ZSM, Table S1, Supporting Information). Species were identified according to the most recent species descriptions (Stauffer et al. 2008; Geiger et al. 2010a), and a key is available from Fig. S1 (Supporting Information).

### Phenotypic differentiation and morphometric analysis of body shape

Landmark-based geometric morphometrics were used to capture information on body shape variation and to compare the assignment to morphological similar groups with the assignment to genetic entities. A modified protocol from two other studies applying the method to Midas cichlids (Klingenberg et al. 2003; Parsons et al. 2003) was applied. For data acquisition the TPS software package (Rohlf & Marcus 1993; Rohlf 2006a&b) was used. Principal components (PCA) and canonical variates analysis (CVA) on the partial warp scores were applied to examine variation in body shape among individuals and species using

PCAGen6p and CVAGen6o (Sheets 2003) after removing non-shape variation via ‘Generalised least squares Procrustes superimposition’.

Since seven individuals could not be assigned to any of the six species *a priori* - possibly due to hybrid origin - a CVA with assignment test (Nolte & Sheets 2005) was applied to explore those individuals’ phenotypic affinities. Robustness of assignment and morphological similarity or cohesiveness of species was evaluated with a Jackknife procedure with 10% and 20% of the individuals treated as ‘unknowns’ in 1000 resampling trials. A multivariate analysis of variance (MANOVA, with Hotelling’s pairwise test) on the PCs bearing more than 5% of variance was used to examine among-species differentiation in PAST (Hammer et al. 2001). All individuals used in the molecular genetic analysis were included in the morphometric analysis, except for four *A. flaveolus* that were dissected for other reasons.

**Phylogeny reconstruction**

Extraction of genomic DNA, amplification and sequencing of the mitochondrial control region as well as alignment of mtDNA sequence data, haplotype-frequency calculation and network construction and AFLP genotyping follow the methods outlined in Geiger et al. (2010b). For the tree-reconstruction based on AFLP data the software package TREECON 1.3b was used (Van de Peer 1994) and the Link et al. (1995) distance-measure chosen to compute a matrix based on the binary AFLP matrix. Link’s formula is based on Jaccard’s similarity measure and takes into account only shared and unique bands while absent bands are ignored and remains thus unaffected by homoplasic absent bands (e.g. Bonin 2007).

To assess robustness of the AFLP based phylogenetic hypothesis, and to explore alternative branching-patterns leaf-stability (LS) and lineage-movement (LM) indices for each single taxon and selected clades were calculated in Phyutility v.2.2 (Smith & Dunn 2008). The LS index measures the consistency of each taxon´s position across a chosen number of bootstrap replicates. A value of 1 indicates that the individual’s position in the topology is stable and equal in all examined trees. The LM index calculates attachment frequencies of selected branches 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).

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 containing 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 descendants. Conversely, removal of non-hybrid taxa should not affect bootstrap support of other nodes.

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 & Moulton 2004) as implemented in SplitsTree (Huson 1998).

### Outlier locus detection

In order to discern between effects from neutral processes and selection, a Bayesian method was applied to identify potential candidate loci under selection, implemented in Bayescan 1.0 using default settings (Foll & Gaggiotto 2008). Jeffreys' scale of evidence for Bayes factors (BFs) was applied to identify candidate loci using the strictest criteria ‘decisive’, corresponding to BFs greater 100 and selection posterior probabilities above 99 %. Performance of Bayescan was evaluated with two alternative software programs to identify loci under directional selection: Dfdist (www.rubic.rdg.ac.uk/~mab/stuff/), most commonly used for AFLP markers (Pérez-Figueroa et al. 2010), and SAM (Joost et al. 2007), which performs multiple univariate logistic regressions to test for associations between allelic frequencies and environmental variables (see CCA details below for coding of environmental variables).

## Inference of genetic structure

First, the Bayesian algorithm of Structure 2.2 (Pritchard et al. 2000; Falush et al. 2007) was used to identify the number of differentiated clusters without *a priori* group designation based on the complete AFLP matrix. The admixture-model with correlated allele-frequencies (Falush et al. 2003) was chosen, and $\alpha$ (admixture parameter) and $\lambda$ (allelic frequencies parameter) were set to be inferred from the data. Each run consisted of a burn-in period of 50k followed by 250k iterations 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 11. Runs that did not converge during the burn-in phase were identified by 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 $\Delta K$ from the Structure output 'LnP(D)'.

After inferring the most likely number of genetic clusters the GENSBACK (gb) option in Structure (Pritchard et al. 2000) was used to test for immigrant ancestors. This model makes use of prior population information for each individual assisting the clustering process and calculates the probabilities that each individual was either from its predefined population (here species) or had a parent or grandparent from any other species (i.e. gb=2). Sensitivity of the data to *v* (probability of individual misclassification or mixed ancestry), was evaluated by varying the parameter MIGRPRIOR (0.1, 0.05, and 0.01), as suggested by Pritchard et al. (2000). We thus tested for immigrant ancestors from the last two generations and used for the seven potential hybrids as predefined species those with which they clustered in the NJ tree.

Additionally, we performed Structure runs and PCA as graphical representation of population differentiation on the AFLP matrix containing outlier loci only, and on the presumably neutral AFLP matrix without outlier loci.

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) the six species, (2) the three sampling locations, and (3) three coloration groups. According to their prevalent ground coloration when breeding, individuals were pooled into three groups: 1) *A. astorquii*, *A. zaliosus*, and *A. supercilius* (black), 2) *A. chancho* and *A. globosus* (yellow) and 3) *A. flaveolus* (green).

Loci identified to be under directional selection were removed from the AFLP matrix and AMOVAs as well as F-statistics recalculated as a measure of presumably neutral differentiation. Differentiation between species was estimated using F-statistics on uncorrected p-distances (Weir & Cockerham 1984) as implemented in Arlequin 3.11, and their significance tested by permutating haplotypes among populations as well as generating bootstrap confidence intervals. For the AFLP data set, additionally pairwise $\Phi_{ST}$ based on standard Jaccard coefficient were generated after distance transformation (d=1-s) in FAMD (Schlüter & Harris 2006).

## Stable isotope analysis (SIA)

For an estimation of ecological differentiation with the null hypothesis of no dietary differentiation between the six sympatric L. Apoyo *Amphilophus* species, we analyzed a total of 132 samples presumably covering all major components of the lacustrine food-web.

Muscle samples for stable isotope analysis were taken from a total of 84 *Amphilophus* individuals from all six species and from 16 individuals belonging to all other known fish species from L. Apoyo, except introduced *Oreochromis* spp. (McCrary et al. 2007). Various fish species with different ecological characteristics such as the poeciliid *Poecilia* sp., the atherinid silverside *Atherinella* cf. *sardina*, the predatorious bigmouth sleeper goby *Gobiomorus dormitor,* and the largest cichlid in L. Apoyo, *Parachromis managuensis* were included to estimate the relative position of the *Amphilophus* species in L. Apoyo's food-web. Additionally, we drew plankton samples at two different depths (5-7m and15m) and collected aufwuchs from rocks close to hydrothermal springs (mainly consisting of filamentous green algae, cyanobacteria and attached diatoms) and benthic flocculent material from littoral zones (mainly *Terpsinoë americana*, *Campylodiscus clypeus* and other diatoms). Finally, *Pyrgophorus coronatus*, an abundant snail and important dietary component of certain *Amphilophus* species (McCrary et al. 2008) was included (without shell) as well as *Chara* sp., the only macrophyte and preferred habitat for *P. coronatus* in that lake (Table S2, Supporting Information).

Samples were oven dried at 40℃ for at least 24 h, homogenized with a mortar and pestle, weighed (ca. 0.55 mg for fish muscle tissue and up to 2.4 mg for environmental samples) and loaded into tin cups prior to analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope ratios. All plankton, snail, *Chara*, benthos and detritus samples were decalcified with hydrochloric acid (5 % by volume) for several hours to ensure complete removal of carbonates for $\delta^{13}C$ analyses of organic matter. All $\delta^{15}N$ values are from non-decalcified material. Isotope analyses were performed with an elemental analyzer (NC 2500, Carlo Erba) coupled to an isotope ratio mass spectrometer (Delta Plus, Thermo-Finnigan). Ratios (R) of the heavy ($^{15}N$, $^{13}C$) to the light ($^{14}N$, $^{12}C$) isotope are given as δ values (in ‰) relative to a standard (AIR and VPDB for N and C, respectively) according to the formula $\delta = (R_{sample}/R_{standard} - 1)x1000$. Analytical precision (1 SD of repeatedly measured lab standard peptone) typically was 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$.

$\delta^{13}C$ values of all fish samples were lipid-normalized following the suggestions of Kiljunen et al. (2006) and applying the formula presented by Post et al. (2007) to take into account the varying content of $\delta^{13}C$-depleted lipids. For all *Amphilophus* species-pairs, ANOVA post-hoc comparisons were generated for $\delta^{13}C$ and $\delta^{15}N$ mean signatures. As a proxy for realized niche-breadth we obtained the convex-hull area in the $\delta^{13}C$ / $\delta^{15}N$ bi-plot for all included *Amphilophus* individuals and calculated the ratio between this and each single species convex-hull areas as a measure of degree of specialization (Layman et al. 2007).

### Correlation between genetic, morphometric and ecologic differentiation

Testing for correlation between species-pairs genetic, morphometric and ecologic differentiation we used Mantel Test 2.0 (Liedloff 1999) with 5,000 randomizations and used as input matrices the pairwise $F_{ST}$'s from the complete AFLP data, the Euclidean distances between species pairs mean $\delta^{13}C$ and $\delta^{15}N$ and the Euclidean distances between species pairs mean for each of the first four principal components based on the geometric morphometric data. As graphical representation of the relationships between those matrices, linear regressions based on ordinary least squares were generated in PAST (Hammer et al. 2001) including a 95% confidence band for the fitted line.

Additionally, a canonical correspondence analysis (CCA) was conducted in CANOCO 4.51 to test for associations between the distribution of AFLP loci and predictor variables, treating the genetic data as dependent variables. The statistical framework of CCA provides an efficient way to relate patterns of genetic variation to descriptive variables (Angers et al. 1999). The underlying eigenvalue ordination technique allows direct analysis of the relationships between multivariate 'ecological' data tables (ter Braak 1986; ter Braak & Verdonschot 1995), integrating regression and ordination without assuming linearity in the response of 'species' to environmental variables. Using this approach, we aimed to identify single AFLP loci via CCA that are explicitly related to 'environmental' variables.

Each individual in the CCA was characterised by following variables: species (decomposed into six variables), trophic position (see below), breeding coloration (categorized as black, yellow or green) and principal component scores one to four from the geometric morphometrics. Details for coding of variables are given in Table S3 (Supporting Information) and were the same as used in SAM to detect loci under selection. Relative importance of the explanatory variables was tested by means of the forward selection procedure and discriminatory power assessed by the non-parametric Monte-Carlo permutation test under the full model with 5,000 permutations as implemented in CANOCO.

Since we did not obtain stable isotope ratios for all individuals included in the molecular genetic study, different CCA data sets were created including (1) all genotyped individuals with raw $\delta^{15}N$ and $\delta^{13}C$ values and species mean $\delta^{15}N$ and $\delta^{13}C$ for individuals not included in the SIA, (2) all genotyped individuals with each species' trophic position derived from $\delta^{15}N$ and categorized as three trophic levels, and (3) only those individuals for which both, stable isotope and AFLP data were available.

Additional CCAs were conducted with and without species assignment coded as variables and independently for the AFLP matrix containing the presumably neutral loci only and a matrix containing outlier loci only.

## Results

### Morphometric analysis of body shape

The geometric morphometric analyses support morphological differentiation between the six described species. The first four PCs together explain 75.44 % of variance and a MANOVA on those PCs bearing at least 5% of variance revealed significant differentiation between the six species (global and all pairwise tests $p<0.05$). For each of PC1 to PC4, species mean values were calculated and translated into species-pairwise Euclidean distances later used in Mantel tests. Judged from deformation grids as graphical representation of the variance explained, PC1 (32.5%) is associated with an up- and backward shift of the snout tip, differences in the position of the insertions of the pectoral, pelvic and anal fin, PC2 (22.8%) with a general dorsoventral inflation, PC3 (14.2%) with a down- and backward shift of the snout tip and differences in the position of the insertions of the pectoral, pelvic and dorsal fin and PC4 (6%) with differences in the shape of the caudal peduncle and differences in the position of the insertions of the pectoral, pelvic and anal fin.

The CVA assignment test correctly classified 95.7% of the individuals and species were separated in morphospace along the first four CV axes explaining together 96.92% of morphometric variance among the six species (Fig. S2, Supporting Information). Only *A.*

*chancho* showed considerable overlap in morphospace with *A. flaveolus*. The Jackknife procedure showed that the reassignment test is fairly robust. When treating 10 % as ‘unknowns’ still 79.5 % of the individuals were correctly and significantly assigned, and with 20 % declared ‘unknowns’ still 77.9 % were correctly assigned to their respective species at 5 % significance level. Only two of the seven potential hybrid individuals were unambiguously assigned to one species, the remaining were not significantly or ambiguously assigned (Table 1).

**Phylogenetic relationships**

In terms of a phylogenetic species concept the recovered NJ-phylogeny delivered different but overall low levels of monophyly support for the six L. Apoyo Midas cichlid species. The topology of the NJ-tree based on the Link et al. (1995) distance measure on 2297 AFLP loci contains both, taxonomically homogeneous and heterogeneous clades (Fig. 1).

The single species forming a monophylum is *A. globosus*, whereas all other species are not monophyletic: *Amphilophus chancho* forms a cluster together with seven *A. zaliosus* individuals; *A. astorquii* falls into two main clusters, one including an *A. flaveolus* and being sister to the second *A. zaliosus* assemblage; *A. flaveolus* is situated basal to the Apoyo in-group and falls into several clusters with affinities to *A. supercilius*; the latter species also falls into several clusters, although closely associated ones. Several individuals were not grouped according to our species identification and will be discussed later. Leaf-stability indices (Table S1, Supporting Information) were similarly low for most species and individuals (*A. astorquii*, m=0.55; *A. chancho*, m=0.56; *A. flaveolus*, m=0.55; *A. supercilius*, m=0.58; *A. zaliosus*, m=0.57, without the seven conspicuous *A. zaliosus* individuals which had elevated LS indices m=0.81) except for *A. globosus* (m=0.65). The included potential hybrid individuals did not show conspicuously in- or decreased LS indices.

As a consequence of the recovered topology with rather heterogeneous clades, the phylogeny based tests for homoplasy-excess and lineage-movement where only performed for selected clades and not for species specific groups only, and will not be presented in detail. For the homoplasy-excess test we conducted 36 removal experiments and excluded once at a time either each clade with bootstrap support (BS) >25 %, all individuals of a single species or each of the potential hybrid individuals. Conspicuous results were the increase of BS for the monophyly of *A. globosus* from m=30.2% to 50% upon removal of *A. chancho*, and the monophyletic grouping of the otherwise non-monophyletic *A. zaliosus* upon removal of either *A. chancho* (BS=58%), *A. astorquii* (BS=39%) or *A. supercilius* (BS=29%). This hints to a possible hybridization component in the speciation event of the latter three species.

The lineage-movement exploration showed that the seven conspicuous *A. zaliosus* individuals appeared in 38.8% of 2000 bootstrap replicates outside the whole Midas cichlid species complex as basal sister to *A. lyonsi*. This was only found in 4% for *A. zaliosus* excluding the seven 'aberrant' individuals and in 14% for *A. chancho* but not for any remaining species.

The in-group neighbor-net derived from genetic distances calculated with the Link et al. algorithm (1995) shows the same major groups as the NJ-tree but also indicates that there is conflicting signal at the base of the L. Apoyo radiation as indicated by numerous reticulations (Fig. 2).

Among all 126 mtDNA control region sequences included, 38 different haplotypes were identified that are unique to L. Apoyo. All six sampled species contain haplotypes that are closely (≤ 5 mutations) related to the central haplotype 'A'. Almost half of the 126 individuals (42.06 %) from the six species carry the most common haplotype 'A'. The relationships within the Apoyo Midas cichlids based on mtDNA are complex and all species share a variable amount of mitochondrial haplotypes (Fig. S3, Supporting Information).

## Stable isotope analysis (SIA)

The SIA supports the notion of dietary differentiation among L. Apoyo's *Amphilophus*. The detected range of $\delta^{15}N$ from 3.3 to 8.9‰ suggests that there are at least two trophic levels present in *Amphilophus* from L. Apoyo, given a mean $^{15}N$ enrichment of about 3.4% between trophic levels (e.g. Wada et al. 1987; Post 2002). *Amphilophus globosus* exhibited the lowest $\delta^{15}N$ values (m=3.9‰), *A. astorquii* showed intermediate $\delta^{15}N$ levels (m=5.5‰) and *A. supercilius* had the highest mean $\delta^{15}N$ of all examined species (m=7.8‰) (Fig. 3 & S4, Supporting Information). ANOVA post-hoc pairwise comparisons revealed significant differences in $\delta^{15}N$ between all *Amphilophus* species, except pairs *A. astorquii* / *A. flaveolus* and *A. zaliosus* / *A. supercilius* (Table 2). Rough niche-breadth estimation as percentage of total convex hull area of all *Amphilophus* spp. ranged from 4.5% for *A. zaliosus* to 54.5% for *A. flaveolus* with intermediate values for *A. globosus* (4.8%), *A. chancho* (12.8%), *A. astorquii* (16.6%) and *A. supercilius* (16.0%). Convex hull areas were not correlated with contained number of individuals (r=0.29, p=0.58). The $\delta^{13}C$ of fish tissues were less variable than $\delta^{15}N$ values, but *A. zaliosus* is separated from the other species by most negative and *A. flaveolus* by most positive $\delta^{13}C$ values (Fig. 3). These rather small differences might be related to pelagic (more negative $\delta^{13}C$) versus littoral niches (higher $\delta^{13}C$) of the respective species (Vander Zanden et al. 1999).

### Genetic structure and species cohesiveness with and without outlier loci

Between species' genetic differentiation based on the short mtDNA control region sequence data varied greatly and delivered significant $F_{ST}$'s in nine out of 15 species-pair comparisons (Table 3). The smallest but still significant $F_{ST}$ was calculated between *A. zaliosus* and *A. astorquii* (0.021), the highest between *A. chancho* and *A. globosus* (0.573). Investigation of within- and between-species variation using hierarchical AMOVAs with the mtDNA haplotype data showed that most (~72 %) variation is attributable to within-species differentiation, whereas ~28 % can be explained by between-species differences. Dividing the mtDNA haplotypes into groups according to sample location or coloration groups did not explain any amount of genetic variation (Table 4).

Using Bayescan 1.0 to identify potential candidate loci under directional selection from the AFLP matrix, 49 loci (~2 %) were detected to be influenced by directional selection applying Jeffreys' scale of evidence for Bayes factors with the strictest criteria ‘decisive’ (selection posterior probabilities above 99 %). Within the Dfdist analysis we used two significance levels (99% or 95%) and used as baseline $F_{ST}$ either the average or the trimmed mean $F_{ST}$, both provided by the Ddatacal program (part of the Dfdist package) as suggested by e.g. Pérez-Figueroa et al. (2010) or Caballero et al. (2008). The software detected between one ($p<0.01$, average $F_{ST}$) and 19 ($p<0.05$, trimmed $F_{ST}$) loci that showed higher $F_{ST}$ values than under neutral expectations. Loci detected by Dfdist were always among the 49 hits obtained with Bayescan. The spatial analysis approach with the software SAM identified eight markers as significant with both implemented tests (G & Wald) at a 99% confidence level after Bonferroni correction when species assignment was included and three loci without species assignment coded as variable. Again, all detected loci were contained in the 49 identified by Bayescan. Further analyses of the AFLP data were conducted on both, the complete matrix and on the reduced matrix (without the 49 Bayescan loci), which we refer to as the neutral matrix.

All pairwise $F_{ST}$’s obtained from the complete and the reduced, neutral AFLP matrix were significant and ranged between 0.052 - 0.223 based on pairwise differences and 0.096 - 0.353 based on the Jaccard coefficient for the complete matrix and between 0.036 - 0.141 (pairwise differences) and 0.067 - 0.219 (Jaccard coefficient, Table 5) for the neutral matrix. Both methods delivered highly correlated results ($r=0.99$, $p<0.001$) with the $\Phi_{ST}$’s based on the Jaccard coefficient being on average 42 ± 2.6% higher. Exclusion of the 49 loci detected to be under selection had an equal effect on both measures ($r=0.98$, $p<0.001$): $F_{ST}$’s decreased by 30.8 ± 6.8% and $\Phi_{ST}$’s decreased by 30.3 ± 6.7%. Pairwise $F_{ST}$’s were differently strong influenced by the exclusion of the 49 loci, and a weak correlation between

$F_{ST}$ and decrease was observed ($r=0.63$, $p<0.05$). The effect ranged from a decrease by 42.8% (*A. chancho* / *A. zaliosus*) to 20.9% (*A. astorquii* / *A. flaveolus*).

Strongest differentiation based on all loci was detected between *A. zaliosus* and *A. globosus* ($F_{ST}$ 0.223, $\Phi_{ST}$ 0.353) and smallest, but still significant differentiation between *A. flaveolus* and *A. supercilius* ($F_{ST}$ 0.052, $\Phi_{ST}$ 0.096). The discovered differentiation was not expected from the number of private alleles for each species: *A. zaliosus* (88), *A. supercilius* (53), *A. chancho* (48), *A. astorquii* (32), *A. flaveolus* (24) and *A. globosus* (21).

Results from AMOVAs with the AFLP data were similar to those obtained from mtDNA: genetic variation was partitioned as ~14 % among and ~86 % within species. Grouping samples according to location, trophic level inferred from the SIA or coloration delivered small but significant amounts of variation explained by those groupings (~1.6 %, ~6.5 % and ~6.5 % respectively, Table 6). Excluding the 49 outlier loci (~2% of all loci) had a clear effect on AMOVA outcomes: among species variation decreased by one third while within species variation increased by ~5 %. While AMOVA revealed ~6.5 % of variation between coloration groups using all loci, removal of the 49 selected loci lead to a complete loss of genetic variation explained by this coloration-based grouping and a slight reduction in variance explained by the trophic level. This clear effect based on coloration did not occur when those loci were removed that had been identified by Dfdist or SAM only (Fig. S5, Supporting Information).

Findings from the Bayesian cluster analysis with Structure v2.2 without *a priori* species designation suggest different levels of population integrity of the six species and point to the presence of hierarchical genetic structure. The Bayesian cluster method detected six clusters ($K=6$) to be most likely, which was also confirmed by a second peak of Evanno's $\Delta K$ at $K=6$ (Fig. S6, Supporting Information), roughly corresponding to the six species. Of the 22 *A. zaliosus*, 15 were consistently grouped in one cluster with all *A. astorquii* (Fig. 4, top). Repeating the Structure runs including only the latter two species shows that they do not form a genetically homogeneous group - the clustering method clearly differentiates between the two species - and shows that there are two clusters within *A. zaliosus*, one of which with affinities to *A. astorquii* (Fig. S7, Supporting Information), the other composed of the seven aforementioned conspicuous individuals.

Testing for misclassified or immigrant individuals in Structure with GENSBACK=2 and varying *v* (0.1, 0.05, 0.01) gave highly similar results independently of *v*. Mean proportions of correctly classified individuals (above 95% probability) derived from seven independent Structure runs with varying *v* were for: *A. globosus* 95%, *A. astorquii* 80.2%, *A. chancho* 80%, *A. flaveolus* 64.3% and *A. supercilius* 94%. As in the first analysis, 68% of the *A. zaliosus* where always misclassified as *A. astorquii*. Several individuals were constantly detected as being misclassified or having ancestry in one or more other species (Fig. 4; Tables 1 & 7), and only one of the seven included potential hybrids demonstrated a clear signature of admixed origin while the remaining were mainly assigned to the species with which they clustered in the NJ tree reconstruction (Table 1).

The Structure runs on the reduced AFLP matrix and on the 49 outlier loci matrix without *a priori* species assignation delivered extremely different results. No differentiated, coherent clusters were detected in the neutral AFLP matrix, irrespective of K. Only the seven *A. zaliosus* individuals which also clustered together in the NJ tree showed assignment probabilities around 50 % to a non-Apoyo cluster (plot not shown). Quite to the contrary, including only the 49 outlier loci in the Structure runs was highly informative: 1) *K*=6 was the most likely number of genetically differentiated groups, identical to the outcome with the complete AFLP matrix; 2) all *A. zaliosus* individuals grouped together, while they were split based on the complete matrix; 3) a much higher percentage of individuals showed elevated admixture proportions as compared to the complete AFLP matrix and 4) only 8.9 % (compared to 80.2% based on the complete matrix) of the *A. astorquii* individuals showed assignment probabilities above 95 % to a unique cluster. Averaging over ten runs and all individuals, *A. astorquii* showed the following mean (+/- SD) admixture proportions: 5.97 %

(0.07 SD) *A. chancho*, 31.5 % (0.26 SD) *A. flaveolus*, 6.75 % (0.06 SD) *A. globosus*, 14.83 % (0.24 SD) *A. supercilius* and 10.2 % (0.05 SD) *A. zaliosus*.

As with the Structure analyses on the neutral AFLP matrix and on the 49 outlier loci only, comparison of the two PCA delivered interesting insights: while there are coherent species specific clusters including all *A. zaliosus* individuals in a single cluster derived from the outlier loci, those clusters collapse and largely overlap except for the seven 'aberrant' *A. zaliosus* specimens that group distantly from all remaining Apoyo fishes when analysing the neutral matrix (Fig. S8, Supporting Information).

**Correlation between genetic, morphometric and ecologic differentiation**

While no correlation between genetic and isotopic differentiation was found, there is a moderate correlation between pairwise distances from PC3 of the morphometric data with the pairwise AFLP $F_{ST}$ values (Fig. 5). Congruently, the Mantel statistic was significant for a correlation between Euclidean distances based on PC3 and pairwise AFLP $F_{ST}$ only ($r=0.58$, $p<0.05$). The strongest correlation was detected between the ecologic descriptor based on the SIA and, again, the Euclidean distances based on PC3 from the morphometric data (Fig. 5, Mantel $r=0.89$, $p<0.001$).

In total twelve different CCAs were conducted differing in either the sets of individuals, environmental variables included or genetic data used as dependent variables (Table S4, Supporting Information). Congruent patterns emerging from the comparison of the different CCAs were: including species assignment as variables always accounted for more variation of total inertia as compared to the CCA without species assignment coded (39-45 % vs. 28.7-30.4% 'outlier loci' and 10.9-23.3 % vs. 9-16.5 % 'neutral loci'). Overall performance of CCA ordination as judged from explanatory power was much better for the analyses of the outlier AFLP matrix (m=61% including species assignment and m=73.2% without species assignment) as compared to the analyses of the neutral AFLP matrix (m=32.7% including species assignment and m=42.1% without species assignment). Locus – environment correlations were always higher in the CCA on the outlier AFLP matrix (0.932 – 0.937) than on the neutral matrix (0.798 – 0.895) when species assignment was included. The same pattern emerged without species assignment (‘outlier loci’ 0.859 – 0.886 vs. ‘neutral loci’ 0.764 – 0.883), although not as pronounced. The overall large correlation values above 0.764 tell that we can account for most genetic variation by taking into account the environmental variables.

Factors most strongly and significantly related to loci distribution differed between the CCA approaches (Table S5, Supporting Information), but emerging patterns can be summarized as follows: ‘coloration’ and ‘trophic position’ (also as raw δ15 N) almost always significantly improved the model of the CCA on the outlier AFLP matrix while this was not the case for the neutral matrix; the opposite was found for variables ‘PC3’ of the geometric morphometrics and ‘location’, which were almost always significantly associated with the neutral AFLP matrix and not with the outlier loci distribution. Species assignment included as a variable differed in importance between the CCAs, being always significantly related to the outlier loci distribution for *A. chancho*, *A. globosus* and *A. zaliosus*, but not for the remaining species, and to a smaller extent to the neutral AFLP matrix.

## Discussion

We identified eco-phenotypic covariance structure with neutral and non-neutral genetic differentiation that differentially support a role of both, divergent natural, and sexual selection for shaping current species divergence in L. Apoyo. Body coloration, likely involved in sexual selection (e.g. Salzburger 2009), was identified as potential key character for maintaining species cohesion, while ecological selection judged from the proxies applied yielded a comparatively weak signature. Five out of the six species were found to be polyphyletic and showed substantial levels of gene flow between species. We conclude that the shared alleles partially result from gene flow and not only from ancestral polymorphisms, since otherwise we would anticipate stronger incongruence between genotypic and phenotypic cohesiveness. Within *A. zaliosus* several individuals showed putative allochthonous phylogenetic affinities and levels of neutral differentiation not related to divergent selection within L. Apoyo indicating a past introgression event from outside.

**Natural or sexual selection?**

Speciation is thought to begin with the rise of genomically-localised barriers to gene exchange associated with loci for local adaptation, intrinsic incompatibility or assortative mating (Butlin 2010). In the early stages, gene flow will be reduced at only few loci which likely contribute to the initiation of reproductive isolation. We found that only a small proportion (~2%) of the fragments show highly significant signatures of divergent selection, similarly to what had been reported for allopatric populations or ecotypes (reviewed in e.g. Oleksiak 2010). Although we cannot relate candidate loci to genes, the effect of those loci on partitioning of genetic variance according to body coloration indicates a role of sexual selection on at least part of the detected outlier loci. Since we observed a much weaker effect on variance partitioning based on trophic position, we propose that divergent sexual selection is currently completing speciation and supporting species cohesiveness. Disruptive natural (ecological) selection probably played a more important role in the early stage of the radiation, substantiated by the strongest genetic and ecologic differentiation between benthic *A. globosus* and limnetic *A. zaliosus*. Depending on the species pair considered, both types of selection may act in concert, i.e. resulting in both strong genetic and ecological differentiation (e.g. *A. zaliosus* / *A. globosus*), or one of the two possibilities yielded stronger signatures, i.e. strong genetic and little ecologic differentiation (e.g. *A. zaliosus* / *A. supercilius*) or *vice versa* (e.g. *A. astorquii* / *A. flaveolus*). Noteworthy, we did not find ecologically strong but genetically weakly differentiated species pairs. This underlines the primary importance of natural selection for the coherence of ecological divergence and reproductive isolation. In contrast, divergent sexual selection alone is less likely to lead to ecological divergence and speciation, a pattern already known from cichlids in the East

African rift lakes and other species flocks (e.g. Seehausen & van Alphen 1999; Panhuis et al. 2001; Herder et al. 2008; Martin & Genner 2009). With regard to the relative importance of natural vs sexual selection, the likelihood of sympatric speciation is enhanced when disruptive ecological selection favors sexual preferences for traits that signal local adaptation (van Doorn et al. 2009). Even with substantial gene flow natural and sexual selection work in concert to create local adaptation and reproductive isolation. Although it is possible that coloration in Midas cichlids reflects local adaptation, thus probably representing a realized variant of a 'magic trait', this has to be confirmed experimentally.

## Ecological differentiation

Ecological divergence as deduced from trophic position based on $\delta^{15}N$ is present in L. Apoyo, but does not solely explain maintenance of the observed genetic structure according to the applied gradient analysis (CCA). Similar levels of differentiation were described for five sympatric Lake Malawi cichlid species (Genner et al. 1999), indicating that complete ecological (isotopic) segregation may not be necessary to allow coexistence in sympatry. As aforementioned, normal- and thick-lipped *Amphilophus* in L. Apoyeque were shown to diverge eco-morphologically through disruptive selection (Elmer et al. 2010b), differing slightly in their isotopic signatures. Interestingly, $\delta^{15}N$ varies in a much narrower range as that of *Amphilophus* from L. Apoyo ($\delta^{15}N$ ~ 9 to 11 vs 3.5 to 9) and also the carbon values are outside the ranges reported herein ($\delta^{13}C$ ~ -5 to -11 vs -12 to -20). This reflects markedly different baseline isotopic signatures, and demonstrates stronger ecological differentiation and the presence of more trophic levels or isotopically more diverse basal food sources here. The Apoyeque study documents the potential of *Amphilophus* to rapidly develop morphological variation upon which selection operates. Interestingly, there is a thick-lipped species in Lakes Managua and Nicaragua (*A. labiatus*), and also thick-lipped forms known from Lakes Xiloá, Apoyeque and Masaya, raising the question why there is no such form in L. Apoyo. Since it has been demonstrated in sticklebacks that the occurrence of ecotypes can be predicted by ecological and physical lake attributes (Gow et al. 2008) it is conceivable that Midas cichlid evolution follows a similar predictable form. This could be tested with a comprehensive sample of all known thick-lipped or limnetic phenotypes in conjunction with limnological data. Whether thick lips are due to genetic predispositions (e.g. *A.* cf. *labiatus* derived alleles) that have not been present in the founder population of L. Apoyo or is conditional on lake-specific ecological and physical attributes (e.g. abundance & quality of solidified lava) cannot yet be answered.

**Correlations suggesting divergence promoters**

A common pattern in freshwater-fish radiations is an early split into a limnetic and a benthic morph, as e.g. in sticklebacks. This early split is commonly accompanied by directional selection leading to morphological, physiological or behavioral adaptations to increase niche specific resource utilization (e.g. Rundle et al. 2000). If the radiation in L. Apoyo follows to this pattern, then one would expect a significant correlation between morphological and ecological differentiation. Indeed, there is a strong correlation between phenotypic differentiation based on morphometric PC 3 and diet variation between species ($r=0.89$, $p<0.001$), possibly reflecting the adaptive value of this particular feature. Supporting this idea, PC 3 is correlated with pairwise genetic differentiation ($r=0.58$, $p<0.05$), but interestingly, even stronger with the neutral genetic differentiation ($r=0.65$, $p<0.01$). This tendency also emerged in the CCA, where PC 3 was clearly associated with the distribution of the neutral AFLP loci, and to a lesser extent with the outlier loci, supporting the idea that genetic drift can additively contribute to morphological divergence (Leinonen et al. 2006). The finding is also in congruence with the idea that ecological adaptation promotes neutral divergence by genetic drift, because once adaptations lead to the emergence of partial reproductive isolation, gene flow will also be increasingly reduced at hitchhiking neutral loci (Nosil et al. 2009b; Rice et al. 2011).

However, lack of ecological opportunity to specialize into narrower niches (Losos & Mahler 2010) and/or over-interpretation and overestimation of the ecological divergence proxies used in our study might also explain the weakness of eco-genotypic correlation. Alternative factors such as spatially or temporally differentiated breeding may play a role for keeping trophically similar species separated. This is supported by significant differences in breeding depth and timing in trophically similar species (e.g. *A. chancho* & *A. zaliosus*, McCrary & López 2008), which may signal additional resources under competition (McKaye 1977). Accordingly, interspecific, or initially intra-populational competition drives character displacement, as has repeatedly been proposed and documented for sticklebacks (e.g. Schluter 2003), cichlids (e.g. Cooper et al. 2010), birds (e.g. Sætre et al. 1997; Grant & Grant 2006) and many other systems, rendering resources an important factor for divergence to occur. Various studies have shown that intraspecific competition leads to negative frequency-dependent interactions inducing disruptive selection and increasing adaptive variation (reviewed in Hendry et al. 2009). It has been proposed therefore to accept interspecific competition-driven character displacement as a common means for adaptive radiations (Losos & Mahler 2010), and although this comes as a handy explanation also for the L. Apoyo radiation, we are aware that this has still to be demonstrated. In terms of the speciation continuum the six Apoyo species are surely at different stages along the continuum affected variably by different selective forces.

## Hybrid swarm & syngameon hypothesis

Hybridization and introgression can generate and increase standing genetic variation for new adaptive trait combinations suitable for exploiting resources not utilized previously (Dowling & Secor 1997; Stelkens et al. 2009). The same mechanisms concurrently prevent the loss of genetic variation due to strong selection prevailing in sympatric speciation scenarios and may even induce speciation (Mallet 2007). The product is not necessarily the formation of a hybrid species, but rather an increase in genetic variance with the introgressing form disappearing in a hybrid swarm. Theory indicates that adaptation can be rapid when evolution acts on standing genetic variation instead of relying on the rather rare occurrence of beneficial mutations (Barrett & Schluter 2008; Wolf et al. 2010). Our results strongly suggest the existence of remains of an introgression event in the neutral genetic variance of *A. zaliosus*, hereby having increased standing genetic variation.

Kawata & Yoshimura (2000) modelled speciation as the outcome of hybridization between populations differing in distributions of sexually selected quantitative male traits and female mate preference traits, without hybrid inviability. The probability of speciation was negatively correlated to trait overlap between species, but even large overlaps resulted in speciation. In real populations, drift may counter the frequency-dependent selection favouring extinction of one form, or a hybrid swarm may form (Ritchie 2007). We find support for the latter situation, also predicted by the syngameon hypothesis (Seehausen 2004), as there was no detectable genetic structure based on the neutral AFLP matrix, indicative of a 'common' neutral gene pool (disregarding the allochthonous signal of the seven *A. zaliosus*).

### Signals of ancient introgression?

The seven 'aberrant' *A. zaliosus* solely showed a strong differentiation signal in the Structure runs and PCA based on the neutral AFLP matrix. We argue that those individuals carry alleles from an unknown allopatric source, reflecting a past introgression event. They show levels of neutral differentiation apparently not related to divergent selection within L. Apoyo. Further support for this hypothesis comes from several findings: 1) the group is characterised by four, fixed private alleles while no other species carries any fixed private alleles in our sample; 2) they cluster with the remaining *A. zaliosus* when considering the 49 outlier loci only, but cluster distantly from the whole Apoyo radiation based on the complete and neutral AFLP matrix (PCA & Structure); 3) the lineage-movement procedure revealed a tendency for this group to appear outside the whole Apoyo clade based on bootstrapped data. An alternative explanation to introgression from outside Apoyo is the retention of ancestral alleles that have been lost in all remaining individuals, but it is hard to explain why those alleles should have persisted just in seven individuals of a single species. It is equally unlikely that they had evolved in a subset of *A. zaliosus* individuals only, and perished again in the majority of sampled individuals, also rendering 'hybrid speciation' sensu Mallet (2007) as origin of *A. zaliosus* implausible. We further exclude the possibility that the findings are just due to technical artefacts since the seven individuals where analysed on two different deep-well plates, contain one replicate sample, and the four fixed as well as 22 private alleles stem from different primer combinations. Interestingly, all seven individuals were collected along the western shore of L. Apoyo, their distribution thus significantly deviating from the expected distribution as judged from the remaining sampled *A. zaliosus* ($\chi^2$=12.32, df=2, p<0.001). The observed distribution might be due to philopatric behaviour with respect to breeding site, but also genetic drift might play a role.

The impact of the hypothesized introgression event on the speciation propensity of the L. Apoyo radiation is not fully explorable with our data, since they cannot be related to an annotated genome. The fact that introgressed alleles have apparently increased current neutral variation only, does not necessary preclude a role for introgressed alleles as substrate for selection directly after the putative introgression event. Independent of that, the finding demonstrates the possibility that introgression of allochthonous alleles into seemingly fully isolated crater lake species flocks can take place.

## Current Gene Flow

Which L. Apoyo species are interconnected via gene flow? Although in the phylogenetic reconstruction *A. astorquii* fell into (only) two neighbouring clusters, it is according to Structure, PCA and $F_{ST}$ results the species that hybridized most often. Since it is the most abundant of the six species (McCrary & López 2008) this may also be an effect of stochasticity. It would be interesting to test whether there is a link between the species' genomic mosaic signature and their abundance, but more data on biology and demography are needed to tackle this question. Admixture proportions of *A. astorquii* from *A. flaveolus* were the highest estimated, which seems to be biologically sound since they are ecologically similar, hereby suggesting microhabitat utilization as a factor for reproductive isolation in sympatry. But, since also other species pairs are ecologically similar and breed in direct vicinity, but are nevertheless strongly sexually isolated from each other, divergence in microhabitat use as prominent factor loses attractiveness as explanation. Interestingly, of the seven individuals classified *a priori* as potential hybrids, none grouped clearly with *A. astorquii*, neither morphologically nor genetically. We speculate that, although *A. astorquii* and *A. flaveolus* apparently hybridize more frequently, resulting crosses are phenotypically less conspicuous than hybrid offspring of other crosses as e.g. between *A. flaveolus* and *A. supercilius*.

**Hybridization and Speciation**

Traditionally, animal-hybrids were presumed to have reduced fitness (Reyer 2008), but recently the number of examples where hybridization apparently facilitated speciation and adaptive radiations in animals has increased (Dowling & Secor 1997; Mallet 2007, Salazar et al. 2010). Hybridization may constitute a vector for advantageous alleles between species through backcrossing, or hybrids may show higher fitness relative to their genitors when transgressive segregation combines alleles creating novel hybrid traits (e.g. Rieseberg et al. 1999; Ellstrand & Schierenbeck 2000; Gross et al. 2004; Nolte et al. 2005; Stelkens et al. 2009). It has also been hypothesized that hybridization is adaptive, leading to selection for weak discrimination between con- and heterospecifics under certain environmental conditions (Pfennig 2007) while another extreme consequence of hybridization is despeciation (Seehausen et al. 1997; Seehausen 2006; Taylor et al. 2006). Theoretically, reproductive isolation and functional divergence (i.e. speciation) are possible despite substantial levels of gene flow (Smith et al. 1997; Crandall et al. 2000; Dieckmann & Doebeli

1999; Barreto & McCartney 2008; Nielsen et al. 2009), a likely scenario also in L. Apoyo Midas cichlids.

It has repeatedly been put forward that hybridization is particularly advantageous where new ecological niches are created by changing or newly invaded environments (e.g. Seehausen 2004, Nolte et al. 2005; Dowling & Secor 1997). The colonization of Crater Lake Apoyo likely constituted such a situation, offering apart from reduced competition at least two novel dimensions, i.e. depth and higher water transparency, since the most likely source for the seeding population was L. Nicaragua (Barluenga & Meyer 2004; Geiger et al. 2010b) with mean depth ca. 13m and 0.25 – 0.35m Secchi disk transparency, contrasting L. Apoyo with mean depth ca. 142m and 3.5 – 9.5m Secchi disk transparency (Barlow 1976). We know from the lab and the field that 'gold' coloration of individual Midas cichlids affects mate choice (e.g. Barlow & Rogers 1978; Barlow et al. 1977; McKaye & Barlow 1976; Elmer et al. 2009), demonstrating that visual cues play a role during pair formation in Midas cichlids. An increase in transparency might thus have allowed for changes in mate choice patterns on visual cues. Water turbidity may affect sexual selection by impairing the possibility for visually based mate choice (Engström-Öst & Candolin 2007; Järvenpää & Lindström 2004; Seehausen et al. 1997) and also the reverse 'speciation through sensory drive' has been documented in Lake Victoria cichlids (Seehausen et al., 2008) underpinning the great potential of this mechanism.

In summary, we find no strong indication that any of the six species has originated from 'homoploid hybrid speciation' sensu Mallet (2007), i.e. that hybridization has had a primary role in the origin of one of the six species. If so we would expect to find a similar constant signature of introgression in any of the six species as has been described for the seven 'aberrant' *A. zaliosus* individuals above. However, we demonstrate the existence of various levels of gene flow between species pairs, underlining their incipient status and a potentially non-detrimental or even beneficial role of hybridization.

The observed pronounced genetic, phenotypic and ecological differentiation between *A. globosus* and *A. zaliosus* supports a scenario of primary divergence in those species as opposed to a secondary divergence including a hybridization component for *A. astorquii*, *A. chancho*, *A. flaveolus* and *A. supercilius* as deduced from the homoplasy-excess tests and cluster analyses.

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## Figure legends

Fig. 1 Neighbor-joining tree based on Link et al. distance measure using 2297 AFLP loci with bootstrap values ≥ 50 given above nodes.

Fig. 2 AFLP neighbor-network based on Link’s genetic distances.

Fig. 3 Mean isotopic composition (+/- 95% confidence interval) of L. Apoyo Midas cichlid members only.

Fig. 4 Results of Structure clustering analyses. Top: on complete AFLP matrix for K6 with group information, GENSBACK 2 and MIGPRIOR 0.05; Middle: same as top but on the 49 AFLP outlier loci only; Bottom: on the 49 outlier loci for K6 without group information. Species of sample origin given above, sample IDs of individuals given below.

Fig. 5 Linear regression fit with 95% confidence band for Euclidean distances from species mean principal component three versus pairwise $F_{ST}$ and Euclidean distances from mean $\delta^{15}N$ and $\delta^{13}C$ species signatures with Mantel statistics given. ast: *A. astorquii*; cha: *A. chancho*; fla: *A. flaveolus*; sp1: *A. globosus*; sp.2: *A. supercilius*; zal: *A. zaliosus*.

## Tables

Table 1 Summary table for the seven potential hybrid individuals with IDs used throughout this study. CVA assignment based on Mahalanobis distance; mtDNA haplotype inferred from the parsimony network; position in the NJ tree based on Link's distance measure; Structure results show the consensus from seven runs with varying *v* (0.01, 0.05 and 0.1) and GENSBACK=2; alternative clustering in 2000 bootstrap replicates identified by the lineage-movement procedure (LM).

| ID | 81 | 82 | 83 | 84 | 85 | 86 | 87 |
|---|---|---|---|---|---|---|---|
| CVA | *A.chancho ns* | *A.zaliosus ns* | *A.chancho ns* | *A.chancho*[1] *or A.flaveolus*[1] | *A.flaveolus*[1] *or A.supercilius*[1] | *A.flaveolus*[2] | *A.supercilius*[2] |
| mtDNA | *A.zaliosus* | A | B | A | unique | unique | C |
| NJ cluster | *A.chancho / A.zaliosus* | *A.supercilius* | *A.flaveolus* | *A.flaveolus* | *A.supercilius* | *A.flaveolus / A.supercilius* | *A.flaveolus* |
| Structure | 97-99% *A.astorquii* grandparent | 100% *A.zaliosus* grandparent | 24-68% *A.flaveolus* & 30-66% *A.supercilius* grandparent | 97-100% *A.flaveolus* | 97-100% *A.supercilius* | 99-100% *A.supercilius* | 100% *A.flaveolus* |
| LM | *A.chancho / A.zaliosus* (stable) | *A.supercilius* (stable) | 45% *A.flaveolus*<br>7% *A.supercilius*<br>2% *A.chancho* | 40% *A.flaveolus*<br>19% *A.supercilius* | 54% *A.supercilius* | 28% *A.flaveolus*<br>13% *A.supercilius* | 33% *A.flaveolus*<br>6% *A.astorquii* |

[1] equal distance to each species

[2] significant

ns: not significant

Table 2 ANOVA post-hoc pairwise comparisons between L. Apoyo Midas cichlid species (Tukey HSD allowing for unequal sample sizes with significance levels; ns, non-significant). Top right: significance levels for differences in mean $\delta^{15}N$ species signature; lower left: significance levels for differences in mean $\delta^{13}C$ species signature.

| | *A. astorquii* | *A. supercilius* | *A. flaveolus* | *A. globosus* | *A. chancho* | *A. zaliosus* |
|---|---|---|---|---|---|---|
| *A. astorquii* | | <0.01 | ns | <0.01 | <0.01 | <0.01 |
| *A. supercilius* | ns | | <0.01 | <0.01 | <0.01 | ns |
| *A. flaveolus* | ns | ns | | <0.01 | <0.01 | <0.05 |
| *A. globosus* | ns | ns | ns | | <0.01 | <0.01 |
| *A. chancho* | ns | ns | ns | ns | | <0.01 |
| *A. zaliosus* | ns | ns | ns | ns | ns | |

Table 3 Pairwise $F_{ST}$-estimates based on the mtDNA control region, minimum and maximum values in bold.

| | *A. zaliosus* | *A. globosus* | *A. astorquii* | *A. chancho* | *A. flaveolus* | *A. supercilius* |
|---|---|---|---|---|---|---|
| *A. zaliosus* | - | * | * | * | ns | ns |
| *A. globosus* | 0.032 | - | ns | * | * | * |
| *A. astorquii* | **0.021** | 0.028 | - | * | ns | ns |
| *A. chancho* | 0.514 | **0.573** | 0.530 | - | * | * |
| *A. flaveolus* | -0.009 | 0.037 | -0.003 | 0.502 | - | ns |
| *A. supercilius* | 0.015 | 0.061 | 0.029 | 0.422 | -0.007 | - |

*significant (10,000 permutations)

ns: not significant

Table 4 AMOVA hierarchical genetic analysis of pairwise differences based on the mtDNA control region data.

| Source of variation | *df* | Percentage of variation |
|---|---|---|
| among species | 5 | 28.23* |
| within species | 113 | 71.77* |
| | | |
| among locations | 2 | -0.59 ns |
| among trophic levels** | 2 | -6.28 ns |
| among coloration groups*** | 2 | -7.03 ns |

*significant (10,100 permutations)

**trophic position high (*A. chancho*, *A. zaliosus*, *A. supercilius*), intermediate (*A. astorquii*, *A flaveolus*), low (*A. globosus*)

***groups: black (*A. astorquii*, *A. supercilius*, *A. zaliosus*), yellow (*A. chancho*, *A. globosus*), green (*A. flaveolus*)

ns: not significant

Table 5 Pairwise $F_{ST}$-estimates based on the binary AFLP matrix. First value corresponds to $F_{ST}$ based on pairwise differences according to Weir & Cockerham (1984), second value gives the $\Phi_{ST}$ based on standard Jaccard coefficient after distance transformation (d=1-s). All pairwise $F_{ST}$ comparisons were significant based on 10,100 permutations following sequential Bonferroni correction. Lower left: comparisons based on all 2297 loci; upper right: neutral estimates based on the reduced AFLP matrix excluding the 49 loci identified by Bayescan. Minimum and maximum values in bold.

| | *A. zaliosus* | *A. globosus* | *A. astorquii* | *A. chancho* | *A. flaveolus* | *A. supercilius* |
|---|---|---|---|---|---|---|
| *A. zaliosus* | - | **0.141** / **0.219** | 0.098 / 0.171 | 0.099 / 0.170 | 0.092 / 0.163 | 0.119 / 0.207 |
| *A. globosus* | **0.223** / **0.353** | - | 0.105 / 0.177 | 0.099 / 0.166 | 0.098 / 0.160 | 0.112 / 0.184 |
| *A. astorquii* | 0.134 / 0.233 | 0.142 / 0.238 | - | 0.078 / 0.141 | 0.049 / 0.092 | 0.064 / 0.118 |
| *A. chancho* | 0.173 / 0.293 | 0.148 / 0.252 | 0.101 / 0.182 | - | 0.071 / 0.129 | 0.085 / 0.152 |
| *A. flaveolus* | 0.154 /0.266 | 0.128 / 0.212 | 0.062 / 0.114 | 0.105 / 0.188 | - | **0.036** / **0.067** |
| *A. supercilius* | 0.188 / 0.317 | 0.159 / 0.263 | 0.084 / 0.153 | 0.132 / 0.230 | **0.052** / **0.096** | - |

Table 6 AMOVA hierarchical genetic analysis of pairwise differences based on the complete AFLP matrix with 2297 loci and on the reduced matrix excluding the 49 loci identified as being under selection.

| Source of variation | *df* | Percentage of variation | |
|---|---|---|---|
| | | all loci | neutral loci only |
| among species | 5 | 13.77* | 9.15* |
| within species | 113 | 86.23* | 90.85* |
| | | | |
| among locations | 2 | 1.58* | 1.31* |
| among trophic levels** | 2 | 6.53* | 4.64* |
| among coloration groups*** | 2 | 6.52* | -1.24 ns |

*significant (10,100 permutations)

**trophic position high (*A. chancho*, *A. zaliosus*, *A. supercilius*), intermediate (*A. astorquii*, *A flaveolus*), low (*A. globosus*)

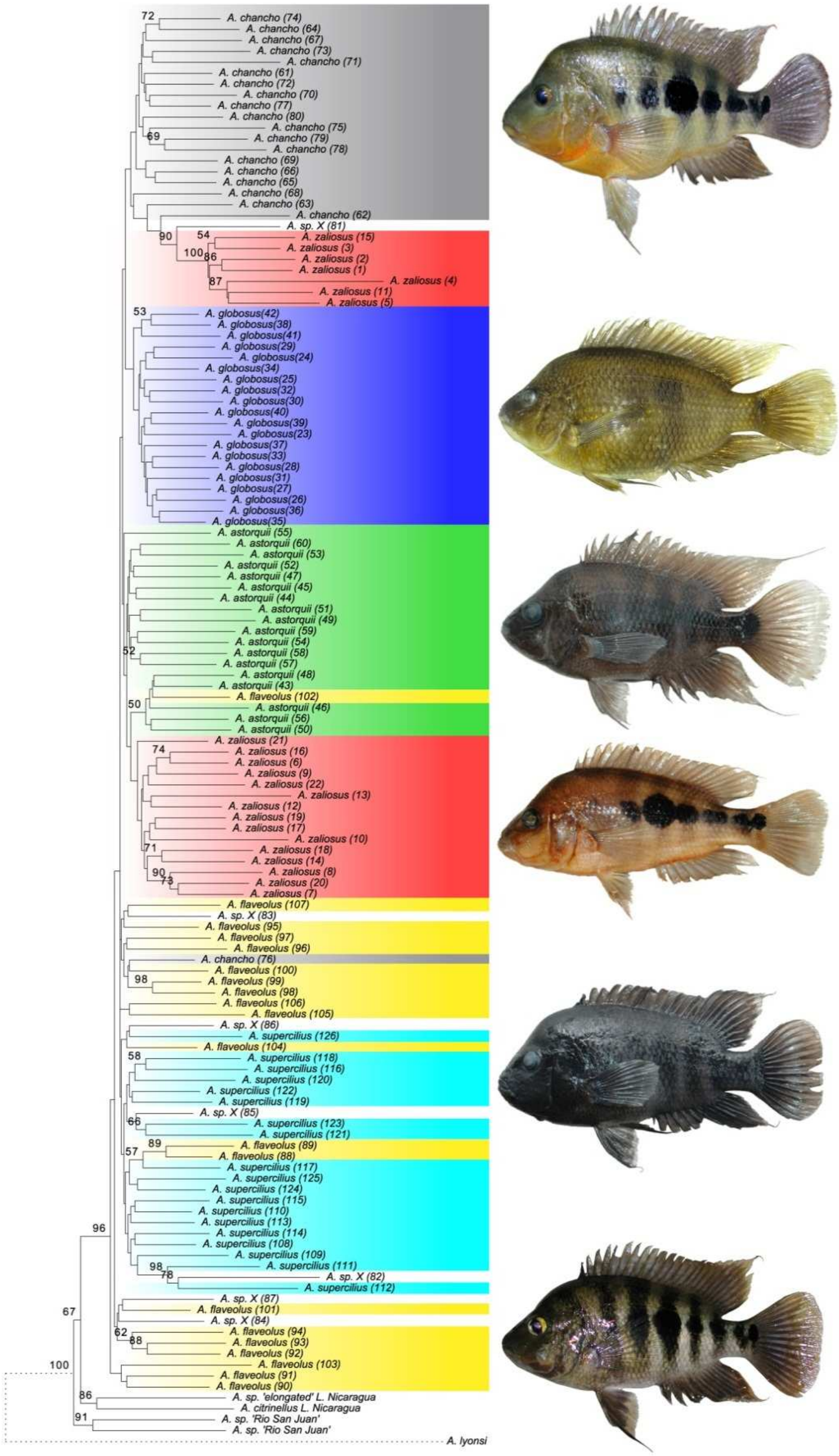
***black (*A. astorquii*, *A. supercilius*, *A. zaliosus*), yellow (*A. chancho*, *A. globosus*), green (*A. flaveolus*)

ns: not significant

Table 7 ‘No immigrant ancestry’ gives the probability that the ancestry of each individual is exclusively in its predefined species, following columns show the probabilities that each individual has the given amount of ancestry in the given source species. Results show the consensus from seven runs with varying *v* (0.01, 0.05 and 0.1) and GENSBACK=2. Position in the NJ tree based on Link’s distance measure, individual’s IDs are those used throughout.

| ID | species | no immigrant ancestry | immigrant | immigrant grandparent | NJ cluster |
|---|---|---|---|---|---|
| 42 | *A. globosus* | 35-83 % | - | 16-61 % *A. chancho* | *A. globosus* |
| 45 | *A. astorquii* | 69-96 % | - | 28 % *A. supercilius* | *A. astorquii* |
| 47 | *A. astorquii* | 27-66 % | - | 30-66 % *A. supercilius* | *A. astorquii* |
| 51 | *A. astorquii* | 40-99 % | - | 43-60 % *A. chancho* | *A. astorquii* |
| 58 | *A. astorquii* | 46-87 % | - | 37-54 % *A. chancho* | *A. astorquii* |
| 62 | *A. chancho* | - | - | 99-100 % *A. zaliosus* | *A. chancho* |
| 63 | *A. chancho* | - | - | 99-100 % *A. zaliosus* | *A. chancho* |
| 68 | *A. chancho* | 0-2 % | - | 98-99 % *A. globosus* | *A. chancho* |
| 76 | *A. chancho* | - | 86-95 % *A. flaveolus* | 12-15 % *A. globosus* | *A. flaveolus* |
| 88 | *A. flaveolus* | - | 100 % *A. supercilius* | - | *A. supercilius* |
| 89 | *A. flaveolus* | - | 98-100 % *A. supercilius* | - | *A. supercilius* |
| 90 | *A. flaveolus* | 16-70 % | - | 30-84 % *A. astorquii* | *A. flaveolus* |
| 102 | *A. flaveolus* | - | 99-100 % *A. astorquii* | - | *A. astorquii* |
| 104 | *A. flaveolus* | 30-98 % | - | 17-33 % *A. supercilius* | *A. supercilius* |
| 105 | *A. flaveolus* | 0-14 % | - | 63-87 % *A. chancho*, 20 % *A. supercilius* | not resolved |
| 106 | *A. flaveolus* | 5-71 % | 10-75 % *A. supercilius* | 12-33 % *A. supercilius*, 13 % *A. astorquii* | not resolved |
| 112 | *A. supercilius* | 0-1 % | - | 99-100% *A. zaliosus* | *A. supercilius* |

897 Fig. 1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A. chancho (62)
A. sp. X (81)
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A. zaliosus (15)
A. zaliosus (3)
100
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A. zaliosus (2)
A. zaliosus (1)
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A. zaliosus (4)
A. zaliosus (11)
A. zaliosus (5)
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A. supercilius (116)
A. supercilius (120)
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A. supercilius (119)
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A. supercilius (123)
A. supercilius (121)
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A. flaveolus (89)
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A. supercilius (110)
A. supercilius (113)
96
A. supercilius (114)
A. supercilius (108)
A. supercilius (109)
98
A. supercilius (111)
78
A. sp. X (82)
A. supercilius (112)
A. sp. X (87)
67
A. flaveolus (101)
A. sp. X (84)
62
A. flaveolus (94)
88
A. flaveolus (93)
A. flaveolus (92)
A. flaveolus (103)
100
A. flaveolus (91)
A. flaveolus (90)
86
A. sp. 'elongated' L. Nicaragua
A. citrinellus L. Nicaragua
91
A. sp. 'Rio San Juan'
A. sp. 'Rio San Juan'
A. lyonsi

898

Fig. 2

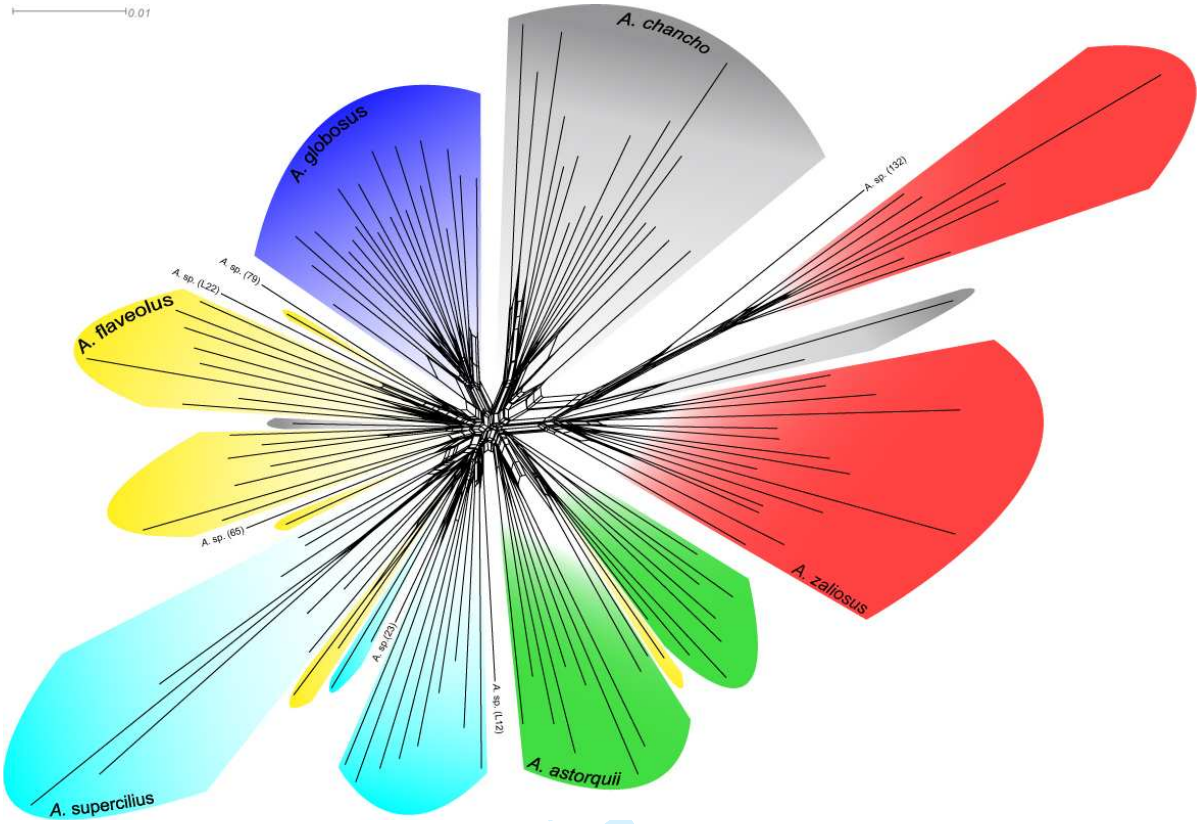

0.01
A. chancho
A. globosus
A. sp. (132)
A. sp. (79)
A. sp. (L22)
A. flaveolus
A. sp. (65)
A. zaliosus
A. sp.(23)
A. sp. (L12)
A. astorquii
A. supercilius

Fig. 3

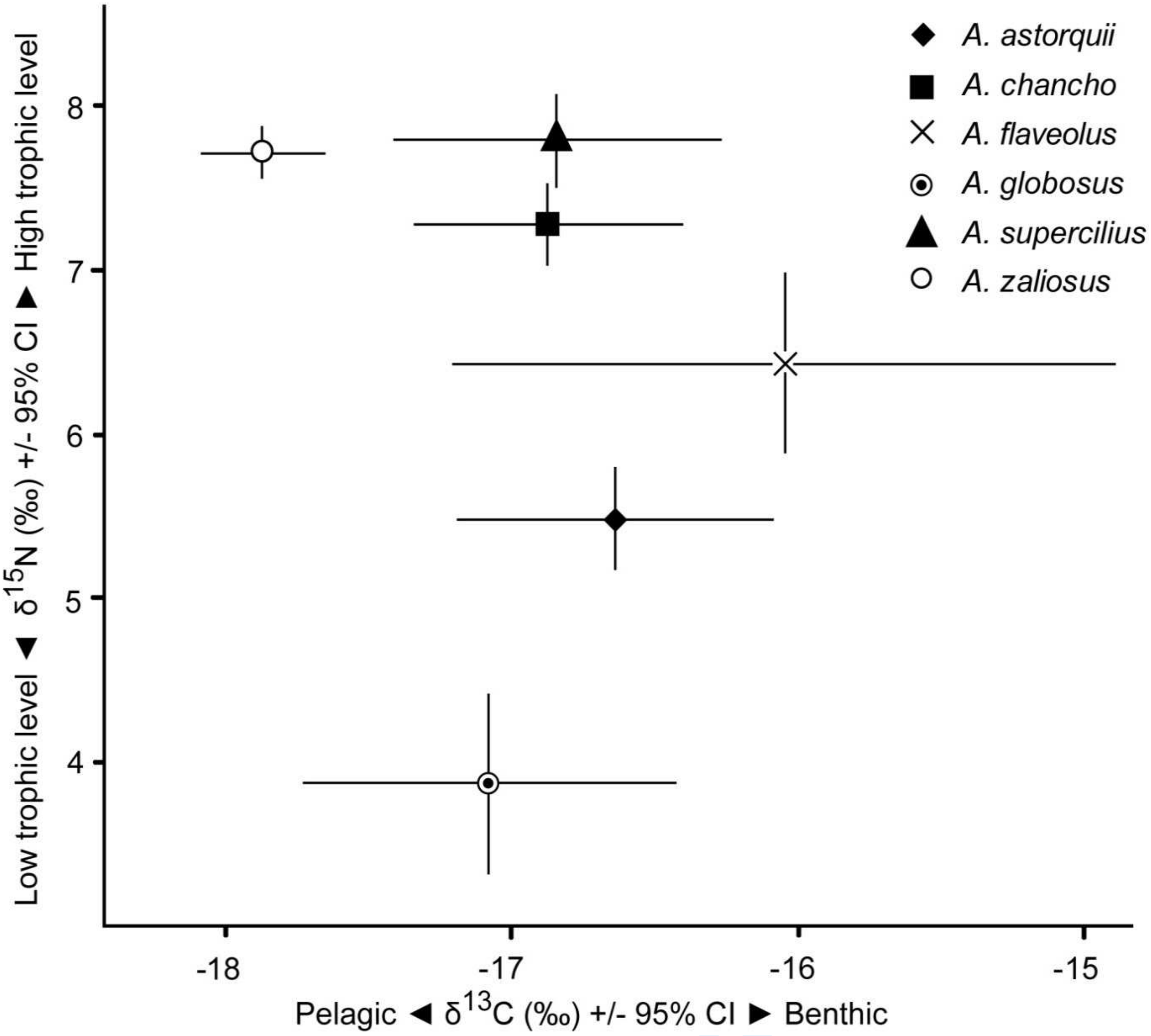

A. astorquii
A. chancho
A. flaveolus
A. globosus
A. supercilius
A. zaliosus
Low trophic level ◄ δ15N (‰) +/- 95% CI ► High trophic level
8
7
6
5
4
-18
-17
-16
-15
Pelagic ◄ δ13C (‰) +/- 95% CI ► Benthic

Fig. 4

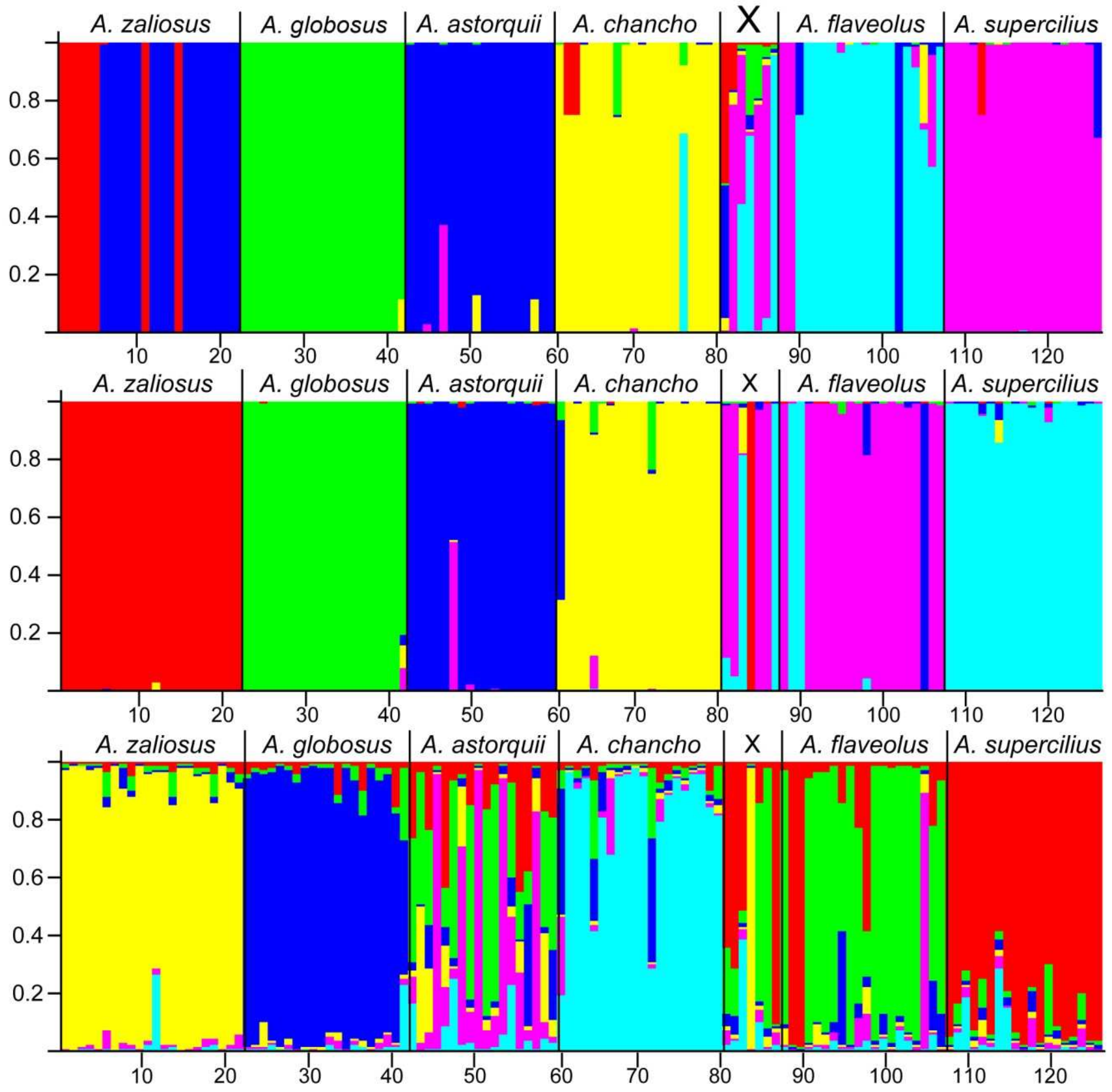
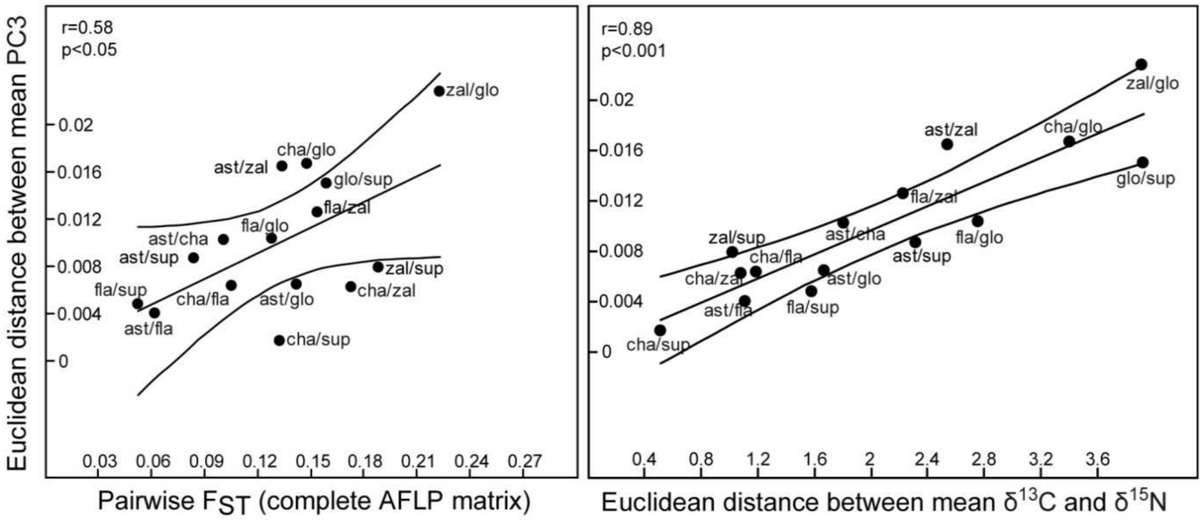

A. zaliosus
A. globosus
A. astorquii
A. chancho
X
A. flaveolus
A. supercilius
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
80
90
100
110
120

Fig. 5


r=0.58
p<0.05
Euclidean distance between mean PC3
0.02
0.016
0.012
0.008
0.004
0
zal/glo
cha/glo
ast/zal
glo/sup
fla/zal
ast/cha
fla/glo
ast/sup
zal/sup
fla/sup
cha/fla
ast/glo
cha/zal
ast/fla
cha/sup
0.03
0.06
0.09
0.12
0.15
0.18
0.21
0.24
0.27
Pairwise $F_{ST}$ (complete AFLP matrix)
r=0.89
p<0.001
0.02
0.016
0.012
0.008
0.004
0
zal/glo
ast/zal
cha/glo
fla/zal
glo/sup
zal/sup
ast/cha
fla/glo
cha/fla
ast/sup
cha/zal
ast/glo
ast/fla
fla/sup
cha/sup
0.4
0.8
1.2
1.6
2
2.4
2.8
3.2
3.6
Euclidean distance between mean $\delta^{13}C$ and $\delta^{15}N$

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## Data Accessibility

-DNA sequences: Genbank accessions GU355718- GU355726; GU355729- GU355733; GU355735; GU355737; GU355739- GU355742; GU355744- GU355748; GU355764; GU355770; GU355850; GU355851; GU362707- G362709; JF784052-JF784149.

-DNA aliquots: stored permanently at ZSM DNA-Bank, available upon request from dnabank@zsm.mwn.de