Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: sexual selection versus ecological diversification

Anthony B. Wilson[1], **Katharina Noack-Kunnmann**[2] **and Axel Meyer**[1*]

[1]*Department of Biology, University of Konstanz, 78457 Konstanz, Germany*
[2]*Department of Vascular Biology, Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*

The growing body of empirical evidence for sympatric speciation has been complemented by recent theoretical treatments that have identified evolutionary conditions conducive to speciation in sympatry. The Neotropical Midas cichlid (*Amphilophus citrinellum*) fits both of the key characteristics of these models, with strong assortative mating on the basis of a colour polymorphism coupled with trophic and ecological differentiation derived from a polymorphism in their pharyngeal jaws. We used microsatellite markers and a 480 bp fragment of the mitochondrial DNA control region to study four polymorphic populations of the Midas cichlid from three crater lakes and one large lake in Nicaragua in an investigation of incipient sympatric speciation. All populations were strongly genetically differentiated on the basis of geography. We identified strong genetic separation based on colour polymorphism for populations from Lake Nicaragua and one crater lake (Lake Apoyo), but failed to find significant genetic structuring based on trophic differences and ecological niche separation in any of the four populations studied. These data support the idea that sexual selection through assortative mating contributes more strongly or earlier during speciation in sympatry than ecological separation in these cichlids. The long-term persistence of divergent cichlid ecotypes (as measured by the percentage sequence divergence between populations) in Central American crater lakes, despite a lack of fixed genetic differentiation, differs strikingly from the patterns of extremely rapid speciation in the cichlids in Africa, including its crater lakes. It is unclear whether extrinsic environmental factors or intrinsic biological differences, e.g. in the degree of phenotypic plasticity, promote different mechanisms and thereby rates of speciation of cichlid fishes from the Old and New Worlds.

Keywords: sympatric speciation; assortative mating; Midas cichlid; *Amphilophus citrinellum*;
mitochondrial DNA; microsatellites

1. INTRODUCTION

A great deal of morphological and behavioural variation exists in natural populations of most species, yet empirical evidence for the role of this variation in establishing genetic differentiation and, ultimately, speciation remains limited (reviewed in Lynch 1989; Coyne 1992; Ricklefs & Schluter 1993). It is clear that allopatric speciation through vicariant events and colonizations has played a key role in the diversification of terrestrial (Bleiweiss 1998) and aquatic animals (Brooks 1950) as well as plants (Ricklefs & Renner 1994). However, even in the absence of geographical barriers, it is thought that variation can lead to partial or complete reproductive isolation between sympatric populations through a reduction in gene flow (Bush 1994).

The challenges inherent in identifying instances of sympatric speciation have contributed to ongoing debates concerning its importance (Mayr 1988; Bush 1994). Research into morphological diversification and speciation in aquatic systems (e.g. McKaye 1980; Meyer 1990*a*,*b*, 1993*a*; Schliewen *et al*. 1994; Pigeon *et al*. 1997; Nagel & Schluter 1998) and islands (see the papers in Grant (1998)) has highlighted the potential for the establishment of reproductive isolation and possible speciation in sympatry. In addition to these empirical studies, a

series of recent theoretical treatments has attempted to identify evolutionary conditions that promote sympatric speciation (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). Although the methodologies underlying these studies differ in several important respects, the models are consistent with classical studies (Maynard Smith 1966) in identifying two key characteristics that are conducive to sympatric speciation. Variation in an ecological trait (such as differences in resource use) is necessary for disruptive selection against intermediates to take place (Losos 2000). Assortative mating drives this disruptive selection and leads the divergent subpopulations along separate evolutionary trajectories, resulting in speciation in as few as 300 generations (Dieckmann & Doebeli 1999).

The adaptive radiations of East African cichlid fishes are well-known for their explosive rates of speciation and spectacular diversity (reviewed in Meyer 1993*a*; Stiassny & Meyer 1999). The cichlid species flock of Lake Victoria is possibly as young as 12 400 years old, suggesting that these species not only formed extremely rapidly, but also within one continuous body of water (Meyer *et al*. 1990; Johnson *et al*. 1996). Recent evidence suggests that both niche diversification (Schliewen *et al*. 1994) and sexual selection (Seehausen *et al*. 1999) have played significant roles in the speciation of cichlid fishes (Meyer *et al*. 1990; reviewed in Meyer 1993*a*). Unfortunately, determining underlying patterns of diversification in cichlids is
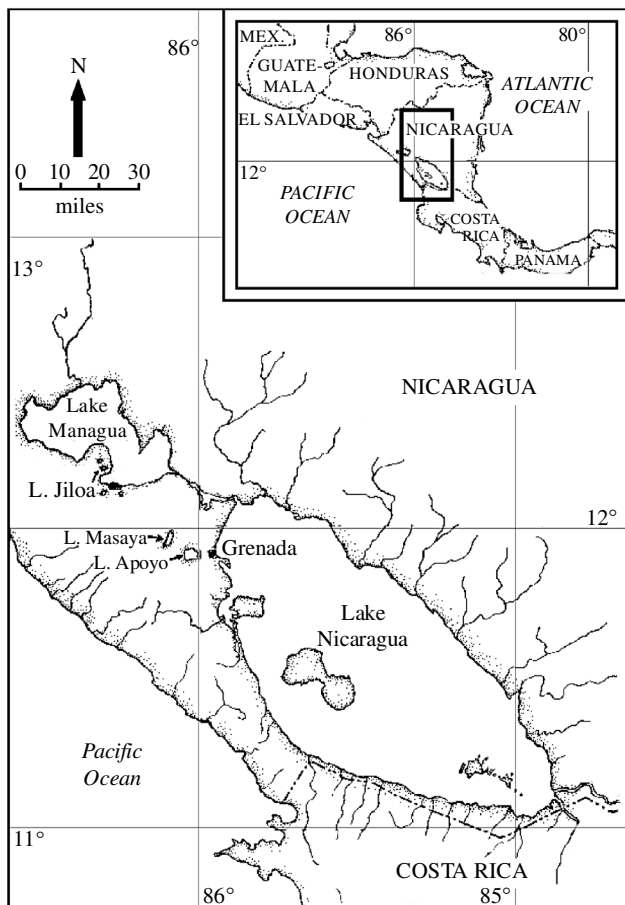
Figure 1. Map of Nicaragua showing the location of the lakes sampled (from fig. 1 in Barlow (1976), reproduced with permission).

Figure 2. Comparative morphology of *A. citrinellum* (adapted from Meyer 1990*b*). (*a*) Variation in pharyngeal jaw morphology (left) and colour morphs (right). (*b*) Association of jaw morphology with colour in *A. citrinellum* from Lake Nicaragua (prepared from table 2 in Meyer (1990*b*)).

complicated by the meagre fossil record, which confounds the elucidation of the historical distribution patterns and dynamics of species formation. Even when historical distributions are known, documenting the genetic effects of historical barriers to gene flow in initiating the speciation process is rarely possible (but see Sturmbauer & Meyer 1992; Rüber *et al.* 1999).

While cichlids from African lakes are highly specialized (Fryer & Iles 1969; Witte 1984), Neotropical cichlids are characteristically generalists with respect to habitat and diet (Kornfield *et al.* 1982; Meyer 1987, 1990*b*), possibly reflecting the relative stability of their Old and New World habitats (Barlow 1976). Long-term field and laboratory studies of the highly polymorphic Neotropical cichlid *Amphilophus citrinellum* (Midas cichlid) (Barlow 1976, 1998; Barlow & Munsey 1976; Barlow & Rogers 1978; Meyer 1989, 1990*a,b*) have yielded extensive insights into its ecology and behaviour, making it an excellent model species for the study of non-geographical isolating factors in tropical lakes. This species lives in the large lakes of Nicaragua (Lakes Managua and Nicaragua) as well as in several small crater lakes (Lakes Masaya, Jiloa and Apoyo) (figure 1).

All *A. citrinellum* individuals start out as normal, protectively coloured, mostly vertically black-and-white striped, young. During their ontogeny, some of the normal morphs, irrespective of sex, lose their melanophores and become gold coloured (figure 2). The age at which the
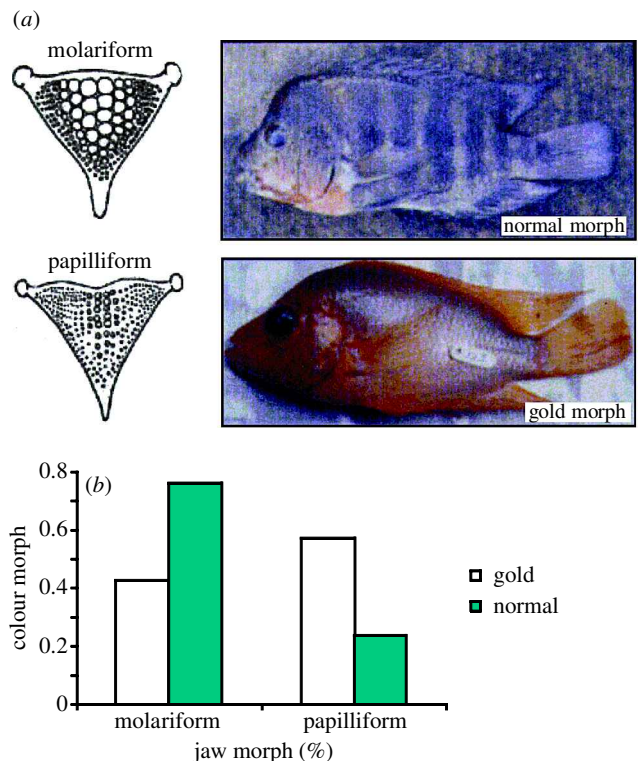
gold morphs undergo this metamorphosis is variable and, in some individuals, it may not occur until after they have reached sexual maturity (Barlow 1976). It has been demonstrated, in both the field (McKaye 1980, 1986) and laboratory (Barlow *et al.* 1977; Barlow & Rogers 1978), that this species shows a strong tendency to mate assortatively with respect to coloration. This assortative mating may be enhanced by the fact that, while the territories of gold morph individuals dominate the benthic zone (where > 50% are gold) (McKaye 1980), normal morphs tend to exclude gold morphs from the limnetic zone during the breeding season (> 90% normal) (McKaye 1980), a pattern of segregation that is commonly observed between divergent ecotypes of fishes such as limnetic and benthic morphs of sticklebacks, sunfish and salmonids (reviewed in Smith & Skulason 1996).

The functional decoupling of the upper and lower pharyngeal jaw in cichlid fishes led to a fundamental shift in function from food transport to food manipulation and preparation and is believed to be partly responsible for their subsequent explosive diversification (Liem 1973). Distinct pharyngeal jaw polymorphisms have been identified in several species of cichlids from both the Old and New Worlds (Greenwood 1965; Kornfield *et al.* 1982; Meyer 1990*a*, 1993*b*) and two distinct pharyngeal jaw morphologies have been found in *A. citrinellum* (Meyer 1990*a*). Papilliform jaw morphs in *A. citrinellum* are characterized by slender, pointed teeth, whereas in molariform morphs the pharyngeal jaws are heavier and the teeth are stouter and sturdier (figure 2) (Meyer 1990*b*). While all fishes are initially papilliform, the frequency of molariform adult Midas cichlids found in

Table 1. *Physical characteristics of the four study lakes and distribution of previously reported colour morphs (Barlow 1976) and pharyngeal jaw morphs (Meyer 1990b) of* A. citrinellum

(Snail abundance (Meyer 1990b) is also included for comparison with the distribution of pharyngeal jaw morphs and is given as the number of snails per 15 cm × 15 cm quadrat.)

	Lake Nicaragua		Lake Apoyo	Lake Masaya	Lake Jiloa
physical characteristics (Barlow 1976)					
surface area (km^2)	7740		21.2	8.4	3.8
maximum depth (m)	50		> 92	82	92
water transparency (Secchi disc) (m)	0.25–0.35		3.5–9.5	3–5	0.23–1.0
colour morphology (Barlow 1976)					
normal (%)	90.7		92.5	91.9	≅ 90
gold (%)	9.3		7.6	8.1	≅ 10
n	1565		79	99	—
jaw morphology (Meyer 1990b)	shore	islands			
molariform (%)	67	7	17	0	50
papilliform (%)	29	93	70	100	49
intermediate (%)	4	0	13	0	1
n	155	40	53	68	136
snail abundance (Meyer 1990b)	n/a	0	75.5	0	111.5

wild populations is highly correlated with the abundance of hard-shelled prey (table 1) (Meyer 1990b). Although molariform fishes are able to crack larger and harder snails than papilliform morphs, their handling time for soft prey is significantly longer (Meyer 1989). This ecological trade-off may help to maintain divergent ecotypes in natural populations and explains their highly different resource usage in nature (Meyer 1990a).

The formation of discrete, adaptive fitness peaks has been shown to be a key intermediate step during the process of sympatric speciation (Doebeli 1996; Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). Both assortative mating (Kondrashov & Mina 1986) and resource polymorphisms (Meyer 1993b; Smith & Skulason 1996) have been implicated as potentially significant factors in sympatric speciation. *A. citrinellum* exhibits both assortative mating on the basis of colour and a trophic polymorphism that is correlated with prey availability. Moreover, these two polymorphisms may be coupled, as a study by Meyer (1990b) identified that, within a single population, 76% of all normal morphs have molariform pharyngeal teeth and 57% of all gold morphs have papilliform jaws (see figure 2). We therefore hypothesized that, if assortative mating is strong and trophic polymorphisms generally enhance reproductive isolation through ecological differentiation, this should result in decreased gene flow, possibly leading to speciation in sympatry.

In an effort to measure and describe population structuring that might be congruent with possible sympatric speciation in lacustrine populations of the Midas cichlid, we sequenced a 480 bp fragment of the most variable segment of mitochondrial DNA (mtDNA), i.e. the control region, and conducted a microsatellite analysis based on four hypervariable microsatellite markers. Previous analyses of these fishes have only identified a single cytochrome *b* mtDNA haplotype in populations from several Central American lakes (A. Meyer, C. H. Biermann and S. Pålsson, unpublished data). The higher evolutionary rate of the mtDNA control region makes it particularly

sensitive to subtle changes in population structuring (Avise *et al.* 1987). Microsatellites are generally also highly variable and have proven useful in previous studies of cichlid populations (e.g. Van Oppen *et al.* 1998; Agnese *et al.* 1999). Our combined microsatellite and mtDNA sequence data analyses were used in an effort to clarify the relative roles of geographical and ecological factors in promoting reproductive isolation within and between four lacustrine populations of *A. citrinellum* in Nicaragua.

2. MATERIAL AND METHODS

(a) *Sample collection and analysis of microsatellite loci and the mtDNA control region*

Fresh specimens of *A. citrinellum* (figure 2) were collected in 1987 from Lake Nicaragua and from three crater lakes, Lakes Apoyo, Jiloa and Masaya (figure 1), which vary in their surface area, depth and water transparency (table 1). Lake Nicaragua samples were purchased from two fish markets in Granada, while samples from the crater lake populations were gill netted from the shoreline of the lakes. Tissues of heart, liver and muscle were stored frozen at −80 °C prior to DNA extraction, which was performed following a previously published extraction protocol (Kocher *et al.* 1989). Identification and characterization of the four microsatellite loci (*Acit* 1–4) was performed as outlined in Noack *et al.* (2000). A 480 bp portion of the mtDNA control region was amplified with primers L15995 (Meyer *et al.* 1994) and H00651 (Kocher *et al.* 1989) under previously published reaction conditions (Kocher *et al.* 1989). Approximately 0.2 µg of Qiagen (Valencia, CA, USA) column-purified product from this polymerase chain reaction (PCR) was cycle sequenced with 5 pmol of primer L15926 and 2 µl Terminator Ready Reaction Mix using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The cycling profile for the sequencing reaction consisted of 25 cycles at 96 °C for 10 s, 40 °C for 5 s and 60 °C for 4 min. Ethanol/sodium acetate-purified cycle sequencing products were analysed on an ABI 377 Automated Sequencer (Applied Biosystems).

Table 2. F_{ST} and R_{ST} estimates over all loci and significance values for all pairwise combinations of lake populations, comparison of colour morphs within lakes and comparison of trophic morphs within lakes

(The microsatellite sample sizes are given in parentheses. Probability values: $^*p < 0.05$, $^{**}p < 0.001$. The F_{ST} estimates were calculated according to Weir & Cockerham (1984) and the R_{ST}-values were calculated following Slatkin (1995) as implemented by ARLEQUIN v. 2.0 (Schneider *et al.* 2000). The significance estimates are based on 10 000 permutations of the data set following sequential Bonferroni correction. The p-value of the test is the proportion of permutations leading to an estimate equal to or larger than that observed (Schneider *et al.* 2000). Kimura two-parameter distance estimates were used to calculate mtDNA F_{ST}-values. See figure 4 for mtDNA population sizes.)

	F_{ST}	R_{ST}	mtDNA F_{ST}
between lakes			
Lake Nicaragua (51)–Lake Jiloa (51)	0.065^{**}	0.051^*	0.141^{**}
Lake Nicaragua (51)–Lake Masaya (15)	0.048^{**}	0.001	0.129^*
Lake Nicaragua (51)–Lake Apoyo (24)	0.183^{**}	0.203^{**}	0.223^{**}
Lake Jiloa (51)–Lake Masaya (15)	0.092^{**}	0.139^*	0.015
Lake Jiloa (51)–Lake Apoyo (24)	0.306^{**}	0.413^{**}	0.320^{**}
Lake Masaya (15)–Lake Apoyo (24)	0.181^{**}	0.184^{**}	0.344^{**}
within lakes separated by colour			
Lake Nicaragua golds (25)–Lake Nicaragua normals (16)	0.033^*	0.065^*	0.315^{**}
Lake Apoyo golds (4)–Lake Apoyo normals (20)	0.003	0.003	0.199^*
Lake Masaya golds (5)–Lake Masaya normals (10)	-0.026	-0.058	0.000
within lakes separated by jaw morphology			
Lake Nicaragua papilliform (19)–Lake Nicaragua molariform (31)	-0.002	0.032	-0.086
Lake Jiloa papilliform (33)–Lake Jiloa molariform (5)	0.015	-0.030	0.067

(b) *Statistical analyses*

(i) *Microsatellites*

In order to ensure an independent assortment of microsatellite markers, exact tests of linkage disequilibrium between microsatellite loci were performed using GENEPOP v. 3.1d (Raymond & Rousset 1995a). The significance of these tests was estimated using a permutation procedure (Raymond & Rousset 1995a). Genetic distances between populations and morphs were measured by calculating both F_{ST} (Weir & Cockerham 1984) and R_{ST} (Slatkin 1995) as implemented by ARLEQUIN v. 2.0 (Schneider *et al.* 2000). The significance of these estimates was tested under the null hypothesis (H_0) of no difference between populations by permuting genotypes between populations (10 000 iterations).

Exact tests of both global and pairwise tests of genotypic equilibrium were performed using GENEPOP v. 3.1d (Raymond & Rousset 1995a) in an effort to clarify the patterns of inter- and intrapopulation differentiation independently. An unbiased estimate of the significance of these tests was calculated through a 10 000 step, 1000 iteration, Markov chain series of permutations (10 000 dememorization steps) of a contingency table of allelic distribution for each population. The significance of the p-values across the six loci was determined using Fisher's probability combination test (Raymond & Rousset 1995b).

Finally, in order to test for deviations from random mating, observed heterozygosities within populations were tested for departure from Hardy–Weinberg expectations using GENEPOP v. 3.1d (Raymond & Rousset 1995a). A 10 000 step, 1000 iteration, Markov chain method (10 000 dememorization steps) was used in calculating an unbiased estimate of the p-value.

(ii) *Control region*

DNA sequences were aligned by eye and a minimum-spanning haplotype network was constructed following a star decomposition search using the parsimony method as implemented by PAUP* v. 4.b3a (Swofford 1998). Geographical and morphological population subdivision was measured using F_{ST} estimates as calculated by ARLEQUIN v. 2.0 (Schneider *et al.* 2000) from a Kimura two-parameter distance matrix based on sequence data. The significance of these estimates was determined by a 10 000 step, 1000 iteration, Markov chain method (10 000 dememorization steps).

The relative significance of within- and between-lake variation in colour morphs was clarified with an analysis of molecular variation (AMOVA). Kimura two-parameter distance estimates were calculated from sequence data and an AMOVA was conducted with ARLEQUIN v. 2.0 (Schneider *et al.* 2000).

3. RESULTS

(a) *Microsatellite data*

(i) *Descriptive statistics and linkage disequilibrium*

The four microsatellite loci detected consistently high levels of intraspecific variation (between four and 26 alleles) for the four populations examined ($n = 141$) with the intralocus heterozygosity averaging 0.488 (± 0.260) (Noack *et al.* 2000). Although exact tests for genotypic linkage disequilibrium between microsatellite loci within populations indicated a single significant p-value (χ^2-test, $p < 0.05$) for 24 pairs of loci tested (4.17%) (data not shown), this value was rendered insignificant following Bonferroni correction. Global tests of linkage disequilibrium calculated from within-population data were not significant at the 5% level (χ^2-test), indicating that all loci segregate independently.

(ii) *Differentiation between lake populations*

Global exact tests of genotypic differentiation indicated significant heterogeneity in gene frequencies among the four lacustrine populations ($p < 0.0001$). In an effort to
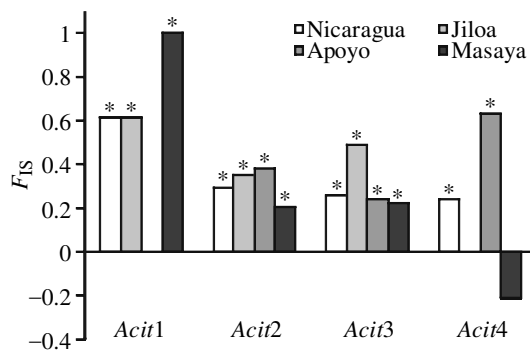
Figure 3. Hardy–Weinberg exact test for each locus in each population calculated by GENEPOP v. 3.1d (Raymond and Rousset 1995*a*). An asterisk indicates significant heterozygote deficiency ($p < 0.05$). All four populations indicated significant deviations from Hardy–Weinberg equilibrium ($p < 0.001$). Note that Lake Apoyo is fixed for a single allele at *Acit* 1.

partition these data further, pairwise estimates of genotypic differentiation were calculated. All six pairwise combinations of lake populations also showed highly significant differences ($p < 0.001$).

Differences in allele frequency distribution translated into highly significant fixation indices among the four lake populations investigated. In order to assess overall genetic differences between pairs of populations, we calculated both F_{ST} and R_{ST} so as to take into account uncertainty over the mode of mutation in microsatellites (Slatkin 1995). When all loci were combined, overall estimates of genetic differentiation between the four populations were highly significant for both F_{ST} ($p < 0.0001$) and R_{ST} ($p < 0.0001$). Pairwise population comparisons indicated significant differentiation between all population pairs except for a single R_{ST} estimate calculated between Lake Nicaragua and Lake Masaya (table 2). Overall, these results indicate that highly significant genetic differentiation exists between the four lacustrine populations of the Midas cichlid.

(iii) *Departures from Hardy–Weinberg proportions*

Comparison of expected heterozygosities with observed values indicated a substantial heterozygote deficit in all four populations (figure 3). Out of a total of 14 population-locus comparisons, 13 showed highly significant departures from Hardy–Weinberg proportions ($p < 0.0001$). When pooling across all four loci, a significant heterozygote deficit was found for each population ($p < 0.0001$), indicating that observed deviations from Hardy–Weinberg equilibrium were due to consistent heterozygote deficits at all loci and not solely the result of deviations at a single locus. Taken together, these results indicate non-random associations of allele frequencies within each lake population, which may be explained by non-random mating in these populations (see § 4).

(iv) *Differentiation between pharyngeal morphotypes and colour morphs*

In order to examine the genetic structure within each lake population, we tested, as far as the abundance of morphs (see table 1) and our sampling allowed, for genetic differentiation between colour morphs and pharyngeal jaw morphs within each lake.

Comparison of the two colour morphs was possible for the Lake Masaya, Lake Apoyo and Lake Nicaragua populations. Comparison of colour morphs in the two crater lakes failed to identify significant genetic differentiation on the basis of microsatellite data, but the mitochondrial data found significant support for genetic separation based on colour for the Lake Apoyo population (table 2). We had a larger sample size for both of the colour morphs (25 golds versus 16 normals) for Lake Nicaragua. Consistent differences between the two colour subpopulations in mtDNA haplotype frequency distributions ($p < 0.05$) and overall F_{ST} (0.033) and R_{ST} (0.065) estimates ($p < 0.05$) supported the separation of the Lake Nicaragua *A. citrinellum* population on the basis of their colour polymorphism.

Tests for differentiation on the basis of jaw morphology were possible for the populations from Lakes Nicaragua and Jiloa. Estimates of F_{ST}, R_{ST} and genotypic differentiation were non-significant for both comparisons (table 2), indicating a lack of genetic differentiation in these populations on the basis of jaw morphology.

(b) *mtDNA control region data*

In order to further explore genetic differentiation, we sequenced a 480 bp fragment of the mtDNA control region from a random subset ($n = 76$) of cichlid individuals, thereby identifying a total of 36 haplotypes (figure 4). While the dominant mtDNA haplotype was found in individuals from all four lakes, clustering of secondary haplotypes largely reflected geographical structuring of populations (figure 4), suggesting independent post-colonization molecular diversification of this mtDNA region.

Quantitative estimates of population structuring based on the mtDNA sequences supported this qualitative interpretation of the data (table 2). F_{ST} estimates based on mtDNA indicated strong partitioning of populations on the basis of geography. In addition, colour morphs within the Lake Apoyo and Lake Nicaragua populations were also significantly differentiated (table 2). In agreement with the microsatellite data, mtDNA provided no statistical support for genetic differentiation on the basis of trophic morphology in any study lake (table 2).

Further investigation of within- and between-lake variation in colour morphs was conducted via a hierarchical AMOVA (table 3). As suggested by the haplotype network, a large proportion of the genetic diversity (18.4%) was found between lakes, while the within-lake variation in colour morphs was responsible for only 2.4% of the total genetic variation (table 3). These results illustrate that the diversification of colour morphs is relatively recent in comparison to lake colonization, implying independent and repeated radiations of colour morphs in each of the lakes.

Overall, our results demonstrate strong population subdivision between the lakes due to geographic isolation. In addition to the between-lake variation, the differentiation between colour morphs in Lakes Apoyo and Nicaragua suggests that assortative mating of cichlids within lakes may also be driving diversification and possibly speciation. The significant positive F_{IS}-values found for each subpopulation (figure 3) suggest further population subdivision, possibly due to non-random
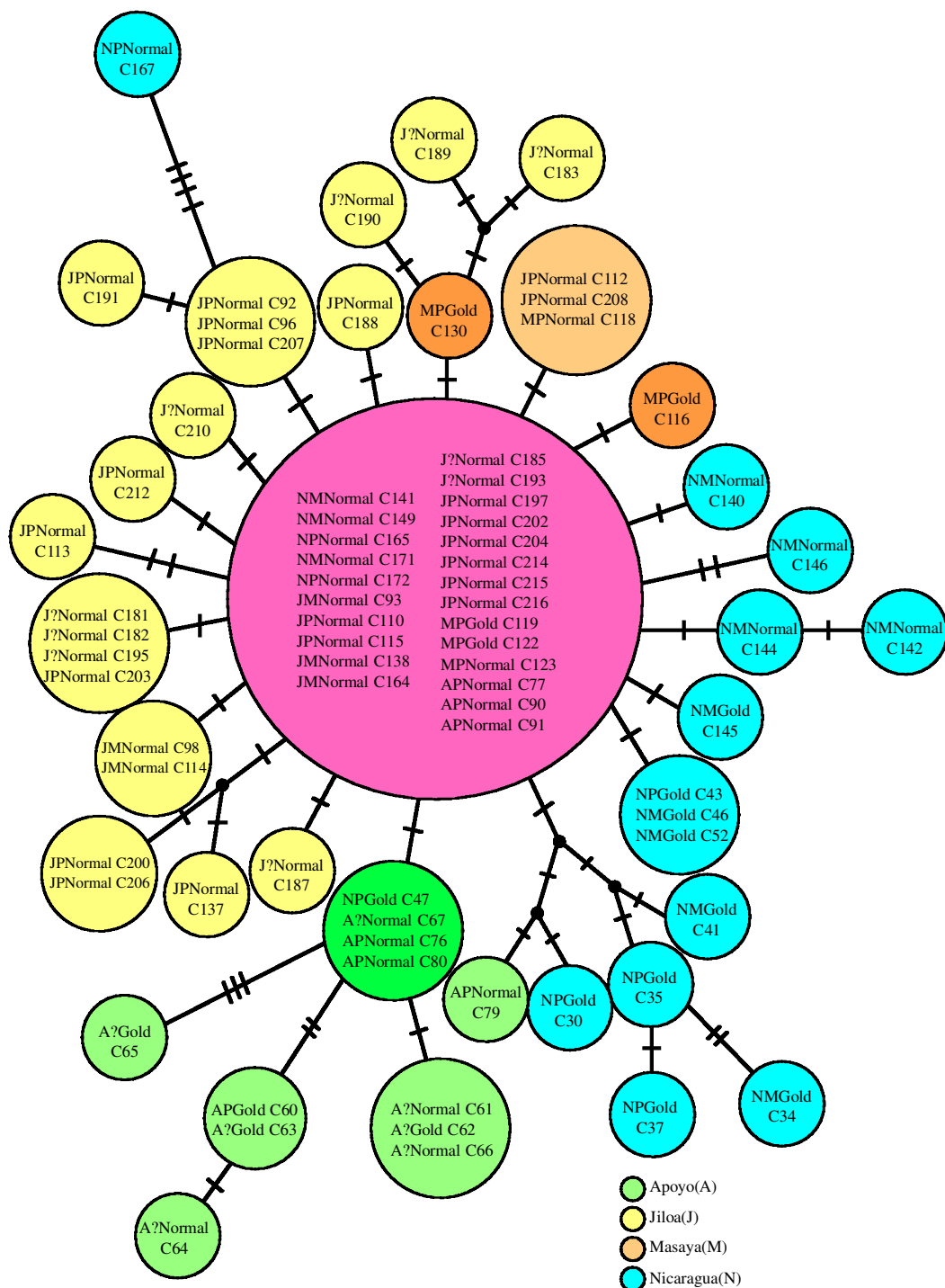
Figure 4. Haplotype network constructed from mtDNA control region haplotype data. The colours of the haplotype groupings reflect geographic affinities. Jaw morphology: P, papilliform jaw morphology; M, molariform jaw morphology; ?, jaw morphology unknown. Colour morphology: Gold, gold morph; Normal, normal morph. The haplotype network was generated following a star decomposition search based on the parsimony algorithm as implemented by PAUP* v. 4.0b4a (Swofford 1998).

mating in natural populations based more strongly on colour differences than on pharyngeal jaw differences.

4. DISCUSSION

Our microsatellite markers and mtDNA sequence data reveal consistently high genetic differentiation between *A. citrinellum* populations from different lakes. This finding is consistent with topographic data indicating that the three crater lakes sampled are highly isolated and are not connected by any current river systems (Barlow 1976). This genetic differentiation also reflects the high degree of morphological variation between *A. citrinellum* populations from different lakes where phenotypic differences have been documented (table 1) (Barlow 1976; Meyer 1990*b*). There was much higher variation in the coloration of gold morphs of Midas cichlids (ranging from white to orange) from more turbid lakes (e.g. Lake Nicaragua) than that found in lakes with clear water (such as from Lake Apoyo) (Barlow 1976), which is possibly indicative

Table 3. *AMOVA hierarchical genetic analysis of populations of colour morphs based on mtDNA control region sequence data*

(The Kimura two-parameter distance estimates were calculated from sequence data as implemented by ARLEQUIN v. 2.0 (Schneider *et al.* 2000).)

source of variation	d.f.	variance	% total
between colour morphs	1	0.0247	2.42
among populations of similar colour morphs	4	0.1877	18.39
within populations	36	0.8081	79.18

Group 1 (normal), Lakes Apoyo, Nicaragua and Masaya
Group 2 (gold), Lakes Apoyo, Nicaragua and Masaya

of an influence of turbidity on the evolution of coloration and sexual selection, as has been suggested for Lake Victoria cichlids from Africa (Seehausen *et al.* 1997). While the variation in gold morphs was much larger in Lake Nicaragua than in Lake Apoyo, the absolute proportion of gold morphs in all four study lakes was remarkably similar (table 1), suggesting that frequency-dependent selection may be contributing to colour metamorphosis. The fundamental differences in water transparency, surface area and depth profiles (table 1) in these lakes offer further opportunities for testing the impact of visibility on sexual selection in cichlid species.

All four *A. citrinellum* populations were found to deviate significantly from Hardy–Weinberg expectations, with substantial heterozygote deficits. These results suggest that some form of within-lake reproductive isolation may be playing a role in the mating patterns of Midas cichlids in Nicaraguan lakes. While our data suggest that assortative mating on the basis of colour may be playing a role in the divergence of populations within Lakes Nicaragua and Apoyo, Schliewen *et al.* (1994) found a different pattern in a species flock of cichlids in Cameroon where trophic specialization has apparently played a key role in diversification in sympatry. This contrast in patterns of diversification in Old and New World crater lake cichlids highlights the complexity of factors promoting sympatric speciation in nature.

The two colour morphs of this species show a strong tendency to mate assortatively with respect to colour (Barlow & Munsey 1976; Barlow *et al.* 1977; McKaye 1980) and, within Lake Jiloa, have also been observed breeding at different depths (McKaye 1980). Our molecular data support these ecological findings, demonstrating significant population structuring of Midas cichlid populations on the basis of coloration (table 2) in both Lake Nicaragua and Lake Apoyo. While Lake Nicaragua had a greater diversity of colour morphs of *A. citrinellum* (see above), it appeared to hold similar levels of genetic diversity of Midas cichlids as those found in Lake Apoyo (table 2), suggesting that, although intrinsic factors may be responsible for the proportion of fishes that undergo metamorphosis (see above), environmental factors (possibly variation in the carotenoid content of the diet) (Witte *et al.* 1997) may contribute to subtle variation in gold morphs. In contrast to Lakes Nicaragua and Apoyo, colour morphs were not strongly differentiated in the smaller Lake Masaya, perhaps suggestive of a

role of lake size (see table 1) on assortative mating in *A. citrinellum*. As the presence of genetic structuring in colour morphs in Lakes Nicaragua and Apoyo may be significant, further study of fishes collected in 1999 from a diverse size range of habitats may help to reveal the significance of microtopographic factors on the mating patterns of Midas cichlids (T. Hrbek, A. B. Wilson and A. Meyer, unpublished data).

The Midas cichlid also exhibits trophic polymorphism of its pharyngeal jaws and the two trophic morphs have been shown to be ecologically separated (Meyer 1989, 1990*a*,*b*). Meyer (1990*b*) argued that, if mate choice and pair formation took place in each trophic morphs' respective habitat, one might expect to find genetic differentiation between the two trophic morphs. However, our data revealed no significant genetic variation between trophic morphs within a subpopulation (table 2). Furthermore, while a previous study of colour and pharyngeal jaw morphs (Meyer 1990*b*) has suggested that the colour morph and pharyngeal jaw morphology covary, the present study provides no genetic evidence to support this hypothesis. While our data fail to support the hypotheses of reproductive isolation on the basis of trophic polymorphisms and their ecological consequences, the significant heterozygote deficiencies in all four lakes suggest that non-random mating may be having a significant impact on the population genetic structure of *A. citrinellum* in Nicaraguan lakes, thereby potentially promoting species-level divergence through sympatric speciation where assortative mating based on colour differences (see above) might lead to reinforcement of ecological variation.

Barlow (1998) presented an argument for explaining why the Midas cichlid has failed to speciate, even in the presence of presumably powerful reproductive isolating mechanisms such as strong assortative mating. A detailed laboratory study of the timing of melanophore loss and consequent colour metamorphosis revealed that, while the majority of juveniles change colour prior to reproductive age (18 months), some adults may not metamorphose until they are two or more years old. Therefore, more slowly metamorphosing, gold individuals could possibly mate with genetically normal individuals for at least one to two reproductive seasons before they breed 'true' to their colour. Even if these aberrant individuals are rare, Barlow (1998) argued that the potential impact of individuals mating with normal morphs first and then with golds later in their life span could be enough to break down genetic structuring based on coloration, even in the presence of generally strong assortative mating in the field, where more than 90% of all pairs are of the same coloration (McKaye 1986).

The high degree of genetic differentiation (0.25–0.60% mtDNA control region sequence divergence within populations) in populations of *A. citrinellum* suggests that, in addition to observed morphological variation within each lake, extensive genetic variation has been achieved within each of the four lacustrine populations since their colonization and has apparently not resulted in speciation. The genetic diversity of *A. citrinellum* is remarkably similar to that found in the cichlids of the Cameroon crater lakes (Schliewen *et al.* 1994), where comparable levels of sequence divergence in the mtDNA control

region (0.59% in Lake Bermin) and population structuring (as quantified by F_{ST}) delineate what are considered to be morphologically well-separated cichlid species flocks (Schliewen *et al.* 1994).

There are several alternative explanations for these contrasting patterns of speciation, one being differences in rates of molecular evolution between Old and New World cichlids (Farias *et al.* 1999). Farias *et al.* (1999) documented higher rates of molecular evolution in geophagine cichlids from South America than those found in lineages of African cichlids. They proposed that radically different climatic histories, in combination with biotic factors, might explain this rate acceleration. If *A. citrinellum* has experienced a similar acceleration in its molecular evolution, the ages of the Nicaraguan crater lake cichlid populations might actually be younger than those of the Cameroon crater lakes, even though their levels of mtDNA sequence divergence are quite comparable.

In addition to biological interpretations of this difference between Neotropical and African patterns of cichlid evolution, there remains a fundamental difference between the largely phylogenetically based studies of African cichlids and the more ecological approach that has characterized the study of Neotropical cichlids such as *A. citrinellum*. With the exception of a long-term ecological study of Lake Victoria cichlids by Witte and co-workers (e.g. Witte *et al.* 1997), many molecular studies of African cichlids (including Schliewen *et al.* 1994) have been conducted on groups for which little ecological data are available. Furthermore, the sample sizes in many of these molecular studies were generally limited to only a few representatives of each putative species (e.g. Meyer *et al.* 1990; Schliewen *et al.* 1994; but see Van Oppen *et al.* 1998), thereby possibly obscuring genetic and ecological variation bridging assumed species boundaries. As highlighted above, the levels of sequence divergence and F_{ST} estimates for *A. citrinellum* were comparable and, in some cases, exceeded those observed in cichlid species from the Cameroon crater lakes, where Schliewen *et al.* (1994) genetically characterized a monophyletic lineage of six endemic species of *Tilapia* in Lake Bermin on the basis of a single representative of each putative species. This significant discordance in experimental approach may be partially responsible for apparent differences in diversification in Old and New World cichlids.

While high levels of phenotypic plasticity and low rates of speciation appear to be common in Neotropical cichlids (Kornfield *et al.* 1982; Meyer 1987, 1990*b*), the reverse is generally assumed for African cichlids (Fryer & Iles 1969; Witte 1984; Meyer 1987; but see Hoogerhoud 1986; Witte *et al.* 1997). While this may also reflect differences in experimental emphasis in the study of African and Neotropical cichlids, the striking contrast in rates of molecular evolution (Farias *et al.* 1999) and apparent mechanisms of speciation may be partly explained by intrinsic differences that have accrued since the divergence of the two groups or, alternatively, reflect dissimilar environments encountered by Old and New World cichlids. The characterization of genetic factors (i.e. speciation genes) (Coyne 1992; Ting *et al.* 2000) possibly underlying observed phenotypic variation in cichlids in their Old and New World habitats will be an important next step in research efforts at bridging the gap between

genotype and phenotype and clarifying the relative significance of intrinsic and extrinsic factors on speciation in aquatic environments.

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