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Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon

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Abstract

Although there is mounting evidence that speciation can occur under sympatric conditions, unambiguous examples from nature are rare and it is almost always possible to propose alternative allopatric or parapatric scenarios. To identify an unequivocal case of sympatric speciation it is, therefore, necessary to analyse natural settings where recent monophyletic species flocks have evolved within a small and confined spatial range. We have studied such a case with a cichlid species flock that comprises five *Tilapia* forms endemic to a tiny lake (Lake Ejagham with a surface area of approximately 0.49 km^2) in Western Cameroon. Analysis of mitochondrial D-Loop sequences shows that the flock is very young (approximately 10^4 years) and has originated from an adjacent riverine founder population. We have focused our study on a particular pair of forms within the lake that currently appears to be in the process of speciation. This pair is characterized by an unique breeding colouration and specific morphological aspects, which can serve as synapomorphic characters to prove monophyly. It has differentiated into a large inshore and a small pelagic form, apparently as a response to differential utilization of food resources. Still, breeding and brood care occurs in overlapping areas, both in time and space. Analysis of nuclear gene flow on the basis of microsatellite polymorphisms shows a highly restricted gene flow between the forms, suggesting reproductive isolation between them. This reproductive isolation is apparently achieved by size assortative mating, although occasional mixed pairs can be observed. Our findings are congruent with recent theoretical models for sympatric speciation, which show that differential ecological adaptations in combination with assortative mating could easily lead to speciation in sympatry.

Keywords: adaptive radiation, assortative mating, speciation, sympatry, *Tilapia*

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Introduction

Molecular genetic analyses of natural species assemblages render it increasingly likely that speciation occurs under sympatric conditions (Schliewen *et al*. 1994; Bush & Smith 1998; Hellberg 1998). Furthermore, there are now theoretical models which show that speciation may occur in sympatry under a variety of realistic conditions (Kondrashov & Mina 1986; Rice 1987; Wilson 1989; Turner & Burrows 1995; Doebeli 1996; Johnson *et al*. 1996; Kondrashov & Shpak 1998; Dieckmann & Doebeli 1999; Higashi *et al*. 1999; Kondrashov & Kondrashov 1999). Finally, controlled field experiments have identified ecological conditions that are responsible for maintaining divergent selective regimes for closely related species living in sympatry (Rice & Salt 1990; Heath & Roff 1996; Schluter 1996a,b; Rundle *et al*. 2000). However, although there are many examples of

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closely related sympatric sister taxa, there are no data sets available linking evidence for monophyly and strictly sympatric origin of a given taxon pair with data describing simultaneous ecological and genetic divergence. This is especially true for the numerous postglacial fish assemblages, whose members often differ considerably in aspects of their ecology, morphology and life history, but where convincing scenarios for monophyly and sympatry are difficult to make. Therefore, although existing data suggest that ecological sympatric speciation has played an important role for their evolution, strong evidence for this is still missing. This is a general problem for any study trying to provide evidence for sympatric speciation, because scenarios in favour of ecological sympatric speciation can usually easily be dismissed by proposing alternative allopatric scenarios. In some putative cases, for example, there is no solid evidence for the sympatric origin of a studied species pair, although they occur in sympatry. In other cases, there is solid evidence for a monophyletic origin of sympatric species assemblages, but the speciation process apparently had been completed for a long period, as in the case of the Barombi Mbo crater lake (Schliewen *et al*. 1994). In those cases, driving forces and patterns of speciation are difficult to reconstruct, because the current ecological and selective conditions might not reflect those which long ago have lead to sympatric divergence. Thus, even though sympatric speciation may occur frequently, it is very difficult for studies in natural settings to rule out any allopatric ghost of the past.

To make ecological sympatric speciation arguments more credible, it is necessary to fill that major gap of evidence by identifying natural populations where separation under sympatric conditions is currently underway or has only recently been completed. Only then will it be possible to infer the selective conditions that have led to the divergence, as the current prevailing ecological conditions would likely reflect those that were existing during the initial phase of divergence. As theoretical models predict that genetically distinct populations may arise within a few thousand generations under sympatric conditions (Doebeli 1996), it seems clear that the focus has to be on extremely young populations to have a chance of identifying such situations.

In our previous study on cichlid species flocks in two volcanic crater lakes of south-western Cameroon, we were able to show that two lakes of different age, Lake Barombi Mbo with an age of 1 million years (Cornen *et al*. 1992), and the younger Lake Bermin (G. Kling personal communication cited in Stiassny *et al*. 1992) harbour morphologically well differentiated and speciose monophyletic species flocks, which probably have arisen under sympatric conditions (Schliewen *et al*. 1994). In the same study we identified an additional remote lake of nonvolcanic origin, Lake Ejagham, which harboured five phenotypically discernible, albeit very similar cichlid forms of the substrate breeding tilapiine cichlid genus *Tilapia*.

Lake Ejagham is approximately 18 m deep and completely isolated from the neighbouring river-system, and because it has no inflowing streams and its single outlet (Amarafú) has a waterfall several meters high, any migration of fish from the river into the lake is prevented. The lake is tiny and covers an area of only 0.49 km^2. The majority of the lake bottom (approximately 0.35 km^2) is covered with flocculent mud, which renders only the inshore areas suitable for reproduction of the substrate breeding tilapias (Fig. 1). The ecology of the immediate inshore area is influenced by the fringing rainforest, which causes massive input of allochthonous plant material, insects and their aquatic larvae and the permanent presence of at least six species of piscivorous birds. With increasing depth and, therefore, increasing distance from the shore, leaves, twigs, branches and trees become rare on the lake floor, while the underlying sandy bottom becomes exposed until the interior mud zone is reached. Apart from the five different *Tilapia* (*Coptodon*) ssp., the lake is inhabited by two undescribed *Sarotherodon* ssp. (Cichlidae), one cyprinodont (*Aphyosemion gardneri lacustre*), one poeciliid (*Procatopus aberrans*) and one cyprinid (*Barbus* cf. *callipterus*). According to Livingstone the lake is 'most likely a solution basin produced by groundwater dissolving carbonates out of the marine sediments that are supposed to underlie the lake, but it might possibly be a maar …'. On the basis of

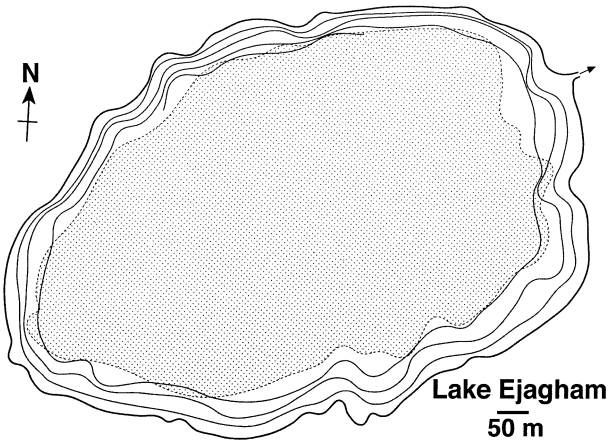

Fig. 1 Bathymetric map of Lake Ejagham based on 53 metered SCUBA transects which were laid around the complete lake shore at an approximately 50 m distance to each other (further details see methods). Solid lines indicate the outline of the lake basin and the 3 m, 6 m, 9 m and 12 m depth contours. The maximum depth of the lake is 18 m. Lake Ejagham covers an area of approximately 0.49 km^2 with a maximum diameter of approximately 1020 m. The central area (shaded) is completely covered with flocculent mud of organic origin, which renders only the periphery of the lake (approximately 0.15 km^2) suitable for substrate brooding *Tilapia*. The bottom of the inshore area is characterized by leaves and branches, followed by open sandy areas with increasing depth. Stones are exposed in low frequency in all depths, especially in steeper areas, but not in the interior mud zone.

sediment cores, he further supposes 'that it is older than 5000 years and probably originated during the last glaciation, which lowered world sea level and generated relatively dry climates' (quotations from personal communication, 9 April 1999).

Using a combined data set of partial mitochondrial D-Loop and cytochrome *b* sequences, we were previously able to show that all studied Ejagham-Tilapias were closely related to *Tilapia* sp. *aff. guineensis* '*Cross*', which represents an undescribed *Tilapia* species of the subgenus *Coptodon* living in the Cross River drainage near Lake Ejagham. In addition, all Ejagham-*Tilapia* shared the same cytochrome *b* haplotype (Schliewen *et al*. 1994). The question remained open as to whether the observed phenotypic diversity of Ejagham-Tilapias is the result of phenotypic plasticity or polymorphism within a single panmictic gene pool; or alternatively, whether this represents a situation where ongoing or recently completed speciation has preceded detectable mitochondrial genetic divergence among those forms. Among them, two phenotypes were, at first glance, indistinguishable, but occurred in two different size-classes of reproductively active animals. According to their morphology, they are referable to the single described taxon of that lake, *T. deckerti*. This apparently closely related pair of phenotypes (referred from now on to as *T.* cf. *deckerti 'large black'* and *T.* cf. *deckerti 'little black'*) seemed to be the ideal candidate to study processes leading to sympatric divergence under natural conditions. To obtain data that would serve to fill the mentioned gap in previous data sets, we used a combination of ecological and behavioural field methods on the one hand, and molecular genetic and phenetic analyses on the other. By those means we could test the hypothesis for both, the monophyly of the pair, as well as its partial or even complete reproductive isolation. This, in combination with the investigation of patterns of resource use, mating and life history, sheds light on the selective forces responsible for their sympatric divergence.

Materials and methods

Identification of the Tilapia *forms of Lake Ejagham in the field*

Tilapia cf. *deckerti*. The target pair of forms, *T.* cf. *deckerti* was differentiated from all other *Tilapia* (*Coptodon*) and therefore from all Ejagham-*Tilapia* by its slender body form, acutely pointed snout and, when not breeding, by a typical yellow-green colouration (Fig. 2a,b). Breeding individuals were invariably completely black with iridescent blue lips and never larger than 12 cm. Breeding fish were classified as *T.* cf. *deckerti 'little black'* (Fig. 2b) and *T.* cf. *deckerti 'large black'* (Fig. 2d) according to their size, which was estimated using a size standard (see SCUBA methods below). Non-breeding juveniles, subadult and adult fish could be classified according to their size, but it was not straightforward to assign them to either *'little black'* or *'large black'*. However, small deepwater fish (Fig. 2a) appeared to have larger eyes than similar sized *T.* cf. *deckerti* from the shallow water (Fig. 2b). Juveniles of *T.* cf. *deckerti* could be differentiated from all other juvenile cichlids in the lake by their extremely elongated '*Tilapia*-mark' at the base of the dorsal fin (Fig. 2e). Qualitative feeding observations suggest that the small deepwater fish primarily feed on planktonic organisms in the open water column, while inshore fish, in addition, pick on small particles from the substrate and feed on allochthonous matter (probably insects) from the water surface.

T. sp. *'jewel'*. As 'jewel' we identified all breeding animals with a comparatively compact body, for which the following three criteria fitted: always under approximately 15 cm in length, intensive yellow body colouration with or without black vertical bars, breeding site always above 2 m water depth.

T. sp. *'dark jewel'*. As 'dark jewel' we identified all breeding animals with a comparatively compact body, which were larger than approximately 15 cm and which had an overall dark but not completely black colouration, but had at least one black ventral body portion. They bred exclusively in excavated caves below 5 m water depth. However, in the pilot phase of the study (Spring 1993) two small pairs of small dark *Tilapia* were identified at approximately 10 m depth, which were only 10 cm long, and similar to *T.* sp. '*dark jewel*' and which bred under the few stones available in the deep part of the lake. Those animals were not found again in the two study phases of 1994 and 1995. However, they were included separately in the morphometric analysis for completeness as '*dark jewel/jewel**'.

T. sp. *'predator'*. As 'predator' we identified all breeding or nonbreeding animals larger than approximately 20 cm (total length), which had almost no yellow colouration on the lateral body portions and which bred exclusively in the shallow inshore region above 2 m. They excavate large nest-pits under large branches or logs. In the pilot phase (Spring 1993), one animal that resembled a *T.* sp. *'predator'* male was found breeding with *T.* sp. *'dark jewel'* female in a cave of approximately 8 m depth. This animal, and two morphologically aberrant shallow water specimens are included in the morphometric analysis as 'predator*'.

SCUBA transects

In order to study the complete lake basin, 53 SCUBA transects of 50 m in length and at a distance of approximately 50 m from each other were laid almost perpendicular to the complete lake shore. This was done in the dry

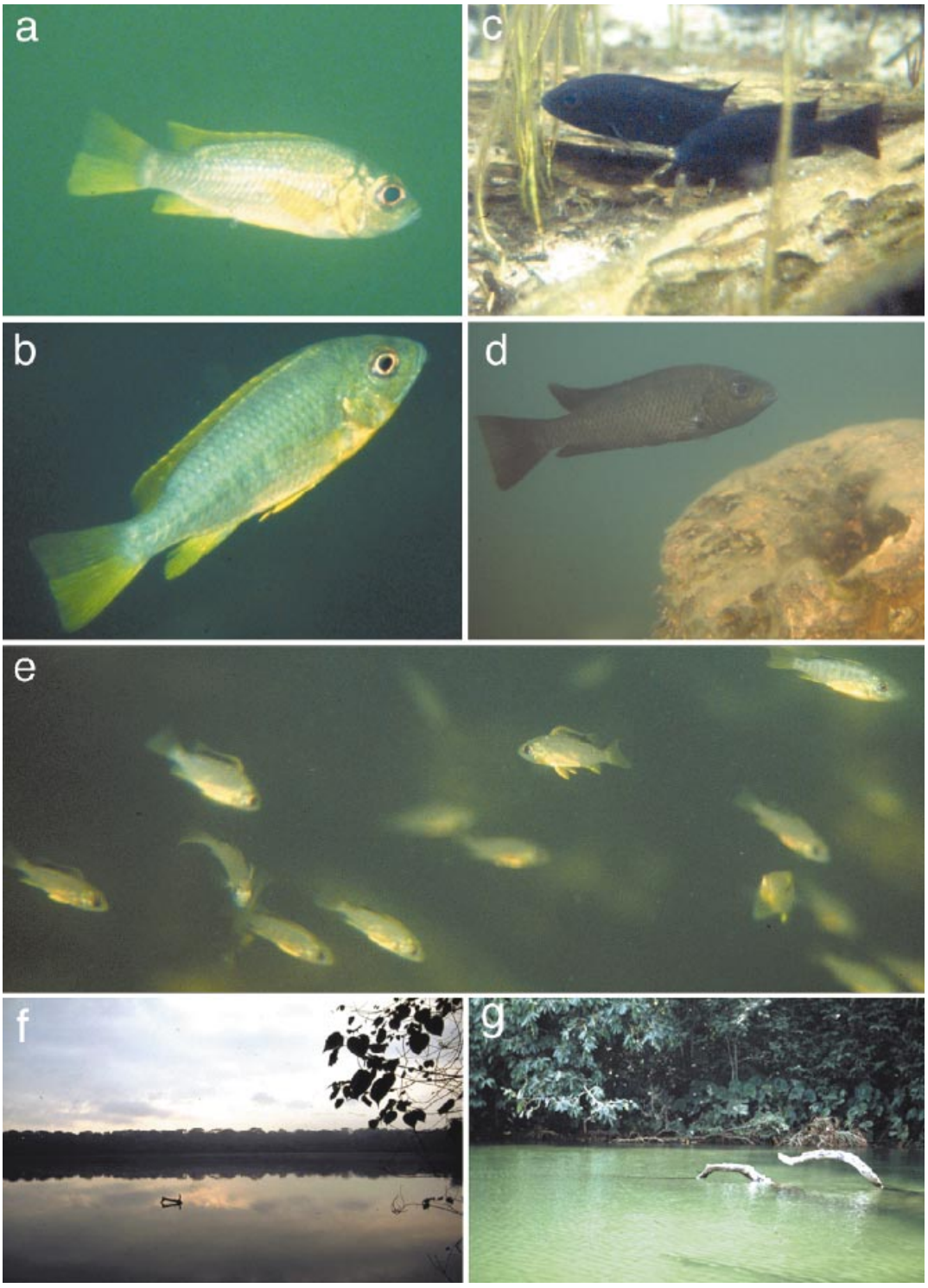

Fig. 2 Underwater photographs of *Tilapia* cf. *deckerti* forms and photographs of the lake. (a) Non-breeding individual photographed in approximately 11 m depth out of 'swarms of little'. Note the comparatively large eyes in comparison with a shallow water individual shown in (b). (b) Non-breeding individual of a 'large' *T.* cf. *deckerti* in the shallow water region. Note the small eye in comparison with the deep water individual shown in (a). (c) Breeding pair of *T.* cf. *deckerti 'little black'* photographed in less than 1 m depth. (d) Breeding female of *T.* cf *deckerti 'large black'* in front of a loghole in less than 1 m depth, which contains recently spawned eggs (not visible). (e) *'juvenile swarm'* in approximately 3 m depth. Note the very long black '*Tilapia*-mark' at the posterior base of the dorsal fin, which is unique among *Tilapia* for *T.* cf. *deckerti* juveniles. (f) View across the lake and (g) view of the lake shore — note that the shallow inshore region is fringed by dense forest, which provides allochthonous food (e.g. fallen insects), as well as shelter and structure by fallen wood.

(February to April 1994) and once in the wet season (August to September 1995). Each transect consisted of two ropes that were marked every meter and were fixed at a distance of 1 m to each other so that a complete transect area covered 50 m^2. Two transects were used simultaneously: one was laid down towards the centre of the lake bottom first. Meanwhile, the second transect was positioned. Investigation of the first transect started at least 1 h after the second one had been laid. Transects began at a depth of approximately 1 m and ended after a distance of 50 m at a variable depth depending on the slope of the lake floor. In addition to the transects, the complete inshore area above 1 m was investigated once in the dry and once in the wet season to complement the ecological and ethological transect data.

Depth distribution data were recorded for all *T.* cf. *deckerti*, occurring within the transect (nonbreeding animals) or within an area that extended laterally 1 m of the two transect lines (breeding pairs). Data were obtained between 9 am and 5 pm, while slowly swimming from the end of the transect towards its beginning. *T.* cf. *deckerti* individuals were classified according to size-class, reproductive state and association. The size-class categories were estimated by comparing them visually with a 7-cm stick and labelled as: 'juveniles' (smaller than approximately half of the stick length), 'little' (shorter than the stick, but longer than half of it) and 'large' (longer than the stick). The 7 cm stick was used after having measured *Total Length* and *Standard Length* of several hundred breeding and nonbreeding fish in the field (see Sample collection). Their reproductive state was labelled as 'breeding' for all those animals that were black and territorial. Breeding individuals were in addition classified as breeding on the 'soil' or in a 'loghole' of a fallen tree, and it was noted whether there was another breeding pair of *T.* cf. *deckerti 'little black'* or *'large black'* visible within sight (approximately 2–4 m). All freely moving individuals exhibiting a yellow-green colour were 'nonbreeding'. Association was categorized as 'swarms' (at least a dozen individuals within a short distance to each other — compare Fig. 2e), 'single' (maximum of five individuals within a short distance to each other) and 'pairs' (two black 'breeding' individuals).

Sample collection

All fishes used in morphometric and genetic analyses were collected, using a fine-meshed gill-net (25 m long, 6 mm mesh-size). Non-breeding animals were driven by one diver into the net while a second diver encircled them with the net and brought the catch to the surface, where it was transferred into a bucket. Breeding fishes were usually collected by a single diver using two hand-nets or a smaller gill-net (10 m long, 6 mm mesh-size). Collections of breeding *T* cf. *deckerti 'little black'* include animals from both the shallow inshore zone above 1 m, and from deeper zones down to 10.5 m in depth. Tissue samples for DNA analysis were either obtained from muscle tissue of the tail-musculature, or from fin-samples and were directly transferred into 96% ethanol reagent grade *Total Length*, *Standard Length* and *Weight* of each fish was recorded immediately after capture using a ruler (accuracy 1 mm) and a small field scale (accuracy 0.1 g). In some cases, ripe eggs were removed and counted from females which had an extended genital papilla indicating that they were ready to spawn.

Laboratory methods

Genomic DNA was extracted from approximately 5 mm^2 of ethanol-preserved fin-tissue or a piece of muscle with similar dimensions using a SDS-proteinase K/salt-chloroform extraction method. Polymerase chain reaction (PCR) conditions and sequencing of the first 200 bp of the D-loop are described in Schliewen *et al*. (1994). Microsatellites were developed as described in Tautz (1989) and Rassmann *et al*. (1991). Microsatellites were amplified (30 cycles) with the following standard temperature profile: 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 90 s elongation at 72 °C. Loci US-758/773, US-780/783 and US-781/784 (all perfect AT repeats) were then run on a polyacrylamide-sequencing gel (4 or 6%), dry blotted for 1 h on a HYBOND membrane (Amersham) and visualized by probing the blot with a ^{32}P-labelled simple-sequence oligonucleotide $(AT)_8$. UNH002 and UME002 (perfect CA repeats) were visualized on an ABI sequencer after amplification with dye-labelled primers. Primer sequences are: locus US-758/773 5′ ATCAGCACGTCATCTGCATGAG and 5′ GCAAAGCAAAGCTGAGAAACAA; locus US-780/783 5′ TAAGTTCCATGCACCGAGATA and 5′ TATGGGAACCTGTGAATGTGAG; locus US-781/784 5′ GAGCGAAACCTGAACAGAATAC and 5′ AGAGCCTGCTGGGGACAAGAGT; locus UNH002 5′ TTATCCCAACTTGCAACTCTATTT and 5′ TCCATTTCCTGATCTAACGACAAG; locus UME002 5′ TCAGAGTGCAATGAGACATGAAT and 5′AATTTAGAAGCAGAAAATTAGACG.

Analysis of genetic data

Initially, all D-Loop haplotypes were subjected to phylogenetic analyses. Parsimony trees were determined for those haplotypes using the heuristic search routine of the maximum parsimony program PAUP 3.1.1 (Swofford 1993). A parsimony network was constructed from the resulting trees, showing all possible single mutational steps among the haplotypes and inferred nodes. The sequences were then subjected to the analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) using the program ARLEQUIN version 1.1 package (Schneider *et al*. 1997), to determine PHIST (an analogue for haplotypic data of Wright's F_{ST}) as an

estimator of the overall genetic distances among all Ejagham-*Tilapia* forms and to assess genetic distances among pairs of populations ('forms').

Similarily θ, another analogue of F_{ST} was estimated from the microsatellite data according to Weir & Cockerham (1984) using another implementation in the program ARLEQUIN version 1.1 (Schneider *et al*. 1997). Observed and expected heterozygosities of microsatellite allelic diversity was calculated with the program GENEPOP 3.1.

P-values, i.e. the probabilities that PHIST or θ are not larger than 0, were determined using permutations ($n = 1000$ for PHIST, $n = 10000$ for θ). All *P*-values for pairwise calculations were Bonferroni-corrected for adjusting the level of significance for the number of pairwise multiple comparisons.

Morphometric measurements and analysis

Measurements were taken from breeding animals only, except those from the riverine. The 12 measurements were taken with a digital caliper (Mahr Meßtechnik) to 0.1 mm accuracy. Except for one measurement (DORPEL) they were measured as described in Barel *et al*. (1977) on the left side of the fish body: *total length* (TL), *standard length* (SL), *head length* (HL), *eye length* (EYEL), *snout length* (SNL), *preorbital depth* (POD), *interorbital width* (IOW), *caudal peduncle depth* (CPD), *upper jaw length* (UJL), *lower jaw length* (LJL), *lower jaw width* (LJW), *pectoral fin length* (PEL). The measurement of body height was 'distance from beginning of dorsal fin to beginning of pelvic fin' (DORPEL). Morphometric data were log-transformed and subjected to a principal component analysis (PCA) especially designed to remove size effects, i.e the 'sheared' PCA (Humphries *et al*. 1981). This was necessary in order to remove size related effects on shape among groups of individuals of nonovelapping size-classes as in the case in *T.* cf. *deckerti 'little black'* and *'large black'*. In this analysis the first principal component (PC I) integrates size-related variation, whereas the PC II, PC III and following components are theoretically size-free. The sheared PCA was performed using the DOS-routine SHEAR by Norm McLeod available on the internet (http://www.life.bio.sunysb.edu/morph/softmisc.html).

Measuring of scale circuli for relative age estimation

In tilapiine cichlids, scales show a pattern of circular rings (circuli), which are laid down at regular intervals. The age estimation is based on the fact that circuli are laid down regularly in a cichlid fish (every second to third day in the tilapiine *Oreochromis niloticus*), which means that two fish with scales that have the same number of circuli are approximately of the same age (Doyle *et al*. 1987). That this interpretation is not only valid for *Oreochromis*, but also for *Coptodon*, is supported by data from *T. (Coptodon) zillii* of the River Niger (Daget 1956). Once circuli are laid down, they remain unchanged over time, except that the interior portion of the scales may granulate, e.g. that the interior rings fuse and disintegrate. Therefore, the number of circuli laid down can be used to estimate age. We used this method to compare relative age of *T.* cf. *deckerti 'little black'* and *'large black'*, since we did not have animals of known age. Three scales were removed from preserved breeding *T.* cf. *deckerti 'little black'* or *'large black'* individuals, namely the third, fourth and fifth rostral scales directly above the lateral line on the right side of the body. The scales were cleaned with KOH and photographed later under a microscope (50×) and the pictures were printed on a videoprinter. Only scales that allowed good readings (e.g. with a low degree of interior granulation) were used in the analysis. However, due to prevalent interior granulation and the consequently irregular start of ring formation, rings were counted only after a fixed distance (1 cm on the videoprint) from the focus of the scale. This distance in the interior part of the scale corresponded to between 18 and 21 rings, as evidenced by comparison with ungranulated scales.

General statistical methods

Standard statistical tests were performed using SPSS for UNIX (SPSS Inc., Chicago). Comparison of two linear regressions for significant difference were performed using the software SsS (Rubisoft Software GmBH, Puchheim, Germany), after testing for homoscedasticity and other necessary prerequisites of the data set. The mathematics used in that program are taken from Zar (1996). Pairwise multiple comparisons were Bonferroni-corrected according to Chandler (1995).

Results

Monophyly of Tilapia *cf.* deckerti 'little black' *and* T. *cf.* deckerti 'large black'

According to molecular phylogenetic analysis, *Tilapia* cf. *deckerti* clearly is a member of the *Tilapia* subgenus *Coptodon* (Schliewen *et al*. 1994). Currently there are 23 recognized species within the subgenus *Coptodon* (Thys van den Audenaerde 1971; Stiassny *et al*. 1992). Both forms of *T.* cf. *deckerti* that are studied here are characterized by a slender fusiform body, an acute mouth and a black breeding colouration (Fig. 2). In addition, the typical round dot in the posterior portion at the base of the dorsal fin ('*Tilapia*-mark') is extended to a longitudinal stripe in juveniles of *T.* cf. *deckerti* (Fig. 2e). None of these characters are shared by any other *Tilapia* (*Coptodon*) (Thys van den Audenaerde 1968, 1971; Stiassny *et al*. 1992). Therefore, these characters are unique within *Tilapia* (*Coptodon*), and in this combination,

unique within cichlid fishes. Hence, they can be regarded as synapomorphies of that taxon pair and therefore support their monophyly.

In addition, a morphometric comparison of all Ejagham-*Coptodon* and the closely related *T.* sp. *guineensis 'Cross'* was performed by calculating 'size-free' sheared principal components II and III from morphometric characters (see methods). In a plot of PC II to PC III both forms of *T.* cf. *deckerti* (*'little black'* $n = 20$; *'large black'* $n = 20$) show almost no overlap with other *Tilapia* forms of Lake Ejagham [*T.* sp. *'dark jewel'* $n = 21$; *T.* sp. *'jewel'* $n = 28$, *T.* sp. *'predator'* $n = 24$, small aberrant *T.* cf. *'dark jewel/jewel*'* $n = 3$, aberrant *T.* sp. *'predator*'* $n = 3$ and the *T. guineensis 'Cross'* ($n = 14$)], but have higher factor loadings for PC II than all other forms, except for *T.* sp. *'predator'*. However, this is the only predatory *Coptodon* known, which is easily distinguished by its colouration, adult body size and breeding behaviour. The morphometric data therefore support the monophyly of both forms of *T.* cf. *deckerti.*

Morphometric differentiation of T. *cf.* deckerti *phenotypes*

To search for morphometric characters that differentiate *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'*, a second sheared PCA was performed analysing only males and females of these two forms (Fig. 3). The highest loading factor for the most discriminating PC III was detected for the measurement EYEL, confirming the overall impression that *T.* cf. *deckerti 'little black'* had larger eyes than *T.* cf. *deckerti 'large black'* (Fig. 2). We investigated similarity in this discriminating measurement for a much larger sample size of nonbreeding deepwater animals (out of 'swarms of little') as well as shallow-water animals on the one hand, and breeding *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* on the other hand. We found that the measurements from *T.* cf. *deckerti 'little black'* animals resemble 'deepwater' more than nonbreeding 'little' from the shallows, while breeding *T.* cf. *deckerti 'large black'* animals fall into the variation of 'shallow'. (Fig. 4). Assuming that EYEL is at least partially genetically determined (Hofmann & Hausberg 1993), we conclude that the majority of breeding *T.* cf. *deckerti 'little black'* are most likely recruited from big-eyed deep-offshore animals, whereas the majority of breeding *T.* cf. *deckerti 'large black'* animals predominantly come from small-eyed individuals that grew up in the shallows.

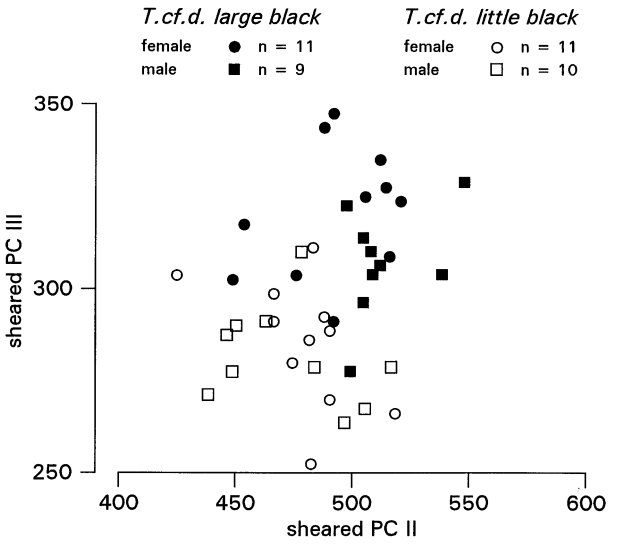

Fig. 3 Plot of sheared principal components II and III calculated from 12 morphometric measurements (see methods) of breeding *Tilapia* cf. *deckerti 'little black'* (n females = 10, n males = 10) and *T.* cf. *deckerti 'large black'* (n females = 11, n males = 9). PC-values of both forms are overlapping for PC II and III. However, *T.* cf. *deckerti 'large black'* on average has higher values for PC III. According to factor loadings contributing to PC II and PC III, respectively, the measurement *Eye Length* contributes most to PC III (data not shown).

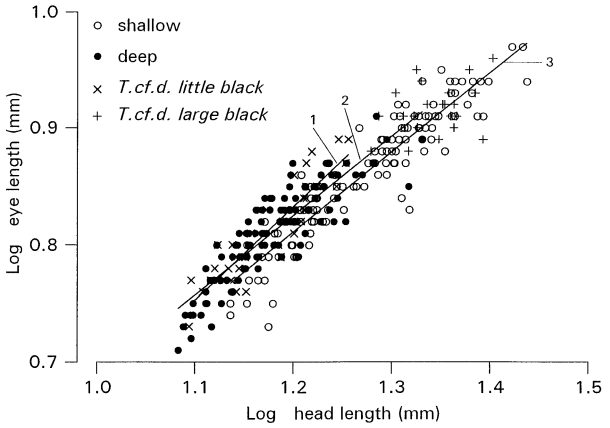

Fig. 4 Linear regressions of *Eye Length* vs. *Head Length* for four discernable groups of *Tilapia* cf. *deckerti.* Breeding *T.* cf. *deckerti 'little black'* (regression line 1: $r = 0.911$; Pearson's $P < 0.001$, two-tailed; $n = 39$); nonbreeding *T.* cf. *deckerti* collected in the deep below 8 m (regression line 2: $r = 0.876$; Pearson's $P < 0.001$, two-tailed; $n = 107$); nonbreeding *T.* cf. *deckerti* from the shallow area above 6 m (regression line 3: $r = 0.930$; Pearson's $P < 0.001$, two-tailed; $n = 124$); *T.* cf. *deckerti 'large black'* ($r = 0.362$; Pearson's P n.s., two-tailed; $n = 22$). The regressions between *T.* cf. *deckerti 'little black'* (regression line no. 1) and nonbreeding *T.* cf. *deckerti* collected below 8 m (regression line no. 2) neither differ significantly in their slopes nor for their y-intercepts (P = n.s.). However, these two each differ significantly from y-intercepts of the regression from nonbreeding *T.* cf. *deckerti* from the shallow area above 6 m ($P < 0.001$) (regression line no. 3). Unfortunately, *T.* cf. *deckerti 'large black'* regressions could not be compared with the regressions from the other forms, as their regression was not significant because of too small sample size. Comparison between regressions of the other data was possible, since they differed not significantly from a normal distribution ($P < 0.05$), were not autocorrelated ($P < 0.05$) and homoscedasticity was fullfilled ($P < 0.05$).

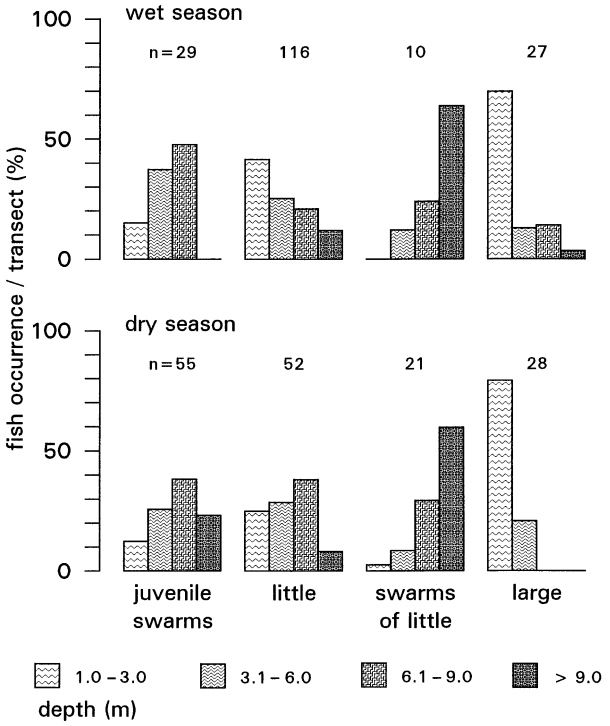

Fig. 5 Depth distribution of four categories of nonbreeding individuals of *Tilapia* cf. *deckerti* (therefore not unequivocally assignable to either *T.* cf. *deckerti 'little black'* or *T.* cf. *deckerti 'large black'*) plotted as fish occurrence per SCUBA transect in per cent along two different sets of 53 transects, once in the wet and once in the dry season (further details see methods). The four columns each represent four depth zones, as indicated at the bottom. 'juveniles' are < 4 cm, 'little' are 4–7 cm (comparable to *T.* cf. *deckerti 'little black'*) and 'large' are > 7 cm (comparable to *T.* cf. *deckerti 'little black'*). In order to compare depth-distributions of all four size-classes, pairwise comparisons using Fishers exact probability tests were performed on absolute numbers of fish occurrence in two depth classes (shallow zone from 1 to 6 m and a deep zone deeper than 6 m) over all transects ($n = 106$) in both seasons. The results show that niche differentiation according to size-classes of nonbreeding *T.* cf. *deckerti* becomes apparent as the fish grow. The depth distributions for the different size classes are significantly different from each other (at least $P < 0.01$ after standard Bonferroni correction for six comparisons), with the exception for the distribution-differences between 'juvenile swarms' and 'little', and 'juvenile swarms' and 'swarms of little', which were significantly different at the $P < 0.05$ level only before Bonferroni correction.

Depth distribution

Different size-classes of nonbreeding individuals used different depths zones in the lake. Juveniles (< 4 cm) are found only in large aggregations mainly in the medium and deep zones ('juvenile swarms' in Fig. 5, compare also Fig. 2e), but never in the immediate inshore region above 1 m water depth. A differentiation of habitat use according to depth zones becomes apparent for medium sized animals (4–7 cm in length; equivalent to *'little black'* sized individuals). On the one hand, large swarms of dozens to hundreds of individuals are found almost exclusively in the deep zone, including the offshore region over the mud ('swarms of little' in Fig. 5). They feed in the open water column close to the lake bottom. On the other hand, single swimming individuals of the same size class are found in all depth zones, including the very shallow regions ('little' in Fig. 5). Finally, 'large' (Fig. 5) individuals (> 7 cm; equivalent in size to *T.* cf. *deckerti 'large black'*) occur almost exclusively in the very shallow inshore region where they can be observed feeding on allochthonous material from the surface, as well as among the leaf litter, over bare sandy soil and wooden debris. In order to test for differential depth-distributions of all four size classes, pairwise comparisons using Fisher's exact probability tests were performed on absolute numbers of fish occurrence in two depth classes (shallow zone from 1 to 6 m and a deep zone deeper than 6 m) over all transects ($n = 106$) in both seasons. The results confirm that differential depth occurrence according to size classes becomes apparent as the fish grow. The depth distributions for the different size classes are significantly different from each other (at least $P < 0.001$ after standard Bonferroni correction for six comparisons), with exception for the distribution differences between 'juvenile swarms' and 'little', and 'juvenile swarms' and 'swarms of little'.

The distribution of breeding *T.* cf. *deckerti* showed a similar pattern. The combined data from SCUBA transects and the investigation of the immediate inshore region showed that *T.* cf. *deckerti 'large black'* pairs occur almost exclusively in the very shallow region, while *T.* cf. *deckerti 'little black'* occurred in high numbers in all depth ranges. The overall breeding activity is elevated in the wet season for *T.* cf. *deckerti 'little black'* ($P < 0.001$, χ^2 test). In addition, *T.* cf. *deckerti 'large black'* are outnumbered by *T.* cf. *deckerti 'little black'* in the shallow region by a factor of approximately 10 (Fig. 6). Therefore, *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* occurred in full syntopy in the shallow inshore zone. Syntopy of *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* was further evidenced by the fact that virtually all observed breeding pairs of *T.* cf. *deckerti 'large black'* were within visible neighbourhood of a *T.* cf. *deckerti 'little black'* pair.

Lenght/weight relationship, growth and age

Because the large size-class of nonbreeding *T.* cf. *deckerti* occurred only in the shallow water, but 'little' size classes in the shallows as well in the deep area, we assumed that alternative selective regimes may prevent fishes from the deep zone to grow bigger than 'little'. Therefore, we tested the hypothesis, that similar-sized 'little' animals have a lower or higher length/weight relationship, depending whether they were collected in the deep water or in the shallows, respectively. Only specimens collected in the dry

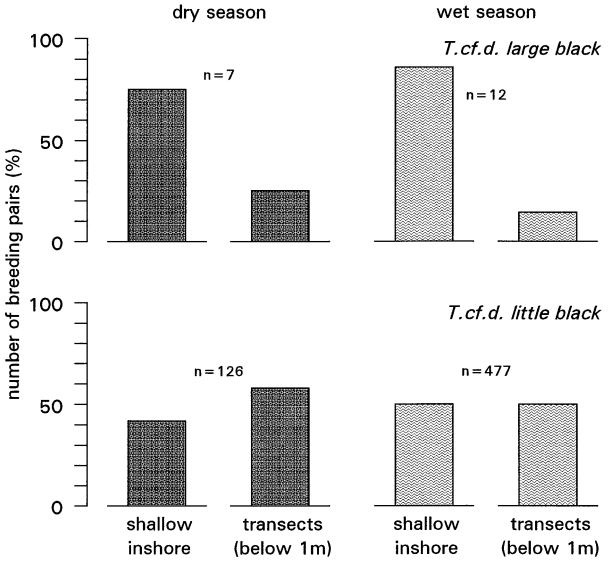

Fig. 6 Depth distribution and seasonality of breeding in *Tilapia* cf. *deckerti*. In addition to the transects, the complete shallow region above 1 m depth was screened for breeding pairs once in the dry and once in the wet season, and compared with breeding activity below 1 m along the transects (further details see methods). The combined data show that *T.* cf. *deckerti 'large black'* pairs occur almost exclusively in the very shallow region, and that overall breeding activity is elevated in the wet season for *T.* cf. *deckerti 'little black'* ($P < 0.001$, χ^2 Test). In addition, *T.* cf. *deckerti 'large black'* are outnumbered by *T.* cf. *deckerti 'large black'* in the shallow region by a factor of approximately 10.

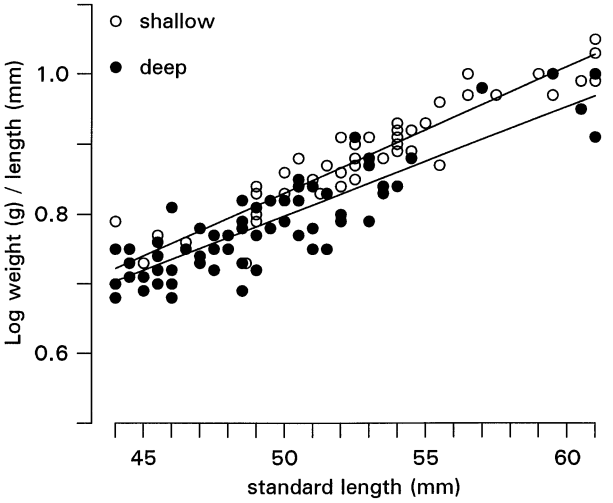

Fig. 7 Comparison of linear regressions of body weight with standard length of approximately equally sized *Tilapai* cf. *deckerti* from the shallow water ($r = 0.887$, Pearson's $P < 0.0001$, two-tailed, $n = 43$) and from the deep water ($r = 0.941$, Pearson's $P < 0.0001$, two-tailed, $n = 61$). The two regressions ($y = -0.069337 + 0.017986x$ and $y = 0.020542 + 0.015539x$ are significantly different in their y-intercepts ($P < 0.0001$). Individuals from the deep water are on average significantly lighter.

season were compared, because at that time less breeding activity takes place, which may restrict normal feeding activity. For the same reason, all animals which showed signs of reproductive activity (dark or black colouration, expanded genital papillae) were excluded. The analysis shows that similar sized animals from the deep water (Fig. 7) were comparatively lighter than those from the shallows. This indicates that foraging efficiency, food availability or the hydrodynamic environment support different length/weight relationships for *T. deckerti* in the two areas and, accordingly, points to different growth potentials for *T.* cf. *deckerti* living in the two different habitats.

That ecological conditions determine maximum size in *T.* cf. *deckerti* is clearly shown for the case of two individuals of *T.* cf. *deckerti 'little black'*, which were brought into an aquarium in the laboratory in October 1994. After 4 years the fish grew from 6.85 cm and 5.9 cm to 20.5 cm and 19.2 cm, respectively. The larger fish, a male, had attained a weight of 129.4 g, thereby showing a ninefold higher weight than any *T.* cf. *deckerti* collected in the field (maximum total length 10.3 cm with 14.6 g).

Because two different size classes of otherwise similar fish may result from alternative life history strategies or simply from different age, we tested the null hypothesis that the *T.* cf. *deckerti 'large black'* are of the same age as *T.* cf. *deckerti 'little black'*. This hypothesis was refuted by the results of scale circulus readings (see methods), which showed that 33 of 35 individuals of *T.* cf. *deckerti 'little black'* (n females = 18, n males = 17; individuals from all depths zones) had scale circulus readings between 25 and 55, whereas all *T.* cf. *deckerti 'large black'* (n females = 17, n males = 14) had readings higher than 55.

Size assortative mating of distinct size classes

During SCUBA obervations along the transects and in the shallow inshore region we observed more than 622 breeding pairs either referable to *T.* cf. *deckerti 'little black'* ($n = 603$) or *'large black'* pairs ($n = 19$) and estimated their length with the size-standard of 7 cm (see methods). Almost all of the pairs had mated size assortatively. The only exception concerns five mixed pairs, in each case between a large male and a small female, which were found during the dry season outside of the transects. To test the hypothesis of size assortative mating within distinct size-classes, we measured TL in 45 breeding pairs that were caught *in situ*. *T.* cf. *deckerti 'little black'* pairs were chosen randomly from all depths zones, while we tried to collect all *T.* cf. *deckerti 'large black'* pairs because of their scarcity. Figure 8(a) shows that these fall into two nonoverlapping size classes, proving size assortative mating, which was evident not only among forms, but also within *T.* cf. *deckerti 'little black'* (see legend Fig. 8). It is noteworthy in this context, that our sample did not contain

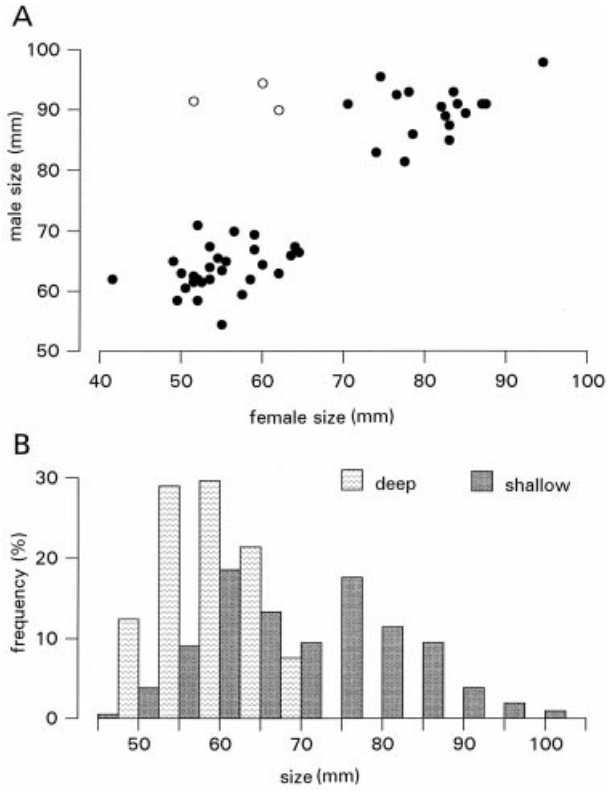

Fig. 8 Size and size assortative mating in *Tilapia* cf. *deckerti*. (a) Relationship of male and female body size (*Total Length* 'TL') of 45 breeding pairs, each filled circle representing one pair. Body size is positively correlated between paired males and females over all pairs ($r_s = 0.824$, $P < 0.0001$). Even within *T.* cf. *deckerti 'little black'* positive assortative mating is evident (Pearson's correlation $r = 0.444$, $P < 0.05$, $n = 28$). In addition to these netted pairs, all pairs occurring up to a distance of 1 m from the transects, as well as in the inshore area above 1 m depth were classified with the help of a 7-cm size-standard as *T.* cf. *deckerti 'little black'* pairs ($n = 603$) or *T.* cf. *deckerti 'large black'* pairs ($n = 19$). Along the transects no 'mixed pairs' were located. However, five pairs from the dry season which were observed outside of the transects could be located, showing that size assortative mating is strong, but not complete. Three of these five mixed pairs were netted and are included into the plot (open circles), but were not included in the statistical analysis, since they were not collected randomly. (b) Size frequency histogramm of nonbreeding *T.* cf. *deckerti* netted during the dry season in the deep water below 4 m ($n = 228$) or in the shallow water above 4 m depth ($n = 157$). The data show that mixed pair combinations or pairs of intermediate size are theoretically possible in the shallow water, were all size classes occur simultaneously.

a single pair of intermediate size, although intermediately sized animals were present in high quantities in the shallows as shown in Fig. 8(b).

Brood site choice of T. *cf.* deckerti

T. cf. *deckerti* are substratum spawners, which lay eggs in different kinds of holes. In the shallow inshore area they had the choice to spawn either in crevices or on the soil (under branches, leaves or small stones), or in elevated logholes of fallen trees. In deeper water the choice was not possible because logs big enough to have holes suitable for breeding were found only in the immediate inshore area. We compared choice of the brood site for *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* pairs, which bred in the shallow inshore area above 1 m where there is an abundant choice for different breeding sites. We found that it differed significantly between the forms (Fisher's Exact Test $P < 0.00001$). *T. cf deckerti 'large black'* pairs almost exclusively spawned in logholes (14 out of 15 pairs), while only few *T.* cf. *deckerti 'little black'* pairs used logholes (15 out of 291 pairs).

Mitochondrial genetic differentiation

As the previous analysis of cytochrome *b* sequences from these forms did not show signs of differentiation, we focused on a stretch of the hypervariable D-loop region, which was sequenced from 158 individuals including all forms in the lake, as well as from the most closely related *Tilapia* species in the surrounding river system, *T.* cf. *guineensis 'Cross'*. This yielded 17 different haplotypes (Table 1), which can be connected in a parsimony network via single mutational steps (Fig. 9). The previously sequenced haplotypes from Lake Bermin and Lake Kotto (Schliewen *et al*. 1994) are not identical to any of the haplotypes found here, but are more or less closely related (Fig. 9). Three internal haplotypes of the network are most frequent and each is shared among at least four of the five Ejagham forms, as well as the most closely related river species. It seems likely, therefore, that these shared mitochondrial lines represent founder lineages that were introduced into the lake by the colonizing cichlid population (Crandall & Templeton 1993). The haplotypes within the lake differ by, at most, two nucleotides from these presumed founder lineages, suggesting that they have evolved very recently. Similar low levels of D-loop sequence divergence were found previously among 14 cichlid species in Lake Victoria (Meyer *et al*. 1990), which has been dated to be only 12 400 years old (Johnson *et al*. 1996), and for the *Alcolapia*-cichlid species flock, no older than approximately 9000 years (Seegers *et al*. 1999). Therefore, the haplotype data set suggests a similar age for the radiation of the *Tilapia* in Lake Ejagham, which is in accord with the geological assumptions (see methods).

An analysis of mitochondrial molecular variance using AMOVA did not yield statistically significant differentiation among *T.* cf. *deckerti 'little black'* and *'large black'*. However, the overall PHIST value indicates a high degree of population substructure among all lake forms (overall PHIST 0.12, $P < 0.0001$). If pairwise PHIST values were calculated for all lake forms and the riverine ancestral species *T.* sp. *aff.*

Table 1 Sequence information for the 200 bp of mitochondrial D-loop sequenced for 158 individuals of Ejagham *Tilapia* and the riverine *T. guineensis 'Cross'*. Bold lower case letters indicate variable positions which were numbered from 1 to 10. Dots indicate invariable positions. The table below provides the information on the 17 haplotypes. It refers to the variable sites 1–10 and shows the haplotype frequency in each group

5′–3′ D-Loop

CTCTGCCTCCAATACATATATGTATTATCACCATTATT**t**TATGTTAAACATATCCTATATATATATACATACAATTA**ag**AAGACATAGACTGAATTCACA
. . . . . 1 . . . . . 23 . . . . . (100 bp)
CATATTTGT**cGa**GAACATTCAATCTAAGA**ga**AACATAAACCAAT**a**AATGATAAATTCCAATA**t**A**t**ATTATAAATGATTAAACGATAGTTTAAGACCGAAC
. . . . . .4 . 5 . . . . . 67 . . . . . .8 . . . . . 9.10 . . . . . (200 bp)

Haplotype No.	Haplotype Sequence Information										Frequency of Haplotypes						
	Pos 1	Pos 2	Pos 3	Pos 4	Pos 5	Pos 6	Pos 7	Pos 8	Pos 9	Pos 10	Little Black	Large Black	Jewel	Dark Jewel	Predator	*T. gui. Cross*	Total
1	T	A	G	C	A	G	A	A	T	T	5	0	4	9	8	6	32
2	T	A	G	C	A	G	A	A	T	G	0	0	0	0	0	1	1
3	T	A	G	C	A	A	A	A	T	C	1	0	0	0	0	1	2
4	T	A	G	C	A	G	A	A	T	C	5	5	9	1	0	4	24
5	T	A	A	C	A	G	A	A	T	C	0	1	0	0	0	0	1
6	T	A	G	C	A	G	A	G	T	T	0	0	0	0	0	1	1
7	T	A	G	C	A	G	A	G	T	T	13	7	13	7	11	1	52
8	T	A	G	C	G	G	A	G	T	T	0	0	0	0	8	0	8
9	T	A	G	C	A	G	A	A	C	T	0	0	5	1	2	0	8
10	T	A	A	C	A	G	A	G	T	T	1	5	2	2	0	0	10
11	T	A	G	C	A	G	G	G	T	T	1	1	0	0	0	0	2
12	T	A	G	C	A	A	A	G	T	T	3	5	0	0	0	0	8
13	T	G	G	C	A	A	A	G	T	T	0	1	0	0	0	0	1
14	T	G	G	C	A	G	A	G	T	T	0	1	0	0	0	0	1
15	T	A	G	T	A	G	A	A	T	T	0	0	0	0	0	1	1
16	T	A	A	T	A	G	A	A	T	C	0	0	0	0	0	1	1
17	A	A	G	C	A	G	A	A	C	C	0	0	0	0	0	5	5
											29	26	33	20	29	21	158

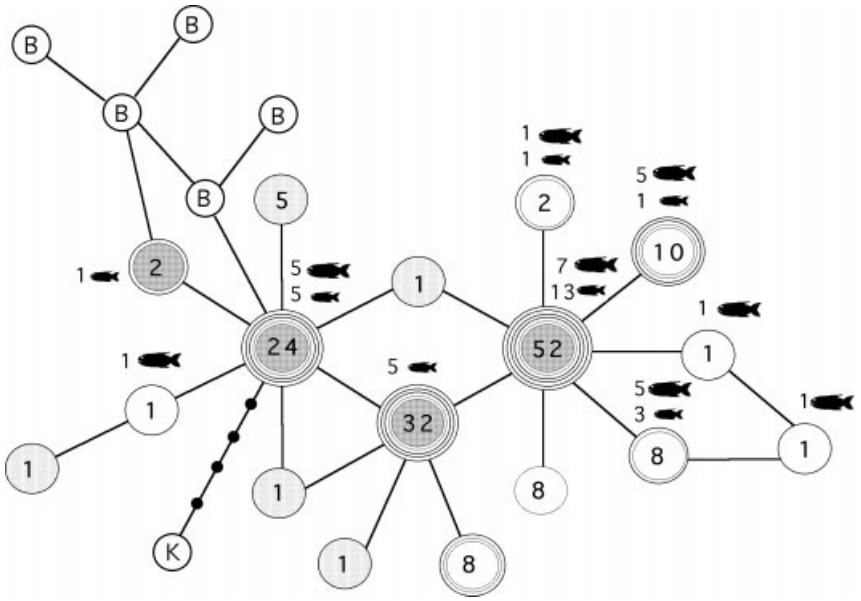

Fig. 9 Parsimony network of the D-Loop lineages found in the five Lake Ejagham *Tilapia* groups, the riverine *Tilapia* species and previously sequenced haplotypes from nearby lakes (Schliewen *et al*. 1994). All animals from Lake Ejaghm were captured in breeding colouration. Each branch represents one mutational step. Haplotypes found exclusively in the river species are shaded lightly, those of the lake forms are white, and haplotypes found in both are darkly shaded. The number of rings around a given haplotype corresponds to the number of *Tilapia* forms in which this haplotype occurred. The numbers within the rings indicated how often the respective haplotype was found among the 158 sequenced. Ten of the 17 haplotypes were found in *T.* cf. *deckerti 'little black'* (small fish symbol) or *T.* cf. *deckerti 'large black'* (big fish symbol). Their absolute frequencies are indicated next to the fish symbols. The smaller rings with the letters indicate the position of the five haplotypes from Lake Bermin (B) ($n = 9$) and the one from Lake Kotto (K) ($n = 1$). The latter is connected to the network by several mutational steps (indicated by black dots). Lake Bermin belongs to the river Cross drainage, while Lake Kotto belongs to a different drainage. The close proximity of the Lake Bermin haplotypes suggests, that the radiation in Lake Bermin is also very young, although the lake itself may be much older than Lake Ejagham.

Table 2 Pairwise θ and Φ_{ST} values among the five lake *Tilapia* forms and the riverine species *T. guineensis 'Cross'*. Except for the latter, all investigated specimens were breeding at the time of capture. *P*-values (the probabilities that θ or Φ_{ST} are not larger than 0 are given in parentheses below the values and were determined using permutations (Φ_{ST} $n = 1000$; θ $n = 10\,000$). N (Φ_{ST}), number of mitochondrial haplotypes sequenced per population; N (θ), mean number of individuals screened at 5 loci and standard deviation per population. All *P*-values for pairwise θ calculations remained significant at the 1% level after standard Bonferroni correction for 15 multiple comparisons. Overall $\theta = 0.14522$ ($P < 0.00001$)

	'Little Black'	'Large Black'	'Jewel'	'Dark Jewel'	'Predator'	*T. g. 'Cross'*
N(Φ_{ST})	29	26	33	20	29	21
N(θ)	24.0 ± 0.0	24.0 ± 0.0	28.2 ± 2.7	19.4 ± 0.5	26.8 ± 0.4	19.6 ± 0.8
						Φ_{ST}
'Little Black'		0.016	0.027	0.031	0.088	0.253
		(0.194)	(0.123)	(0.129)	(0.005)	(< 0.001)
'Large Black'	0.036		0.099	0.102	0.137	0.291
	(< 0.001)		(0.005)	(0.009)	(0.001)	(< 0.001)
'Jewel'	0.108	0.131		0.013	0.123	0.166
	(< 0.001)	(< 0.001)		(0.244)	(0.001)	(0.001)
'Dark Jewel'	0.098	0.133	0.148		0.085	0.267
	(< 0.001)	(< 0.001)	(< 0.001)		(0.028)	(< 0.001)
'Predator'	0.110	0.121	0.142	0.166		0.389
	(< 0.001)	(< 0.001)	(< 0.001)	(< 0.001)		(< 0.001)
T. g. 'Cross'	0.114	0.137	0.079	0.173	0.142	
	(< 0.001)	(< 0.001)	(< 0.001)	(< 0.001)	(< 0.001)	
	θ					

Table 3 Microsatellite variation at five loci in the Lake Ejagham cichlids and the riverine species *Tilapia guineensis 'Cross'*. *n*, number of individuals analysed; A_O, number of observed alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

	Locus UNH002				Locus UME002				Locus 781/784				Locus 758/773				Locus 780/783				Average		
	n	A_O	H_O	H_E	*n*	A_O	H_O	H_E	*n*	A_O	H_O	H_E	*n*	A_O	H_O	H_E	*n*	A_O	H_O	H_E	A_O	H_O	H_E
'Little Black'	24	10	0.67	0.72	24	11	0.75	0.87	24	14	0.92	0.95	24	14	0.96	0.93	24	5	0.50	0.49	10.8	0.76	0.79
'Large Black'	24	6	0.67	0.66	24	8	0.75	0.81	24	13	0.92	0.88	24	13	0.96	0.87	24	5	0.58	0.50	9.0	0.78	0.74
'Jewel'	28	12	0.64	0.76	23	10	0.74	0.87	30	15	0.87	0.90	30	13	0.83	0.86	30	9	0.87	0.83	11.8	0.79	0.84
'Dark Jewel'	19	2	0.63	0.48	19	8	0.68	0.75	19	5	0.74	0.78	20	7	0.70	0.74	20	3	0.55	0.57	5.0	0.66	0.66
'Predator'	27	11	0.70	0.86	27	9	0.78	0.77	26	8	0.77	0.74	27	15	0.70	0.82	27	3	0.37	0.46	9.2	0.66	0.73
'Cross'	20	16	0.85	0.91	20	22	0.85	0.95	20	17	1.00	0.95	20	21	1.00	0.96	18	17	0.89	0.95	18.6	0.92	0.94

Table 4 Distributions of allele frequencies for five microsatellite loci in the two taxa *Tilapia* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'*

Locus UNH002	**Alleles**																	
	256	259	260	262	266	268	274	276	283	291	299							
'little black'	0.208	0.021	0.125	0.479	0.021	0.000	0.021	0.063	0.021	0.021	0.021							
'large black'	0.500	0.000	0.042	0.292	0.021	0.104	0.042	0.000	0.000	0.000	0.000							
Locus UME002	**Alleles**																	
	189	199	201	203	205	207	209	211	213	215	217	235						
'little black'	0.021	0.021	0.021	0.146	0.000	0.188	0.208	0.125	0.104	0.021	0.021	0.125						
'large black'	0.000	0.104	0.042	0.042	0.042	0.292	0.208	0.250	0.000	0.000	0.000	0.021						
Locus 780/783	**Alleles**																	
	92	94	96	98	100	102	104	106	108	112	114	118	120	122	124	126	128	
'little black'	0.083	0.125	0.083	0.063	0.063	0.000	0.021	0.000	0.021	0.021	0.104	0.083	0.042	0.146	0.021	0.125	0.000	
'large black'	0.021	0.146	0.083	0.000	0.021	0.021	0.042	0.021	0.021	0.000	0.000	0.125	0.104	0.125	0.250	0.000	0.021	
Locus 781/784	**Alleles**																	
	102	104	106	108	110	114	116	118	122	126	128	130	132	134	136	138		
'little black'	0.104	0.104	0.167	0.063	0.042	0.000	0.000	0.021	0.042	0.042	0.063	0.063	0.063	0.042	0.104	0.083		
'large black'	0.021	0.146	0.021	0.000	0.042	0.042	0.021	0.021	0.000	0.021	0.208	0.083	0.125	0.229	0.021	0.000		
Locus 753/773	**Alleles**																	
	92	100	102	104	108	110	112											
'little black'	0.042	0.708	0.104	0.083	0.000	0.000	0.063											
'large black'	0.000	0.688	0.104	0.146	0.021	0.042	0.000											

guineensis 'Cross', they were always higher and statistically significant than any pairwise comparisons among lake forms, which supports the idea of isolation of the lake from the river (Table 2). Pairwise tests were rarely significant among lake forms, except for *T.* sp. *'predator'*. However, the lowest of all values was calculated for the two forms of *T.* cf. *deckerti*, supporting their close relationship. Consequently in an UPGMA-distance tree based on PHIST-distance calculated with AMOVA, the two *T.* cf. *deckerti* cluster as sister taxa as expected (not shown).

Estimation of gene flow using microsatellite allelic diversity

To investigate whether the two forms are not simply representatives of a single highly polymorphic species, but are genetically separated, we have typed them for five microsatellite loci. Descriptive data of microsatelllite allele diversity are shown in Tables 3 and 4. Unbiased estimates of Hardy–Weinberg exact *P*-values for each single locus and over all loci by the Markov chain method as implemented in the program GENEPOP (version 3.1.d) did not reject the null hypothesis of no heterozygote deficit for both *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'*. However, if both populations where pooled, there was a significant global heterozygote deficit ($P < 0.05$) as expected for populations with strongly reduced gene flow. In addition, some of the low frequency alleles showed fixed differences in our sample (Table 4). Applying Weir and Cockerham's (1993) *F*-statistic to these data, we find a significant genetic separation of all groups (pairwise comparisons see Table 2; estimated overall $\theta = 0.12$, $P = < 0.0001$) again showing that gene flow among them is restricted. Pairwise comparisons yielded low, but highly significant F_{ST} values for all pairwise tests after Bonferroni-correction (Table 2). The lowest values again were obtained for the target pair of forms *T.* cf. *deckerti 'little black'* and *'large black'*. Consequently in an UPGMA distance tree based on θ distance calculated with AMOVA, the two *T.* cf. *deckerti* cluster as sister taxa as expected (not shown).

Discussion

The study of phenotypic, ecological, behavioural and genetic differentiation of the *Tilapia* cf. *deckerti* pair was initiated in order to investigate whether this pair of forms might be in the process of sympatric speciation and whether the pattern of sympatric differention may lead to the identification of possible selective forces driving it.

Monophyly of the T. *cf.* deckerti *pair*

Establishing monophyly of closely related sister taxa embedded within a group of other closely related taxa of recent origin is notoriously difficult (Albertson *et al*. 1999). In fact, this difficulty has lead to the situation, that despite a considerable amount of data collected on sympatrically occurring postglacial fish species pairs, e.g. sticklebacks, not a single case of unquestionable sympatric speciation could be substantiated without doubts (Taylor & McPhail 1999). Our evidence for the monophyly of the studied taxon pair is based both on morphological and genetic analyses. The comparison with all other *Tilapia*-species of the subgenus *Coptodon* showed that the *T.* cf. *deckerti* pair represents two highly aberrant representatives of that subgenus, which are characterized by a suite of synapomorphic characters. Their black breeding colouration and the elongate *Tilapia*-mark are each unique for the subgenus *Coptodon* and have no obvious ecologically selected value. This suggests that these character states are synapomorphies and not the result of convergent natural selection. Again, their slender fusiform body form and small pointed snout is unique among *Coptodon*, although one may argue that they are the result of convergent selection for life in the open water column. However, we regard this interpretation as unlikely, since in the only other known *Tilapia* (*Coptodon*) species assemblage in Lake Bermin, open-water species have also evolved, but these have a deeper body and a less pointed snout (Stiassny *et al*. 1992).

The genetic data supporting the monophyly of the pair are weaker because they are based not on unique character states, but on frequencies of haplotypes and microsatellites. However, the extremely close PHIST and θ distances for both data sets support the notion that *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* are more closely related to each other than to any other Ejagham cichlid or the potential ancestor from the Cross River system with which Ejagham-*Tilapias* share mitochondrial haplotypes.

We therefore regard the evidence provided for the monophyly of both *T.* cf. *deckerti* forms as sufficient. In fact, their phenotypic and genetic proximity rather questions their status of belonging to two distinct gene pools rather than representing merely a single polymorphic species.

Do the two forms of T. *cf.* deckerti *represent members of distinct gene pools?*

Sympatric speciation is the process by which one founder population splits into two distinct populations, and which eventually evolve independently from each other, despite of their maintained sympatric occurrence. To detect possible cases of incipient sympatric speciation, it is therefore necessary to show that two phenotypically distinguishable populations of monophyletic origin have started to evolve along distinct evolutionary pathways. We used genetic, morphological and behavioural correlates of gene flow to investigate whether *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* already belong to gene pools with their own genetic integrity.

The AMOVA of mitochondrial haplotype diversity indicated that this is the case, but the statistical support for that conclusion was not significant. θ values calculated from the allelic diversity of microsatellites were also very low but showed statistically significant differentiation. This suggests that there is not enough gene flow between the forms to result in homogenization of the gene pool. However, there is a small likelihood that statistically significant, but very low θ values can still be obtained by chance, i.e. as a sampling artefact (Ruzzante *et al*. 1998). We have, therefore, looked for independent evidence for reduced gene flow among the two forms, by analysing the morphological and behavioural characters that are likely to be genetically influenced.

In the morphological comparison of *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* we found that the groups differed significantly in an ecological key trait, namely EYEL. Interestingly, this characteristic is one of the most prominent that differentiates deepwater from shallow water species among different but closely related species in other cichlid species flocks (Huber *et al*. 1997). This suggests that eye size in cichlids is a naturally selectable and therefore heritable characteristic. From studies of regressive eye evolution in subterranean fishes, it was shown that EYEL is a characteristic with high heritability (Hofmann & Hausberg 1993). If one accepts that eye size is at least partially heritable in *T.* cf. *deckerti*, the statistically significant differences in eye size between *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* support the genetic distinctiveness of the forms.

Finally, the two forms differed significantly in their preference for different brood sites. In the shallow water zone, where both *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* had the choice of breeding in logholes, almost all *T.* cf. *deckerti 'large black'* individuals bred in logholes, but only a small fraction of *T.* cf. *deckerti 'little black'*. This significant difference cannot be explained by competition for breeding sites as is known for other substrate-brooding cichlids (McKaye 1977), because logholes that had been

used before, remained unoccupied despite ongoing breeding activity of *T.* cf. *deckerti 'little black'*. It rather points to alternative brood site preferences of the two forms.

In summary, the evidence from microsatellite allele frequencies, morphology and behaviour supports the hypothesis that *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'* indeed belong to different gene pools. The low θ values could indicate that a small amount of gene flow still occurs between the forms, but this could also be explained by the very recent common ancestry.

Are the two forms of T. *cf.* deckerti *ecologically different?*

Non-breeding individuals of *T.* cf. *deckerti* from the ecologically different deep and shallow waters of Lake Ejagham differ significantly in EYEL, length/weight-relationship and swarming behaviour. In addition, large individuals, in contrast to small ones, were restricted to the shallow inshore region. Unfortunately, we were not able to assign those nonbreeding fish unambiguously to either *T.* cf. *deckerti 'little black'* or *'large black'*. However, the analysis of similarity of the characteristic that contributed most to the morphological differentiation between *'large black'* and *'little black'*, i.e. EYEL, showed that the deepwater fish resemble similar sized *T.* cf. *deckerti 'little black'* more than *T.* cf. *deckerti 'large black'*. This result does not prove, but renders it very likely, that the breeding animals of *T.* cf. *deckerti 'little black'* are recruited from the bigeyed deepwater animals. In addition, the fact that deepwater fish were significantly lighter than similar sized animals from the shallows, shows that the two forms either experience different patterns of food availability or foraging efficiency in the two habitats.

Interestingly, large eyes and small adult body size are known to occur as a specialization for feeding on small prey, a trend that is generally found in planktivorous cichlids of the deep offshore region (Huber *et al*. 1997). Thus, it seems likely that *T.* cf. *deckerti 'little black'*-like animals are specialized to exploit the offshore resources with predominantly small food particles more efficiently than *T.* cf. *deckerti 'large black'*-like animals (Galis & de Jong 1988). Further, the fact that large individuals are never found in the deep zone highlights the ecological inferiority of that habitat for larger *T.* cf. *deckerti*. On the other hand, large adult body size in *T.* cf. *deckerti 'large black'* may be better for exploiting the inshore resources, which offer larger benthic macroinvertebrates and allochtonous food, such as insects falling from the surrounding trees, as found for various other fishes (Wainwright & Richard 1995; Hjelm *et al*. 2000). Finally, larger adult size was recently shown to be advantageous for shallow water fish, since predation by piscivorous birds, which are common in the inshore region of Lake Ejagham, affects bigger fish less than smaller ones (Krause *et al*. 1998).

Therefore, the combined evidence shows that deep and shallow habitats most likely represent alternative selective regimes for *T.* cf. *deckerti*, which is reflected in significant differences of ecologically relevant traits among individuals from the two depth-zones. The two forms of breeding *T.* cf. *deckerti* are most likely recruited from ecologically different stocks. The evidence presented here is fully in line with other examples of intralacustrine niche differentiation and correlated morphological diversification.

Alternative life histories, size-assortative mating and the evolution of reproductive isolation

Theoretical analysis shows that bimodal quantitative characteristic distributions both for characteristics determining resource exploitation, as well as those determining mate choice, are important prerequisites for sympatric speciation (Kondrashov & Mina 1986; Doebeli 1996; Dieckman & Doebeli 1999). However, to initiate genetic separation, evolution of assortative mating between the different forms is also necessary.

In some well studied cases such as the fruitfly *Rhagoletis*, this might be a direct consequence of the use of different host plants for feeding, i.e. exclusive mating in the different habitats (Bush 1992). However, this is clearly not the case for *T.* cf. *deckerti 'little black'* and *T.* cf. *deckerti 'large black'*. Breeding pairs of both size classes were found directly adjacent to each other, both in the dry and the wet season. Although *T.* cf. *deckerti 'large black'* breeds almost exclusively in the immediate inshore area above 1 m water depth and *T.* cf. *deckerti 'little black'* breeds in all depth zones, *T.* cf. *deckerti 'little black'* pairs still outnumber *T.* cf. *deckerti 'large black'* pairs in the inshore area (Fig. 6). This shows that assortative mating cannot be the direct consequence of mutually excluding feeding/habitat preferences. Instead, it seems likely that other factors are responsible for assortative mating. We have identified two potential factors that may contribute to assortative mating, i.e. age and size at reproduction and differential brood site choice.

In life history theory, optimal age and size at reproduction is the result of a trade-off of intrinsic and extrinsic ecological conditions, e.g. individual resource use efficiency and patterns of resource availability, respectively (Roff 1982). For example, life history theory predicts that if a population experiences problems to reach a size class where it could breed with higher fecundity, it will choose to breed at a relatively small size, a phenomenon called 'stunting' in fish (Heath & Roff 1996). Our analyses suggest that optimal age and size at reproduction is different between the two ecologically different forms of *T.* cf. *deckerti*. Their different length/weight relationship and age of reproducing individuals suggests that the deepwater fish are limited in growth because they have a significantly lower weight than similar sized animals from the shallows. In addition,

if *T.* cf. *deckerti 'little black'* animals are brought into the aquarium and fed ad libitum, they can easily grow to the size of *T.* cf. *deckerti 'large black'* animals, proving that their optimal size for reproduction in the lake is ecologically and not genetically constrained. Size is known to play an important role for mate choice in substrate breeding cichlids and many other groups of fish (Keenleyside *et al*. 1985; McKaye 1986; Lamprecht & Rebhan 1997; Trifenbach & Itzkowitz 1998). Thus, alternative optimal size classes at reproduction, which may correlate with alternative resource use, would directly lead to reproductive isolation, if mate choice patterns lead to stringent size assortative mating.

The second candidate factor which could strengthen assortative mating, is the alternative brood site choice in the shallows, where both forms co-occur when breeding. This factor is most likely not the direct consequence of alternative food resource use. Rather, it is the result of independent evolution of preferences between the two forms and therefore may be the result of reproductive characteristic displacement leading to increased reproductive isolation.

The occurrence of a few mixed pairs could indicate that these animals can still interbreed, but normally avoid doing so. However, since five mixed pairs were identified among only 19 *T.* cf. *deckerti 'large black'* pairs, this would have to be considered to be a high degree of hybridization, which should normally suffice to homogenize the gene pools between the forms. On the other hand, because significantly differentiated gene pools exist nonetheless (see above), we have to infer that the offspring of such mixed pairs have a reduced fitness under conditions of direct competition. They may even be among the medium sized animals that apparently do not take part in reproduction. This interpretation would be in line with the evidence for divergent selection described above, and has similarly been shown for comparable species pairs in temperate lakes (Lu & Bernatchez 1998). A direct test of reduced hybrid fitness, however, is not possible because there are no morphological characteristics which could be used to assign unequivocally a hybrid status to a given animal under natural conditions. This is a problem which one will presumably encounter in any study where one looks at the very first stages of the differentiation of new forms. However, the indirect test of reduced hybrid fitness, namely the demonstration of the virtual absence of gene flow between the forms, should be considered to be the more significant one, at least with respect to the long-term consequences of the speciation process.

An entirely different explanation for the occurrence for these mixed size pairs would be that the small females in these pairs represent genuine *T.* cf. *deckerti 'large black'*, but at a younger age. Eye size measurements did not help to resolve this because of the statistical fluctuations and the small sample size. However, if this were true, it would imply that the separation of the forms is already so complete that size assortativeness alone is not the most important criterion for forming breeding pairs. Although we cannot strictly refute this hypothesis, we consider it unlikely, since the mixed pairs were only found in the dry season, although the class 'little', which would have been the source of these animals, is more abundant in the wet season (Fig. 5). Furthermore, none of the mixed pairs used a loghole for breeding, which is atypical for the *T.* cf. *deckerti 'large black'* form.

Conclusion

Divergent selection on quantitative traits determining efficient exploitation of inshore and offshore resources is well known from previous studies in fish (Schluter 1993; Robinson & Wilson 1994; Robinson *et al*. 1996; Rundle *et al*. 2000). Similarly, the tendency for assortative, especially size assortative mating is known from many fish species including substrate brooding cichlids (McKaye 1986; Keenleyside *et al*. 1985; Lamprecht & Rebhan 1997; Trifenbach & Itzkowitz 1998) but also closely related pairs of postglacially differentiated sympatric fish assemblages (Nagel & Schluter 1998). We have described here a similar situation, but in contrast to previous studies, for the first time of an unambiguously monophyletic pair of fish forms.

Given that the combination of factors identified here are by no means rare in natural populations (Schluter & McPhail 1992; Schluter 1996b), we expect that it should be possible to identify many further situations of ongoing sympatric speciation. In these, it should be possible to study the hypotheses on the selective forces driving sympatric speciation, as well as the applicability of the respective alternative models (Dieckman & Doebeli 1999; Higashi *et al*. 1999; Kondrashov & Kondrashov 1999; Gavrilets 2000). Candidates will typically be found in recently colonized and isolated areas such as islands and lakes that offer a variety of new niches, especially volcanic crater lakes and postglacial lakes that often harbour closely related fish species pairs of unresolved phylogenetic relationships, which differ in resource utilization as well as size and age of reproduction (Schliewen *et al*. 1994; Orr & Smith 1998). In addition, it seems also possible that many other cases of polymorphic aquatic and even terrestrial animals eventually will exhibit similar patterns of incipient sympatric speciation after colonization events (Steinfartz *et al*. 2000). A particularly pertinent case was recently described for introduced salmons in Lake Washington, where it was shown that the first signs of reproductive isolation between two morphotypes could already be traced after a dozen generations (Hendry *et al*. 2000). The increasing number of results on fast divergence under at least potentially sympatric conditions, in combination with appropriate

models, will likely lead to a broader acceptance of a sympatric paradigm of speciation, which might provide better explanations for the existence of species, subspecies and races of many taxa than allopatric or parapatric paradigms.

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