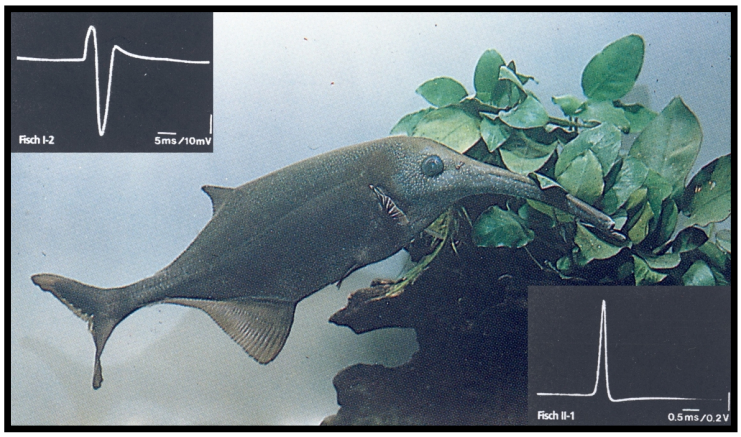
Institut für Biochemie und Biologie

Evolutionsbiologie/Spezielle Zoologie

Adaptive radiation, speciation, and reproductive isolation in African weakly electric fish

(Genus *Campylomormyrus*, Mormyridae, Teleostei)



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1 Introduction

1.1 Systematic and zoogeography of the mormyrid genus *Campylomormyrus*

The mormyrid weakly electric fish (Mormyridae) are endemic to Africa. They comprise one of the most diverse clades of freshwater fish from Africa and the single largest group of electric fish (Alves-Gomes & Hopkins, 1997). Mormyrids belong to the Osteoglossomorpha, considered one of the phylogenetically basal groups of extant teleosts (Lauder & Liem, 1983). Including 180 of the 199 living osteoglossomorpha species, the African Mormyridae are by far the most diverse group within this archaic superorder (Lavoué & Sullivan, 2004). Together with their sister taxon, the monotypic Gymnarchidae, they form the Mormyroidae. Amongst other characters, the monophyly of Mormyroidae is supported by the derived (synapomorphic) presence of electric organs, matched electroreceptors and a greatly enlarged cerebellum (Taverne, 1972). Furthermore, the monophyly of the Mormyroidae as well as the sister relationship between Gymnarchidae and Mormyridae is confirmed by molecular data (see Fig. 1, Alves-Gomes & Hopkins, 1997; Sullivan *et al.*, 2000). However, at and near the species level, the existing morphological and molecular data sets support conflicting phylogenies (Lavoué *et al.*, 2000; Sullivan *et al.*, 2000).

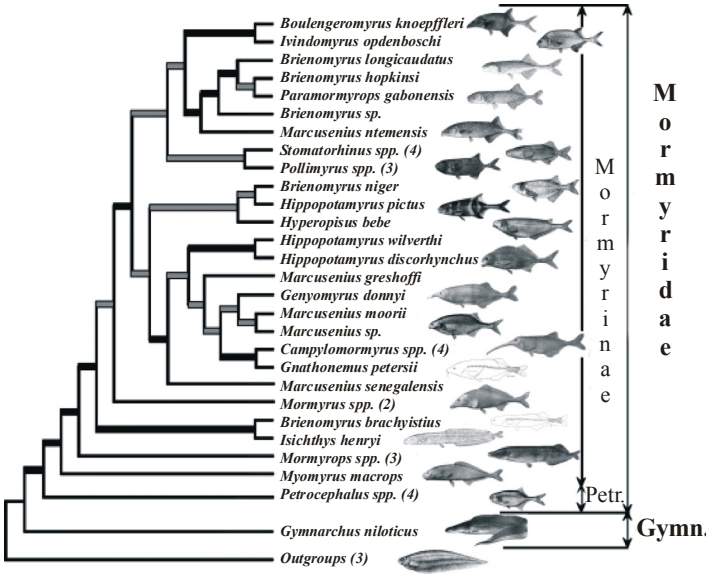

Fig. 1 Proposed relationships of the mormyroid genera based on molecular data (Lavoué *et al.*, 2003). Outgroups were notopterid fish. Black thick branches correspond to well-supported relationships and grey thick branches correspond to weakly supported relationships according to Sullivan *et al.* (2000). Numbers in parentheses refer to the number of examined species for the corresponding monophyletic genera. Abbreviation: Petr. = Petrocephalinae; Gymn. = Gymnarchidae.

At the level of single genera, very little data are available about phylogenetic relationships and processes that might have caused the huge diversification we currently observe in the group. This is especially true for the genus *Campylomormyrus,* whose systematics is extremely puzzling. Based on the analysis of morphological characters, the number of described species fluctuated through the years from 16 (Taverne, 1972) to three (Roberts & Stewart, 1976) and again to 14 (Poll *et al.*, 1982). Most of the species considered nowadays as valid are endemic to a single river system, the Congo and its tributary streams (Fig. 2). Some of them can be found throughout the whole basin, others are restricted to certain areas (Luapula River/Lake Moero or Kasai River). *C. phantasticus* is the only species not present in the Congo Basin, being limited to the Sanaga River (Cameroon). Finally, *C. tamandua* is the most widely distributed species and the only one whose range extends across different river systems including Congo, Volta, Niger, and Tchad/Shari (Gosse, 1984). So far, only two species (*C. numenius and C. tamandua*) have been included in molecular phylogenies (Sullivan *et al.*, 2000; Lavoué *et al.*, 2003).

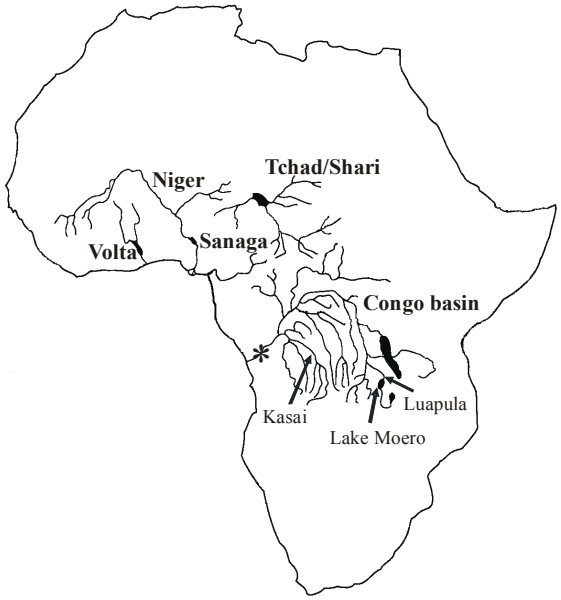

Fig. 2 Geographic location of the African river systems in which *Campylomormyrus* occurs. Most of the species are endemic to the Congo Basin.
* indicates the sampling location Brazzaville/Kinshasa.

1.2 Function of weak electricity in fish

There are several groups of fish with muscles (nerves in case of the knifefish family Apteronotidae) modified into specialized electric organs. The ability to generate electricity from these organs evolved several times independently in the marine electric rays and skates, in the African freshwater Mormyridae and Gymnarchidae, in the South American gymnotiform knifefish (including the electric eel), in several siluriform catfish (including the strongly electric catfish), and in the marine electric stargazers (Moller, 1995). Many species (i.e. marine electric ray, African electric catfish, South American electric eel) are able to

generate strong electricity (between 50 V and 800 V), which delivers sufficient tension and current to serve as a defence mechanism as well as a predatory weapon (Kirschbaum, 1992). In contrast, weakly electric fish discharge electricity at a low voltage (about 1 V) and developed alternative usage of this resource.

African weakly electric fish are able to generate and detect weak electric fields for object detection, orientation, and communication. They detect objects and analyze their electrical properties by measuring distortions in a self-produced electrical field. This process is called active electrolocation (Lissman & Machin, 1958; Bastian, 1994; von der Emde, 1999). During active electrolocation the weakly electric fish can perceive three-dimensional depths and - as a consequence - determine distances (von der Emde, 1999; Schwarz & von der Emde, 2000). Beside this, the electric organ discharge (EOD) of mormyrid fish plays an essential role in social communication (Hopkins & Bass, 1981; Kramer & Kuhn, 1994 (*Campylomormyrus*); Werneyer & Kramer, 2002). The EOD is species-specific (Bass, 1986; Kramer & Kuhn, 1994; Crawford & Huang, 1999) and species recognition based on the species-specific EOD has been proven (Hopkins & Bass, 1981; Moller & Serrier, 1986). It also acts as an indicator of individual identification and discrimination (Crawford, 1992; Friedman & Hopkins, 1996; Crawford & Huang, 1999; Paintner & Kramer, 2003). Furthermore, many mormyrid species show sex differences in EOD at least in the breeding season (Hopkins & Bass, 1981; Westby & Kirschbaum, 1982; Crawford, 1991; Landsman, 1993; Friedman & Hopkins, 1996; Kramer, 1997; Crawford & Huang, 1999). Consequently, mormyrids discriminate between the EODs of conspecifics and heterospecifics and between those of males and females. This discrimination is based on the temporal pattern of the EOD, i.e., the duration and shape of the EOD waveform (Hopkins & Bass, 1981). Therefore, EOD plays a key role in pair formation, mating and social attraction (Bratton & Kramer, 1989; Crawford, 1991; Kramer & Kuhn, 1993). As an effective prezygotic isolation mechanism, the EOD might have been of paramount importance for speciation during the adaptive radiation of mormyrid fish (Sullivan *et al.*, 2002). Previous work aimed at correlating the EOD mode and the phylogeny throughout the entire Mormyridae (Lavoué *et al.*, 2000; Sullivan *et al.*, 2000; Lavoué *et al.*, 2003). However, the importance of EOD as a factor during speciation itself awaits elucidation. As a feature for species recognition, EOD is potentially an important isolation mechanism, which can promote speciation during an adaptive radiation. Due to the possible assortative mating induced by different EOD types, even scenarios based on sympatric and/or parapatric speciation are imaginable.

Apart from species differences, the EOD also changes during ontogeny within species: Larvae of the mormyrid species investigated so far possess a larval electric organ, which produces EOD very different from adults' EOD (Kirschbaum, 1977; Westby & Kirschbaum, 1977, 1978; Denizot *et al.*, 1982). Later in ontogeny, the larval electric organ degenerates and is substituted by the adult's electric organ, which is located in the caudal peduncle (Kirschbaum, 1981). This adult organ initially produces a juvenile EOD, which – in some species - will later on change into the adult EOD. Underestimating or even neglecting this phenomenon might potentially have biased some of the previous studies that tried to take advantage of differences in EODs to clarify the taxonomy of the group. As an example, Schugardt & Kirschbaum (2002) were able to demonstrate that the descriptions of species-specific EODs in *Campylomormyrus* reported in Lovell *et al.* (1997) are comprised by the fact that they had measured juvenile or intermediate EODs for some specimens and sex-specific EODs for adult males and females in other specimens.

1.3 Adaptive radiation in mormyrids

Adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage. It involves the differentiation of a single ancestor into an array of species that inhabit a variety of environments and that differ in the morphological and physiological traits used to exploit those environments. The process includes both speciation and phenotypic adaptation to divergent environments (Schluter, 2000). Adaptive radiation is the outcome of divergent natural selection arising from differences between environments and competition for resources. Many extraordinary examples of adaptive radiations are typical for oceanic archipelagos (the Galapagos finches and tortoises, the Hawaiian honeycreepers, silverswords, and *Drosophilas)* or the East African lakes (cichlids). Adaptive radiations in lacustrine fish are especially well documented (Schliewen *et al.*, 2001; Saint-Laurent *et al.*, 2003; Ostbye *et al.*, 2005). Conversely, almost nothing is known about adaptive radiations in rivers. The same holds true for a consequence of radiation in fish, the so-called fish species flocks (i.e. speciose monophyletic groups with restricted distributions). Again many examples are known from lakes, but examples from rivers are rare. Among the former, lacustrine cichlid species flocks are probably the most renowned and well studied (e.g., Schliewen *et al.*, 1994; Salzburger & Meyer, 2004). Recently, evidences arose suggesting that such explosive speciation phenomena are not limited to lacustrine environments. Sullivan *et al.* (2002; 2004)

proposed weakly electric fish belonging to the Mormyridae as potential model organisms to study species flock evolution in rivers. Restricted distribution, a criterion for species flocks, is hard to prove for mormyrids living in big river systems, which are barely accessible at all points. However, restricted distribution is not a requirement for adaptive radiation, as it is not one of the four criteria defined by Schluter (2000) to detect adaptive radiation. The four criteria are: (1) a recent shared origin of all members of the radiation (common ancestry), (2) an accordance between diverse genetically based phenotypic traits and their divergent environment (phenotype-environment correlation), (3) a benefit of the specific phenotypic trait in its correlated environment (trait utility), and (4) a relatively high rate of lineage splitting (rapid speciation). These four criteria can easily be applied to riverine species. Though it might be hard to definitively prove that mormyrids form a riverine fish species flock, nevertheless mormyrids seem to be very promising candidates to study the phenomenon of adaptive radiation in river systems.

Typically, adaptive radiation either follows a colonization of a new environment or an evolution of a key innovation (Simpson, 1953; Schluter, 2000). Weak electricity could have been such a key innovation that caused the radiation of the African weakly electric fish. As already stated, mormyrids alone include more than 90% of all extant osteoglossomorpha. The acquisition of weak electricity could have opened new ecological opportunities to these fish and, consequently, a new path for evolution. At the same time, the diversification in the EOD might have caused strong assortative mating and, therefore, promoted speciation. There is theoretical evidence for sympatric speciation driven by sexual selection (van Doorn *et al.*, 1998; Doebeli & Dieckmann, 2000; Kirkpatrick & Ravigne, 2002; van Doorn *et al.*, 2004) as well as an increasing number of case studies, especially in fishes (Seehausen & van Alphen, 1999; Lande *et al.*, 2001; Mendelson, 2003; Barluenga *et al.*, 2006). Additionally, the importance of sexual selection for maintaining reproductive barriers between species has been demonstrated (Seehausen *et al.*, 1997). Assuming strong assortative mating based upon the EOD characteristics, sexual selection could have favoured speciation, even in sympatry. However, Barluenga *et al.* (2006) proposed the following criteria for a firm corroboration of the sympatric origin of different species: (1) a sympatric distribution of the most closely related species, (2) genetic evidence for the reproductive isolation among them, (3) their monophyly, and (4) an ecological setting in which allopatric speciation is unlikely. Most of these criteria can also be tested in mormyrids, with the sole exception of the complete exclusion of any allopatric origin of species. This requirement is hard, if not impossible, to prove when dealing with a large river basin like the one considered in this study (Congo

Basin), which had a complex history that could have dramatically altered the connections with adjacent drainages.

1.4 Aims of this study

The major aim of this study is to better understand the relevance of weak electricity in the radiation of African weakly electric fish. *Campylomormyrus* was chosen as a model taxon because of (1) its still unresolved systematics with, possibly, a high number of morphologically very similar yet not described species and, (2) the presence of strikingly divergent electric organ discharge (EOD) waveforms at both intra- and inter-specific levels. Therefore, this genus would offer a unique opportunity to test modes of speciation and achievement of reproductive isolation within a single group.

For a proper understanding of the relationships between speciation and ecological or phenotypic diversifications a robust phylogeny of the genus is an essential pre-requisite. A molecular phylogeny can be considered robust, when unlinked loci (for example a combination of mitochondrial and nuclear genes) yield similar topologies either analyzed separately or combined and when most of the nodes are statistically supported. The reproductive isolation of clades identified by sequence data can be independently confirmed by using microsatellite data. Finally, to assign these groups to nominal species, one can take advantage of quantitative morphometric comparisons with the type specimens of the different species included in the genus.

In order to demonstrate that differences in the EOD waveform played a key role as isolation mechanism during speciation, molecular analyses need to be integrated with observations of the ontogeny of the EOD waveform. Therefore, the maintenance of fish over years is crucial in order to test for congruence between electro-physiological and molecular data, because the EOD might change with maturity. Under the hypothesis that the EOD might be a mechanism promoting isolation, we would expect clearly distinct species-specific adult EODs for mate recognition in closely related species. Under this scenario, the adult EODs should corroborate species definitions but differences should also be particularly prominent between closely related species.

The variation in EOD waveform might have triggered speciation, but must not be the only responsible or causal factor. To test if the radiation in *Campylomormyrus* might have been caused by adaptation to different ecological niches, a close examination of the

morphology is required. If a given feature is associated with the adaptation to a different ecological niche, such a character is expected to vary significantly between distinct species identified by molecular analyses. In this way a possible phenotype-environment correlation could be detected.

In the course of my PhD project, I have used a combination of the approaches mentioned above (molecular data, observations of ontogeny and diversification of EOD waveforms, morphometric analyses of relevant morphological traits) to better comprehend the adaptive radiation of African weakly electric fish and to test the possible roles played by weak electricity and morphological differentiation.

2 Summary of articles

2.1 Summary of article I:

Feulner, P. G. D., Kirschbaum, F. & Tiedemann, R. 2005.
Eighteen microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa.
Molecular Ecology Notes **5**: 446-448.

In this study I developed 18 microsatellite loci specifically designed for the genus *Campylomormyrus.* Microsatellites were initially developed for *Campylomormyrus numenius* and cross species amplification was subsequently tested in additional seven species (*Brienomyrus niger*, *Gnathonemus petersii*, *Hippopotamyrus pictus*, *Mormyrus rume proboscirostris*, and *Petrocephalus soudanensis*). While primers for some mitochondrial (cytochrome *b*, 12S, and 16S rRNA) and nuclear (RAG2, S7 ribosomal protein gene) genes were already available for African weakly electric fish (Alves-Gomes & Hopkins, 1997; Lavoué *et al.*, 2000; Sullivan *et al.*, 2000; Lavoué *et al.*, 2003), no microsatellites have been isolated so far. Contemporary to my work, Arnegard *et al.* (2005) developed five microsatellite loci for another mormyrid genus (*Brienomyrus)*. To successfully complete this work, I entirely performed the lab experiments, isolated the loci, designed the primers, and tested their applicability. I was also able to demonstrate their cross species amplification in the seven closely related species tested for the study. It is important to note that the number of microsatellite loci (18) I've been able to isolate and characterize is well above the number of loci usually developed in analogous studies on non-model organisms. Because these loci are moderately till highly polymorphic (expected heterozygosity ranging between 0.31 and 1.00), they can be used for various applications from population studies to pedigree analyses.

The contributions of the different authors were as follows:
I performed all the lab work, analyzed the data and wrote the manuscript. F. Kirschbaum provided all the samples. R. Tiedemann participated in the discussion of the results and the preparation of the manuscript.

2.2 Summary of article II:

Feulner, P. G. D., Kirschbaum, F., Schugardt C., Ketmaier V. & Tiedemann, R. 2006. Electrophysiological and molecular genetic evidence for sympatrically occuring cryptic species in African weakly electric fish (Teleostei: Mormyridae: *Campylomormyrus*). Molecular Phylogenetics and Evolution **39**: 198-208.

For this study 40 sympatrically occurring specimens of *Campylomormyrus* were maintained during an extended period of time (from two to ten years). Schugardt & Kirschbaum (2002) provided a basis for my further monitoring of morphological as well as electrophysiological changes during ontogeny. In addition, I screened all the specimens for sequence polymorphisms at multiple unlinked loci (mitochondrial cytochrome *b*, S7 ribosomal protein gene, and four flanking regions of unlinked microsatellite loci for a total of 2222 base pairs sequenced). To successfully use these different markers within *Campylomormyrus*, I had to optimize experimental conditions for each of the markers. I found a perfect match between the identification of samples either as *C. tamandua* or *C. numenius* based on morphological characteristics and the molecular data. All *C. tamandua* showed a common electric organ discharge (EOD) type, regardless of age and sex. Contrary to this, in *C. numenius* one identical EOD waveform observed in all juveniles differentiated into three different male adult EOD types. Two of these EOD types formed well-supported clades in the phylogenetic analysis. To clarify relationships within *C. numenius*, I took advantage of the microsatellite loci, which I applied as an independent line of evidence to affirm classification into three groups. The correct assignment and the high pairwise F_{ST}-values support the hypothesis of three reproductively isolated groups within *C. numenius*. The combination of these observations supported the idea that the genus *Campylomormyrus* comprises a set of cryptic species living in sympatry. Furthermore, the findings of dramatic EOD changes at maturity point towards the importance of this mechanism for species recognition in the course of mating. Even if direct evidence from behavioural experiments is still lacking, this is the first indication of species-specific mate recognition based on the EOD waveform in the genus *Campylomormyrus*. Assuming strong assortative mating based upon EOD characteristics, sexual selection could have triggered speciation, possibly even in sympatry.

The contributions of the different authors were as follows:

I performed all the lab work, analyzed the data and wrote the manuscript. C. Schugardt and F. Kirschbaum began the observation of ontogenetic changes in EODs, later on I also performed this part. V. Ketmaier helped with the phylogenetic analyses. Together with R. Tiedemann and F. Kirschbaum, he also took part in the discussion of data and the preparation of the manuscript.

2.3 Summary of article III:

Feulner, P. G. D., Kirschbaum, F., Mamonekene V., Ketmaier, V. & Tiedemann, R. 2006. Adaptive radiation in African weakly electric fish (Teleostei: Mormyridae: *Campylomormyrus*): a combined molecular and morphological approach. Journal of Evolutionary Biology submitted.

In this study I combined analyses of molecular and geometric morphometric data to elucidate the following three issues. First, I could build up a robust phylogeny of 106 *Campylomormyrus* specimens, occurring in sympatry in the area near Brazzaville/Kinshasa (Congo; Fig.1). For this purpose I performed a field expedition to Brazzaville (Republic of the Congo) and conducted an extensive sampling in the area. To my knowledge this is the first comprehensive molecular phylogeny for the genus *Campylomormyrus*, including at least six distinct clades living in sympatry. Such a robust phylogenetic hypothesis is an essential pre-requisite for a better understanding of the relationship between speciation and ecological/phenotypic diversification in this group. Second, due to a morphological comparison with the type material of all the known species deposited at the Royal Museum of Central Africa (MRAC Tervuren, Belgium) a taxonomic classification of our samples was possible. Due to an assignment test based on a canonical variates analysis (CVA) of the type material, the identified phylogenetic clades were associated with described species. Third, according to a CVA of the sympatrically occurring specimens, I could figure out that most of the variation between the phylogenetic clades is due to differences in the elongated trunk-like snout. In this way I was able to link under a single unifying framework taxonomy, morphology and ecological traits. With differences in the morphology of the trophic apparatus, I indeed identified a feature that suggests possible ecological differences among the species. Because these fish feed on insect larvae, which burrow in holes and interstitial spaces at the bottom of rivers, differences in the trunk shape will determine differential accessibility to food sources. Considering also the result of the previous article, these results suggest that the (adaptive) radiation in the genus *Campylomormyrus* may have been caused by the diversification of the snout, which, in turn, allowed a different exploitation of food sources. Such an ecological diversification, coupled with the already identified differences in the electric organ discharge (EOD) waveform, may well be amongst the main phenomena responsible of the wide diversity displayed by this group of electric fish. More explicitly, this

indicates that the adaptive radiation of the African weakly electric fish was triggered by the EOD as a reproductive isolation mechanism and caused by ecological adaptation to different food sources.

The contributions of the different authors were as follows:
I performed all the lab work, analyzed the data and wrote the manuscript. V. Mamonekene supported me during my field trip to Brazzaville (Republic of the Congo). His support has been crucial to the success of the samplings. I also performed the morphometric analysis on my own and examined all the museum material (MRAC, Tervuren, Belgium) including the type specimens. V. Ketmaier helped with the phylogenetic analyses. Together with R. Tiedemann and F. Kirschbaum, he also took part in the discussion of data and the preparation of the manuscript.

3 Discussion

3.1 Phylogeny of sympatrically occurring *Campylomormyrus* species

To frame broad evolutionary questions, a robust phylogenetic hypothesis is an essential pre-requisite. This is the first study aimed to produce a phylogenetic hypothesis for the genus *Campylomormyrus*. In this study we concentrated on the *Campylomormyrus* species occurring in sympatry near Brazzaville/Kinshasa (Fig. 2). This was due to our overall aim to better understand the adaptive radiation of these sympatrically occurring species. To build up a robust phylogenetic hypothesis, we established different marker systems (mitochondrial cytochrome *b* and nuclear S7 ribosomal protein gene) for the genus *Campylomormyrus* (Feulner *et al.*, 2006a). Additionally, we used our self-established microsatellite loci (Feulner *et al.*, 2005) as an independent line of evidence to prove reproductive isolation between the identified phylogenetic clades. This way we found convincing evidences for the presence of at least six sympatrically occurring *Campylomormyrus* species (Feulner *et al.*, 2006b).

The most distantly related clade, clade F (Fig. 2, Feulner *et al.*, 2006b; Fig. 4, Feulner *et al.*, 2006a), can be unambiguously attributed to *C. tamandua.* This is supported by multiple morphological comparisons with the type material of all to-date described species included in the genus (Feulner *et al.*, 2006b). Furthermore, *C. tamandua* is characterised by its peculiar colour pattern of striking yellow bands (Bigorne, 1990). As a result of our ontogenetic observations, we also know that this species is uniform over time in its morphology (unlike *C. numenius*; see below), as well as in its electric organ discharge (EOD) waveform (Fig. 2A, Feulner *et al.*, 2006a). Nevertheless, looking at the phylogenetic trees (Fig. 2, Feulner *et al.*, 2006b; Fig. 4, Feulner *et al.*, 2006a) *C. tamandua* exhibits a large amount of intra-group genetic heterogeneity, comparable to that observed within other distinct clades (e.g. clade A and B). Such a heterogeneity is mainly due to sequence variations of the mitochondrial cytochrome *b* gene (Fig. 3, Feulner *et al.*, 2006a). However, the mean expected heterozygosity based on microsatellite data differs not significantly from the values displayed by the other clades (Table 3, Feulner *et al.*, 2006b). In addition, at the microsatellite level, this clade resulted to be quite homogeneous, as visually evident in the STRUCTURE analysis, whereas all other clades show a much greater degree of heterogeneity (Fig. 3, Feulner *et al.*, 2006b). Therefore, our data strongly suggest that *C. tamandua* is a single interbreeding species. One possible explanation for the high within-species sequence variation at the

mitochondrial level might rely on the wide geographic distribution of this species. Apart from the Congo Basin, it also occurs in the Niger, Tchad/Shari and Volta river systems (Gosse, 1984). Possibly, the population size of this species might be large enough to maintain a number of distinct mitochondrial lineages, though being homogeneous at the microsatellite loci. Obviously, there might be a variety of historical and/or ecological scenarios behind this hypothesis. To properly clarify this issue, additional samplings from the entire distribution range of *C. tamandua* would be necessary, which was clearly beyond the scope of this study.

Basal in the phylogenetic tree, there is a single specimen (K15) and a clade comprised of three specimens (clade E), both with poorly supported or unresolved placements (Fig. 2, Feulner *et al.*, 2006b). By morphological comparison with the type material, K15 is assigned to *C. bredoi* and clade E to *C. elephas* (Feulner *et al.*, 2006b). The assignment of K15 to *C. bredoi* is problematic, because this specimen shows some particular features, e.g. a relatively narrow elongated caudal peduncle. Furthermore, *C. bredoi* is only known from the headwater region of the Congo River and has never been found in the section of the river we sampled (Lower Congo). An extensive and focused sampling in the area coupled with an accurate analysis of taxonomic characters would help to clarify the status of this single specimen, which could possibly be a new species. Clade E is made up of three individuals and is clearly monophyletic. Whether these three specimens represent a single interbreeding group or not is hard to evaluate. The high genetic divergence within the clade and the slight but consistent morphological differences among the individuals suggest that they could represent different species, though forming a monophyletic group.

Clade D is genetically clearly differentiated and can be unambiguously identified as *C. tshokwe* on morphological grounds. This clade is sister to a large group of individuals initially attributed to *C. numenius*. Our genetic analyses detected a large amount of genetic heterogeneity within this *C. numenius* group that could be easily split into at least three clades (A, B, and C). Interestingly, all juvenile specimens of this group possess a uniform morphology and EOD waveform, while they develop distinct differences in either traits at maturity (Fig. 2B and 4, Feulner *et al.*, 2006a). Our observations of the EOD development during growth, of the EOD differences between adult morphs, and in light of the correct assignment of the different morphs based on microsatellites to clades identified by unlinked sequence data, all support the hypothesis of the presence of three cryptic species hidden within the group. Taking advantage of the multiple comparisons we performed between adult individuals of the group and the museum type material, we were able to attribute clades to the following species: clade A to *C. rhynchophorus*, clade B to *C. numenius*, and clade C to *C.*

compressirostris (Feulner *et al.*, 2006b). But while morph A and B show radical changes towards clearly distinct male adult EOD types and form well supported phylogenetic clades, morph C does not form a single monophyletic clade. This is at odds with our microsatellite data, as they indicate a single "morph C" species, with a high degree of genetic heterogeneity within it (Table 1, Feulner *et al.*, 2006a), possibly due to a larger effective population size. In addition, fish of this species show little change in their morphological characteristics during growth compared to the other two morphs (A and B) and their ontogenetic change in EOD waveform is only slight (Fig. 2B and 4, Feulner *et al.*, 2006a). In addition, four individuals (K13, K14, K16, K42) sampled during a field trip to Brazzaville, Republic of the Congo, turned out to represent an additional morph within clade C. The distinctiveness of these four individuals is supported by microsatellites but is not visible in the phylogenetic analysis based on sequence data. Unfortunately, we were not able to record EODs from these four fish, nevertheless morphological analyses assigned them to *C. curvirostris* (Feulner *et al.*, 2006b).

Our phylogeny suggests that clade A and B might have emerged from the heterogeneous clade C. The non-univocal results we obtained on the remaining individuals placed in the clade C may indicate that the differentiation within this group is not completed yet. This view is supported by the fact that neither morphology and EOD nor genetic data (though including a variety of markers with different evolutionary rates) have been able to consistently recover an unambiguous pattern of relationships. Interestingly, clades A and B considerably differ from clade C in their morphology (Fig. 5a, Feulner *et al.*, 2006b) as well as in the EOD waveform (Fig. 2B and 4, Feulner *et al.*, 2006a). This is not the case with the clearly distinct clade F (*C. tamandua*). This species does not show such pronounced differences. Its EOD is quite similar to that of individuals belonging to clade C. These evidences strongly support our initial hypothesis (see point 1.4) that if the difference in the EOD is an important mechanism promoting reproductive isolation, such a difference should be more prominent among closely related species.

3.2 Electric organ discharge (EOD) as reproductive isolation mechanism

It has been hypothesised that characteristics of the EOD waveform function as a reproductive isolation mechanism in mormyrids (Moller & Brown, 1990; Arnegard *et al.*, 2005). In our comprehensive analysis, we could evaluate the stability of the EOD waveform in the genus *Campylomormyrus* over time after maturity, even under changing environmental

conditions such as water conductivity (Feulner *et al.*, 2006a). Behavioural studies to explore individual signal and pattern recognition and well-controlled breeding experiments are still lacking for this genus. Nevertheless, species recognition based on species-specific EODs has already been proven for other mormyrid genera (Hopkins & Bass, 1981; Moller & Serrier, 1986) and there is already first knowledge of the reproductive strategy of *Campylomormyrus* (Kirschbaum & Schugardt, 2002). Furthermore, there are numerous behavioural studies stressing the importance of the EOD in the context of social communication (Hopkins & Bass, 1981; Bratton & Kramer, 1989; Crawford, 1991; Kramer & Kuhn, 1994 (*Campylomormyrus*); Scheffel & Kramer, 2000; Werneyer & Kramer, 2002; Hanika & Kramer, 2005). In contrast to a study on the genus *Brienomyrus* (Arnegard *et al.*, 2005), we found convincing genetic evidence for the existence of reproductive isolation among morphs displaying different EOD types. These results are based on the phylogenetic analyses of sequence variation at different independent markers and are furthermore confirmed by a microsatellite analysis of 16 unlinked loci (Feulner *et al.*, 2005; Feulner *et al.*, 2006b; Feulner *et al.*, 2006a). For the genus *Brienomyrus,* an EOD analysis demonstrated that allopatric taxa tend to show more overlaps in their EOD waveforms than sympatric taxa (Arnegard & Hopkins, 2003). This is a first indication that signal distinction might be enhanced among sympatric species, relative to the allopatric ones. We found a similar trend when we compared genetic data and EOD differentiation. The monophyletic group consisting of clades A, B, and C shows a pronounced difference in their EODs. On the other hand, *C. tamandua,* a genetically distinct species, possesses a EOD waveform very similar to that of clade C (Fig. 2B and 4, Feulner *et al.*, 2006a). This finding is consistent with our hypothesis that the EOD might function as a reproductive isolation mechanism among these clades (A, B, and C). Furthermore, the ontogenetic shift in EOD observed especially in adult males underscores the importance of this trait for mate recognition (Fig. 2B and 4, Feulner *et al.*, 2006a). This might be a first indication that clades A, B, and C split from each other in sympatry, while *C. tamandua* might have originated in allopatry. This hypothesis is in agreement with the current distribution of these taxa (Fig.1 and Table 1, Feulner *et al.*, 2006b). Nevertheless, behavioural studies and breeding experiments as well as samplings of *C. tamandua* in geographically distant (allopatric) locations are still needed to clarify this issue.

Whether EOD divergence is the cause or the effect of speciation remains a point still to be evaluated. Either it might have played a key role as reproductive isolation mechanism during a possible sympatric speciation, or it might have diverged after speciation driven by other factors. Nevertheless, there are evidences from other studies supporting our idea of

reproductive isolation via assortative mating based on EOD characteristics. In cichlids, colour-assortative mating is the causal factor promoting reproductive isolation (van Oppen *et al.*, 1998; Wilson *et al.*, 2000; Maan *et al.*, 2004; Salzburger *et al.*, 2006). Furthermore, the heritability of female preference in cichlids (Haesler & Seehausen, 2005) and the importance of sexual selection for maintaining reproductive barriers between species (Seehausen *et al.*, 1997; Salzburger *et al.*, 2006) have also been demonstrated. Similarly, there are theoretical studies stressing the importance of sexual selection during speciation (van Doorn *et al.*, 1998; Kirkpatrick & Ravigne, 2002). At the same time, there is a general consensus that sympatric speciation by sexual selection alone is unlikely (Arnegard & Kondrashov, 2004; Kirkpatrick & Nuismer, 2004; van Doorn *et al.*, 2004). Consequently, a proper evaluation of additional factors that might also have been involved in the potential sympatric speciation of weakly electric fish is required.

3.3 Morphology as an indicator for adaptation to different ecological niches

Taking advantage of a morphometric analysis based on eleven landmarks we could demonstrate that in *Campylomormyrus* phylogenetic clades recovered by genetic data also differ in their morphology (Fig. 4 and 5, Feulner *et al.*, 2006b). All these phylogenetic clades, which possess unique EOD types, show a clear and consistent differentiation in their shape morphology. The pattern of morphological differentiation nearly perfectly matches the patterns we recovered for EOD and genetic variation. Once again, clades A and B are more explicitly differentiated than *C. tamandua* (Fig. 5a, Feulner *et al.*, 2006b), strengthening our hypothesis that a combination of allopatric and sympatric speciation processes might be responsible for the differentiation we presently observe in the genus. Visualization of the shape differences among clades reveals that these are almost entirely confined to changes in the trunk-like snout. Variations are apparent in the length and in the angle relative to the body axis (Fig. 4b, Feulner *et al.*, 2006b). *Campylomormyrus* feed on insect larvae which burrow into, or hid within interstitial spaces and holes in clay sediments of river channels (Marrero & Winemiller, 1993). Consequently, different shapes of the snout might have led to differential exploitations of food sources. Testing this hypothesis would require the analyses of stomach contents, which could not be accommodated in the present study. We nevertheless believe that the variation in the shape of the snout we have evidenced might at least be a first (though indirect) indication of the presence of different feeding strategies in *Campylomormyrus*.

We therefore hypothesise that, besides sexual selection, also ecological factors (i.e. niche shifts) played an important role during the adaptive radiation of these fish. When reproductive isolation evolves as a consequence of resource-based divergent natural selection and resource competition, the process can be regarded as ecological speciation (Schluter, 1996a,b). In ecological speciation processes, barriers to gene flow evolve between populations as a result of ecologically-based divergent natural selection (Schluter, 2000; Rundle & Nosil, 2005). There is an increasing amount of evidence, especially in fish, suggesting that trophic differentiation is the main factor behind ecological speciation (Denoel *et al.*, 2004; Horstkotte & Strecker, 2005; Kahilainen & Ostbye, 2006); other studies could also correlate such phenotypic differences to genetic divergence (Gislason *et al.*, 1999; Schliewen *et al.*, 2001; Barluenga *et al.*, 2006; Kidd *et al.*, 2006). Moreover, there is theoretical evidence suggesting a prominent role for ecologically driven speciation in sympatry (Dieckmann & Doebeli, 1999), as well as case studies, which demonstrate that ecological rather than geographic separation has resulted in the evolution of reproductive barriers (Hollander *et al.*, 2005). Nevertheless, ecological speciation is merely a hypothesis about mechanisms of selection and does not take into account any consideration of the geographical arrangement of populations (Schluter, 1996b). Consequently, the concept of ecological speciation can also be applied to a riverine context as that under study, where species' ranges might either be completely sympatric, fully allopatric or a mixture of these two alternate situations.

3.4 Adaptive radiation in African weakly electric fish

Weak electricity must have been a key innovation for the whole group of mormyrids. Certainly, there has been a rapid and extensive speciation within mormyrids if compared to their sister taxon, the monotypic gymnarchids and to the overall species-poor group of osteoglossomorpha. In contrast to the monotypic gymnarchids, which possess a wave type electric organ discharge (EOD), mormyrids produce a pulse type EOD. This difference might have been one of the causal factors, if not the crucial one, triggering the radiation of the group. The achievement of weak electricity could have opened new ecological opportunities and, consequently, a new path for evolution. We found strong evidence that the mormyrid pulse type EOD potentially functions as an effective reproductive isolation mechanism via assortative mating, even in sympatry. There are models suggesting that sympatric speciation

can be driven by sexual selection alone (Higashi *et al.*, 1999; Lande *et al.*, 2001). Such a scenario is nevertheless questionable, because some ecological differentiations are also required to stabilize the coexistence of incipient species through frequency-dependent selection (Arnegard & Kondrashov, 2004; Kirkpatrick & Nuismer, 2004; van Doorn *et al.*, 2004). Sexual selection would promote the splitting of species, whereas niche differentiation prevents the extinction of species by competitive exclusion (van Doorn *et al.*, 1998). Phenotypic differences evolving in sympatry are primarily the outcome of markedly different selection pressures in alternative habitats; this especially holds true when competition for food is involved (Schluter, 1996a). For the genus *Campylomormyrus,* we could identify these phenotypic differences in the length and relative angle of the trunk-like snout. A link between this variation and a possible displacement of the trophic niche seems straightforward. Therefore, we identified not only a possible mechanism for sexual selection, but also a phenotypic feature on which disruptive selection could act. According to Kondrashov & Kondrashov (1999) sympatric speciation from disruptive selection and assortative mating is even possible, when variability of fitness and mate choice depends on different, independently inherited quantitative traits. This seems to be the case for *Campylomormyrus*. Here, the two independent traits would be the shape of the snout and the EOD characteristics, which in turn depend on the morphology and physiology of the electrocytes that constitute the mormyrid electric organ (Bass, 1986; Arnegard & Hopkins, 2003). Apart from that, there are well studied examples demonstrating that different ecological adaptations in combination with assortative mating may lead to sympatric speciation (Schliewen *et al.*, 2001; Barluenga *et al.*, 2006). Sympatric speciation is hard to prove in a riverine context, especially in a basin as vast as the Congo, where migration events from nowadays-unconnected river systems might have occurred sometimes in the past. Due to the intrinsic characteristics of the riverine system we investigated, we are not in the position to assume an exclusively sympatric origin for our species, as required by Barluenga *et al.* (2006) criteria (see point 1.3). Nevertheless, we believe that this study provides a convincing scenario for a parapatric, if not fully sympatric origin of some of the *Campylomormyrus* species we analysed. Overall, our findings support the view that disruptive selection can be a potent driving force of speciation also in parapatry, even though adaptive speciation requires ecological contact between diverging lineages and is therefore often equated with sympatric speciation (Doebeli & Dieckmann, 2003; Doebeli *et al.*, 2005). But even though sympatry could not be satisfactorily shown, we found a convincing agreement with Schluter's four criteria for an adaptive radiation (Feulner *et al.*, 2006b): (1) The genus *Campylomormyrus* is clearly characterised by its own morphology and

our phylogenetic analyses (Feulner *et al.*, 2006b), as well as previous studies based on an higher numbers of mormyrid genera (Alves-Gomes & Hopkins, 1997; Lavoué *et al.*, 2000; Sullivan *et al.*, 2000; Lavoué *et al.*, 2003) confirmed its monophyletic status (criterion of "common ancestry"). (2) All species identified on genetic grounds significantly differ in an important morphological trait, i.e. their trophic apparatus. This is a first indication for a correlation with their feeding habitat (criterion of "phenotype-environment correlation"). (3) Because the morphological difference is pronounced in the shape of the snout, an impact with the accessibility of specific food sources is supposable (criterion of "trait utility"). (4) Compared to contemporary clades, there is a high rate of lineage splitting both in the mormyrids in general, in reference to the Gymnarchidae and the Osteoglossomorpha, as well as within the genus *Campylomormyrus*, especially in the group composed of clades A to D (criterion of "rapid speciation"). In conclusion, this study provides a first robust phylogenetic hypothesis for the genus *Campylomormyrus*. This hypothesis is based on different independent sequence markers and further confirmed by microsatellite data as an independent line of evidence. Additionally, my investigations show convincing evidence for an adaptive radiation within African weakly electric fish triggered by sexual selection. In mormyrid fish sexual selection can act via assortative mating based on EOD waveform characteristics, but the EOD might just be the trigger not the cause for the radiation. We suppose that speciation is caused by disruptive selection of morphological traits related to ecological (trophic) differentiation.

4 Abstract

The ultimate aim of this study is to better understand the relevance of weak electricity in the adaptive radiation of the African mormyrid fish. The chosen model taxon, the genus *Campylomormyrus*, exhibits a wide diversity of electric organ discharge (EOD) waveform types. Their EOD is age, sex, and species specific and is an important character for discriminating among species that are otherwise cryptic. After having established a complementary set of molecular markers, I examined the radiation of *Campylomormyrus* by a combined approach of molecular data (sequence data from the mitochondrial cytochrome *b* and the nuclear S7 ribosomal protein gene, as well as 18 microsatellite loci, especially developed for the genus *Campylomormyrus*), observation of ontogeny and diversification of EOD waveform, and morphometric analysis of relevant morphological traits. I built up the first convincing phylogenetic hypothesis for the genus *Campylomormyrus*. Taking advantage of microsatellite data, the identified phylogenetic clades proved to be reproductively isolated biological species. This way I detected at least six species occurring in sympatry near Brazzaville/Kinshasa (Congo Basin). By combining molecular data and EOD analyses, I could show that there are three cryptic species, characterised by their own adult EOD types, hidden under a common juvenile EOD form. In addition, I confirmed that adult male EOD is species-specific and is more different among closely related species than among more distantly related ones. This result and the observation that the EOD changes with maturity suggest its function as a reproductive isolation mechanism. As a result of my morphometric shape analysis, I could assign species types to the identified reproductively isolated groups to produce a sound taxonomy of the group. Besides this, I could also identify morphological traits relevant for the divergences between the identified species. Among them, the variations I found in the shape of the trunk-like snout, suggest the presence of different trophic specializations; therefore, this trait might have been involved in the ecological radiation of the group. In conclusion, I provided a convincing scenario envisioning an adaptive radiation of weakly electric fish triggered by sexual selection via assortative mating due to differences in EOD characteristics, but caused by a divergent selection of morphological traits correlated with the feeding ecology.

5 Abstract (German version)

Das übergreifende Ziel dieser Arbeit ist das bessere Verständnis der Bedeutung der schwachen Elektrizität für die adaptive Radiation der Mormyriden Afrikas. Das gewählte Modell-Taxon, die Mormyriden-Gattung *Campylomormyrus*, zeigt eine große Vielfalt an elektrischen Entladungsformen. Diese Entladungsformen sind alters-, geschlechts-, sowie artspezifisch und ein wichtiges Unterscheidungskriterium von ansonsten kryptischen Arten. Ich untersuchte die Radiation der Gattung *Campylomormyrus* anhand eines kombinierten Ansatzes aus molekularen Daten (Sequenzdaten des mitochondrialen Cytochrom *b* Gens und des nukleären S7 ribosomalen Protein-Gens, sowie 18 Mikrosatelliten, speziell von mir entwickelt für die Gattung *Campylomormyrus*), Beobachtungen der Ontogenie und der Diversifikation der Entladungsform, sowie morphometrische Auswertungen der Gestalt relevanter morphologischer Merkmale.

Ich erstellte eine erste phylogenetische Hypothese für die Gattung *Campylomormyrus*. Durch meine Mikrosatellitendaten, die als unabhängiger Beweis dienten, konnte ich zeigen, dass die identifizierten phylogenetischen Gruppen reproduktiv isolierte biologische Arten sind. Auf diese Weise konnte ich mindesten sechs Arten nachweisen, die in Sympatrie nahe Brazzaville/Kinshasa (Kongo-Becken) vorkommen. Durch die Übereinstimmung von molekularen Daten und Entladungsformen konnte ich drei kryptische Arten unterscheiden, die sich hinter einheitlichen juvenilen Entladungsformen verbergen, sich aber zu verschiedenen adulten Formen entwickelten. Des Weiteren konnte ich zeigen, dass die adulten männlichen Entladungsformen artspezifisch sind und, dass der Unterschied in der Entladungsform zwischen nah verwandten Arten deutlicher ausgeprägt ist als zwischen entfernter verwandten Arten. Dieses Ergebnis und die Beobachtung, dass sich die Entladungsform bei der Geschlechtsreife ändert, weisen darauf hin, dass die Entladungsform als reproduktiver Isolationsmechanismus dient. In einer morphometrischen Gestalt-Analyse verglich ich das Typen-Material der beschriebenen Arten mit den zuvor ermittelten reproduktiv isolierten Gruppen, um auf diese Weise deren Art zu bestimmen. Überdies konnte ich maßgebliche morphologische Unterscheidungsmerkmale identifizieren. Diese äußern sich hauptsächlich in der Gestalt der rüsselartigen Schnauze, könnten daher mit einer trophischen Spezialisierung einhergehen und eine ökologische Artbildung ermöglichen. Zusammenfassend entwickelte ich, in Übereinstimmung mit anderen Untersuchungen und theoretischen Überlegungen, eine plausible Hypothese einer adaptiven Radiation der schwach-elektrischen Fische Afrikas,

ausgelöst durch sexuelle Selektion. Diese wirkt durch assortative Verpaarung, basierend auf Charakteristika der elektrischen Entladungsform. Verursacht wird der Prozess der adaptiven Radiation jedoch durch divergierende Selektion morphologischer Merkmale, die in Bezug zur Nahrungsökologie stehen.

6 References

Alves-Gomes, J. & Hopkins, C. D. 1997. Molecular insights into the phylogeny of Mormyriform fishes and the evolution of their electric organs. *Brain Behav. Evol.* **49**: 324-350.

Arnegard, M. E. & Hopkins, C. D. 2003. Electric signal variation among seven blunt-snouted Brienomyrus species (Teleostei: Mormyridae) from a riverine species flock in Gabon, Central Africa. *Environ. Biol. Fishes* **67**: 321-339.

Arnegard, M. E. & Kondrashov, A. S. 2004. Sympatric speciation by sexual selection alone is unlikely. *Evolution* **58**: 222-237.

Arnegard, M. E., Bogdanowicz, S. M. & Hopkins, C. D. 2005. Multiple cases of striking genetic similarity between alternate electric fish signal morphs in sympatry. *Evolution* **59**: 324-343.

Barluenga, M., Stolting, K. N., Salzburger, W., Muschick, M. & Meyer, A. 2006. Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature* **439**: 719-723.

Bass, A. H. 1986. Species differences in electric organs of mormyrids: substrates for species-typical electric organ discharge waveforms. *J. Comp. Neurol.* **244**: 313-330.

Bastian, J. 1994. Electrosensory organisms. *Physics Today* **47**: 30-37.

Bigorne, R. (1990) Mormyridae. In: *Fauna des Poissons d'Eaux Douces et Saumâtre de l'Afrique de l'Ouest* (eds. Lévêque, C., Paugy, D. & Teugel, G. G.), pp. 122-184. ORSTOM, MRAC, Paris, Tervuren.

Bratton, B. O. & Kramer, B. 1989. Patterns of the electric organ discharge during courtship and spawning in the mormyrid fish, *Pollimyrus isidori*. *Behav. Ecol. Sociobiol.* **24**: 349-368.

Crawford, J. D. 1991. Sex recognition by electric cues in a sound-producing mormyrid fish, *Pollimyrus isidori*. *Brain Behav. Evol.* **38**: 20-38.

Crawford, J. D. 1992. Individual and sex specificity in the electric organ discharges of breeding mormyrid fish (*Pollimyrus isidori*). *J. Exp. Biol.* **164**: 79-102.

Crawford, J. D. & Huang, X. F. 1999. Communication signals and sound production mechanisms of mormyrid electric fish. *J. Exp. Biol.* **202**: 1417-1426.

Denizot, J. P., Kirschbaum, F., Westby, G. W. M. & Tsuji, S. 1982. On the development of the adult electric organ in the mormyrid fish *Pollimyrus isidori* (with special focus on the innervation). *J. Neurocytol.* **11**: 913-934.

Denoel, M., Schabetsberger, R. & Joly, P. 2004. Trophic specialisations in alternative heterochronic morphs. *Naturwissenschaften* **91**: 81-84.

Dieckmann, U. & Doebeli, M. 1999. On the origin of species by sympatric speciation. *Nature* **400**: 354-357.

Doebeli, M. & Dieckmann, U. 2000. Evolutionary branching and sympatric speciation caused by different types of ecological interactions. *Am. Nat.* **156**: S77-S101.

Doebeli, M. & Dieckmann, U. 2003. Speciation along environmental gradients. *Nature* **421**: 259-264.

Doebeli, M., Dieckmann, U., Metz, J. A. J. & Tautz, D. 2005. What we have also learned: Adaptive speciation is theoretically plausible. *Evolution* **59**: 691-695.

Feulner, P. G. D., Kirschbaum, F. & Tiedemann, R. 2005. Eighteen microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa. *Mol. Ecol. Notes* **5**: 446-448.

Feulner, P. G. D., Kirschbaum, F., Mamonekene, V., Ketmaier, V. & Tiedemann, R. 2006b. Adaptive radiation in African weakly electric fish (Teleostei: Mormyridae: Campylomormyrus): a combined molecular and morphological approach. *J. Evol. Biol.* submitted.

Feulner, P. G. D., Kirschbaum, F., Schugardt, C., Ketmaier, V. & Tiedemann, R. 2006a. Electrophysiological and molecular genetic evidence for sympatric speciation in African weakly electric fishes (Teleostei: Mormyridae: *Campylomormyrus*). *Mol. Phylogenet. Evol.* **39**: 198-208.

Friedman, M. A. & Hopkins, C. D. 1996. Tracking individual mormyrid electric fish in the field using electric organ discharge waveforms. *Anim. Behav.* **51**: 391-407.

Gislason, D., Ferguson, M., Skulason, S. & Snorrason, S. S. 1999. Rapid and coupled phenotypic and genetic divergence in Icelandic Arctic char (Salvelinus alpinus). *Can. J. Fish. Aquat. Sci.* **56**: 2229-2234.

Gosse, J.-P. (1984) Mormyriformes. In: *Check-list of the Freshwater Fishes of Africa* (eds. Daget, J., Gosse, J.-P. & Thys van den Audenaerde, D. F. E.), pp. 63-124. ORSTOM, Paris, MRAC, Tervuren.

Haesler, M. P. & Seehausen, O. 2005. Inheritance of female mating preference in a sympatric sibling species pair of Lake Victoria cichlids: implications for speciation. *Proc. R. Soc. Biol. Sci. Ser. B* **272**: 237-245.

Hanika, S. & Kramer, B. 2005. Intra-male variability of its communication signal in the weakly electric fish, Marcusenius macrolepidotus (South African form), and possible functions. *Behaviour* **142**: 145-166.

Higashi, M., Takimoto, G. & Yamamura, N. 1999. Sympatric speciation by sexual selection. *Nature* **402**: 523-526.

Hollander, J., Lindegarth, M. & Johannesson, K. 2005. Local adaptation but not geographical separation promotes assortative mating in a snail. *Anim. Behav.* **70**: 1209-1219.

Hopkins, C. D. & Bass, A. H. 1981. Temporal coding of species recognition signals in an electric fish. *Science* **212**: 85-87.

Horstkotte, J. & Strecker, U. 2005. Trophic differentiation in the phylogenetically young *Cyprinodon* species flock (Cyprinodontidae, Teleostei) from Laguna Chichancanab (Mexico). *Biol. J. Linn. Soc.* **85**: 125-134.

Kahilainen, K. & Ostbye, K. 2006. Morphological differentiation and resource polymorphism in three sympatric whitefish Coregonus lavaretus (L.) forms in a subarctic lake. *J. Fish Biol.* **68**: 63-79.

Kidd, M. R., Kidd, C. E. & Kocher, T. D. 2006. Axes of differentiation in the bower-building cichlids of Lake Malawi. *Mol. Ecol.* **15**: 459-478.

Kirkpatrick, M. & Ravigne, V. 2002. Speciation by natural and sexual selection: Models and experiments. *Am. Nat.* **159**: S22-S35.

Kirkpatrick, M. & Nuismer, S. L. 2004. Sexual selection can constrain sympatric speciation. *Proc. R. Soc. Lond. B Biol. Sci.* **271**: 687-693.

Kirschbaum, F. 1977. Electric-organ ontogeny - distinct larval organ precedes adult organ in weakly electric fish. *Naturwissenschaften* **64**: 387-388.

Kirschbaum, F. (1981) Ontogeny of both larval electric organ and electromotoneurones in *Pollimyrus isidori* (Mormyridae, Teleostei). In: *Advances in Physiological Sciences* (eds. Szabo, T. & Czeh, G.), pp. 129-157. Pergamon Press, Oxford and New York.

Kirschbaum, F. 1992. Electric fishes. *Aqua Geographia* **1**: 59-70.

Kirschbaum, F. & Schugardt, C. 2002. Reproductive strategies and developmental aspects in mormyrid and gymnotiform fishes. *J. Physiol. Paris* **96**: 557-566.

Kondrashov, A. S. & Kondrashov, F. A. 1999. Interactions among quantitative traits in the course of sympatric speciation. *Nature* **400**: 351-354.

Kramer, B. & Kuhn, B. 1993. Electric signaling and impedance matching in a variable environment - the electric organ of mormyrid fish actively adapts to changes in water conductivity. *Naturwissenschaften* **80**: 43-46.

Kramer, B. & Kuhn, B. 1994. Species recognition by the sequence of discharge intervals in weakly electric fishes of the genus *Campylomormyrus* (Mormyridae, Teleostei). *Anim. Behav.* **48**: 435-445.

Kramer, B. 1997. A field study of African elephantfish (Mormyridae, Teleostei): Alectric organ discharges in *Marcusenius macrolepidotus* (Peters, 1852) and *Petrocephalus catostoma* (Günther, 1866) as related to sex. *J. Afr. Zool.* **111**: 313-341.

Lande, R., Seehausen, O. & van Alphen, J. J. M. 2001. Mechanisms of rapid sympatric speciation by sex reversal and sexual selection in cichlid fish. *Genetica* **112**: 435-443.

Landsman, R. E. 1993. Sex-differences in external morphology and electric organ discharges in imported *Gnathonemus petersii* (Mormyriformes). *Anim. Behav.* **46**: 417-429.

Lauder, G. V. & Liem, K. F. 1983. The evolution and interrelationship of the actinopterygian fishes. *Bull. Mus. Comp. Zool.* **150**: 95-197.

Lavoué, S., Bigorne, R., Lecointre, G. & Agnèse, J. F. 2000. Phylogenetic relationships of mormyrid electric fishes (Mormyridae; Teleostei) inferred from cytochrome b sequences. *Mol. Phylogenet. Evol.* **14**: 1-10.

Lavoué, S., Sullivan, J. P. & Hopkins, C. D. 2003. Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. *Biol. J. Linn. Soc.* **78**: 273-292.

Lavoué, S. & Sullivan, J. P. 2004. Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). *Mol. Phylogenet. Evol.* **33**: 171-185.

Lissman, H. W. & Machin, K. E. 1958. The mechanism of object location in *Gymnarchus niloticus* and similar fish. *J. Exp. Biol.* **35**: 451-486.

Lovell, P. V., Hopkins, C. D. & Harned, G. D. 1997. The diversity of electric organ discharges found in eight species of *Campylomormyrus* electric fish (Mormyridae). *Abstracts of the Annual Meeting of the Society of Neurosciences* **23**: 2.

Maan, M. E., Seehausen, O., Soderberg, L., Johnson, L., Ripmeester, E. A. P., Mrosso, H. D. J., Taylor, M. I., van Dooren, T. J. M. & van Alphen, J. J. M. 2004. Intraspecific sexual selection on a speciation trait, male coloration, in the Lake Victoria cichlid *Pundamilia nyererei*. *Proc. R. Soc. Lond. B Biol. Sci.* **271**: 2445-2452.

Marrero, C. & Winemiller, K. O. 1993. Tube-snouted gymnotiform and mormyriform fishes - convergence of a specialized foraging mode in teleosts. *Environ. Biol. Fishes* **38**: 299-309.

Mendelson, T. C. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: Etheostoma). *Evolution* **57**: 317-327.

Moller, P. & Serrier, J. 1986. Species recognition in mormyrid weakly electric fish. *Anim. Behav.* **34**: 333-339.

Moller, P. & Brown, B. 1990. Meristic characters and electric organ discharge of *Mormyrops curviceps* Roman (Teleostei, Mormyridae) from the Moa River, Sierra-Leone, West Africa. *Copeia* 1031-1040.

Moller, P. 1995. *Electric Fishes History and Behavior*. Chapman & Hall, London.

Ostbye, K., Bernatchez, L., Naesje, T. F., Himberg, K. J. M. & Hindar, K. 2005. Evolutionary history of the European whitefish Coregonus lavaretus (L.) species complex as inferred from mtDNA phylogeography and gill-raker numbers. *Mol. Ecol.* **14**: 4371-4387.

Paintner, S. & Kramer, B. 2003. Electrosensory basis for individual recognition in a weakly electric, mormyrid fish, *Pollimyrus adspersus* (Gunther, 1866). *Behav. Ecol. Sociobiol.* **55**: 197-208.

Poll, M., Gosse, J.-P. & Orts, S. 1982. Le genre *Campylomormyrus* Bleeker, 1874, étude systématique et description d`une espèce nouvelle (Pices, Mormyridae). *Bulletin Institut Royal des Sciences Naturelles de Belgique Biologie* **54**: 1-44.

Roberts, T. R. & Stewart, D. J. 1976. An ecological and systematic survey of fishes in the rapids of the lower Zaire or Congo river. *Bull. Mus. Comp. Zool.* **147**: 239-317.

Rundle, H. D. & Nosil, P. 2005. Ecological speciation. *Ecol. Lett.* **8**: 336-352.

Saint-Laurent, R., Legault, M. & Bernatchez, L. 2003. Divergent selection maintains adaptive differentiation despite high gene flow between sympatric rainbow smelt ecotypes (Osmerus mordax Mitchill). *Mol.Ecol.* **12**: 315-330.

Salzburger, W. & Meyer, A. 2004. The species flocks of East African cichlid fishes: recent advances in molecular phylogenetics and population genetics. *Naturwissenschaften* **91**: 277-290.

Salzburger, W., Niederstatter, H., Brandstatter, A., Berger, B., Parson, W., Snoeks, J. & Sturmbauer, C. 2006. Colour-assortative mating among populations of *Tropheus moorii*, a cichlid fish from Lake Tanganyika, East Africa. *Proc. R. Soc. Biol. Sci. Ser. B* **273**: 257-266.

Scheffel, A. & Kramer, B. 2000. Electric signals in the social behavior of sympatric elephantfish (Mormyridae, Teleostei) from the upper Zambezi river. *Naturwissenschaften* **87**: 142-147.

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T. & Tautz, D. 2001. Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Mol. Ecol.* **10**: 1471-1488.

Schliewen, U. K., Tautz, D. & Paabo, S. 1994. Sympatric speciation suggested by monophyly of crater lake cichlids. *Nature* **368**: 629-632.

Schluter, D. 1996a. Ecological speciation in postglacial fishes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351**: 807-814.

Schluter, D. 1996b. Ecological causes of adaptive radiation. *Am. Nat.* **148**: S40-S64.

Schluter, D. 2000. *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.

Schugardt, C. & Kirschbaum, F. (2002) Nilhechte des *Campylomormyrus numenius*-Formenkreises: Bemerkungen zur Ontogenese des Habitus, der elektrischen Entladung sowie zum Fortpflanzungsverhalten. In: *Verhalten der Aquarienfische* (eds. Greven, H. & Riehl, R.), pp. 137-146. Birgit Schmettkamp Verlag, Bornheim.

Schwarz, S. & von der Emde, G. 2000. Distance discrimination during active electrolocation in the weakly electric fish *Gnathonemus petersii*. *J. Comp. Physiol. A. Sens. Neural. Behav. Physiol.* **186**: 1185-1197.

Seehausen, O., vanAlphen, J. J. M. & Witte, F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* **277**: 1808-1811.

Seehausen, O. & van Alphen, J. M. 1999. Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? *Ecol. Lett.* **2**: 262-271.

Simpson, G. G. 1953. *The Major Features of Evolution*. Columbia University Press, New York.

Sullivan, J. P., Lavoué, S. & Hopkins, C. D. 2000. Molecular systematics of the African electric fishes (Mormyroidea: Teleostei) and a model for the evolution of their electric organs. *J. Exp. Biol.* **203**: 665-683.

Sullivan, J. P., Lavoué, S. & Hopkins, C. D. 2002. Discovery and phylogenetic analysis of a riverine species flock of African electric fishes (Mormyridae: Teleostei). *Evolution* **56**: 597-616.

Sullivan, J. P., Lavoué, S., Arnegard, M. E. & Hopkins, C. D. 2004. AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea: Teleostei). *Evolution* **58**: 825-841.

Taverne, L. 1972. *Ostéologie des genres Mormyrus Linné, Mormyrops Müller, Hyperopisus Gill, Isichthys Gill, Myomyrus Boulenger, Stomatorhinus Boulenger et Gymnarchus Cuvier*

considérations générales sur la systématique des poissons de l`ordre des Mormyriformes. MRAC, Tervuren.

van Doorn, G. S., Noest, A. J. & Hogeweg, P. 1998. Sympatric speciation and extinction driven by environment dependent sexual selection. *Proc. R. Soc. Lond. B Biol. Sci.* **265**: 1915-1919.

van Doorn, G. S., Dieckmann, U. & Weissing, F. J. 2004. Sympatric speciation by sexual selection: A critical reevaluation. *Am. Nat.* **163**: 709-725.

van Oppen, M. J. H., Turner, G. F., Rico, C., Robinson, R. L., Deutsch, J. C., Genner, M. J. & Hewitt, G. M. 1998. Assortative mating among rock-dwelling cichlid fishes supports high estimates of species richness from Lake Malawi. *Mol. Ecol.* **7**: 991-1001.

von der Emde, G. 1999. Active electrolocation of objects in weakly electric fish. *J. Exp. Biol.* **202**: 1205-1215.

Werneyer, M. & Kramer, B. 2002. Intraspecific agonistic interactions in freely swimming mormyrid fish, *Marcusenius macrolepidotus* (South African form). *J. Ethol.* **20**: 107-121.

Westby, G. W. M. & Kirschbaum, F. 1977. Emergence and development of electric organ discharge in mormyrid fish, *Pollimyrus isidori*. 1. Larval discharge. *J. Comp.Physiol.* **122**: 251-271.

Westby, G. W. M. & Kirschbaum, F. 1978. Emergence and development of electric organ discharge in mormyrid fish, *Pollimyrus isidori.* 2. Replacement of larval by adult discharge. *J. Comp. Physiol.* **127**: 45-59.

Westby, G. W. M. & Kirschbaum, F. 1982. Sex-differences in the waveform of the pulse-type electric fish, *Pollimyrus isidori* (Mormyridae). *J. Comp. Physiol.* **145**: 399-403.

Wilson, A. B., Noack-Kunnmann, K. & Meyer, A. 2000. Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: sexual selection versus ecological diversification. *Proc. R. Soc. Lond. B Biol. Sci.* **267**: 2133-2141.

7 Appendix

7.1 Article I:

Feulner, P. G. D., Kirschbaum, F. & Tiedemann, R. 2005.
Eighteen microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa.
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PRIMER NOTE

Eighteen microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa

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Abstract

We describe isolation and characterization of the first microsatellite loci specifically developed for African weakly electric fish (Mormyridae), for the genus *Campylomormyrus*. Seventeen of our 18 loci are polymorphic within the *Campylomormyrus numenius* species complex. The polymorphic loci showed four to 15 alleles per locus, an expected heterozygosity between 0.46 and 0.94, and an observed heterozygosity between 0.31 and 1.00. Most primers also yield reproducible results in several other mormyrid species. These loci comprise a set of molecular markers for various applications, from moderately polymorphic loci suitable for population studies to highly polymorphic loci for pedigree analysis in mormyrids.

Keywords: African weakly electric fish, *Campylomormyrus numenius*, cross-species amplification, microsatellites, Mormyridae

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The endemic African mormyrids comprise the single largest group of electric fish worldwide (Alves-Gomes & Hopkins 1997). Mormyrid fish (Mormyridae) belongs to the Osteoglossomorpha, which is considered as one of the phylogenetically basal groups of extant teleosts (Lauder & Liem 1983). They are characterized by the derived (synapomorphic) presence of electric organs, matched electroreceptors and a greatly enlarged cerebellum, among other characters (Taverne 1972). While primers for some mitochondrial (cytochrome *b*, 12S and 16S rRNA) and nuclear genes (*RAG2*, *S7* ribosomal protein gene) are available (Alves-Gomes & Hopkins 1997; Lavoué *et al*. 2000, 2003; Sullivan *et al*. 2000), no microsatellite studies on African weakly electric fish have been conducted so far. Here, we present the first microsatellite primers specifically developed for mormyrids, especially for the genus *Campylomormyrus*.

Genomic DNA was extracted from dorsal fin clips of 13 fishes of the *Campylomormyrus numenius* species complex, originating from the Congo Basin (Central Africa), using the DNeasy DNA extraction kit (QIAGEN) according to the manufacturer's instructions. In order to assess the cross-species amplification of our new primers, we also obtained genomic DNA from several species of other mormyrid genera: *Brienomyrus niger*, *Gnathonemus petersii*, *Hippopotamyrus pictus*, *Mormyrus rume proboscirostris*, and *Petrocephalus soudanensis*.

A microsatellite-enriched genomic DNA library was constructed from a total DNA extract of a *Campylomormyrus numenius* fin sample according to Hamilton *et al*. (1999) and Paulus & Tiedemann (2003). Genomic DNA was simultaneously restricted with *Nhe*I, *Hae*II, *Rsa*I, and *Alu*I. After treatment with mung bean exonuclease and calf intestinal phosphatase, genomic DNA fragments were blunt-end ligated to SNX linkers and polymerase chain reaction (PCR) amplified with linker primers. Fragments were hybridized with 5′-biotin labelled microsatellite probes ($[GA]_{15}$ and $[GT]_{15}$), conjugated with streptavidin-coated magnetic beads (Dynal), and extracted with a magnetic device. After a further PCR amplification, fragments were restricted with *Nhe*I at a restriction site within the linker, ligated into Bluescript plasmids, and transformed into competent *Escherichia coli* (XL1-Blue MRF′, Stratagene). Recombinants were identified by blue-white-selection,

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Table 1 Characteristics of 18 microsatellites in the genus *Campylomormyrus* and the applicability of primers to other mormyrids

Primer name and sequence (5′–3′)		GenBank Accession no.	Characteristics in *Campylomormyrus*					Amplifiable sizes in other mormyrids (— indicates no PCR product)						
			Repeat sequence	T_a	*n*	No. of alleles	Allele size	H_E	H_O	Gp	Ps	Bn	Mr	Hp
CampGTI35F	GCTTCATAATCCCCATCTTGCT	AJ865352	$(GT)_{15}$	55	5	6	122–138	0.89	0.60	92	—	—	—	—
CampGTI35R	GGGAGAACTTGCACAATCGGT													
CampGTII6bF	GTTTCAATGAAAACGCCAAACG	AJ865353	$(TG)_{18}$	50	13	14	144–188	0.94	0.61	144	—	158	—	148
CampGTII6bR	GCCTGAGCCAGTCCCGTCTGTT													
CampGAI28F	GCCAGCTGTGGGGAACTTCAAA	AJ865354	$(GA)_{12}AA(GA)_5$	55	13	7	173–219	0.82	0.46	175	176	167	185	171
CampGAI28R	GAGCTGCCGACATCCACTTCCG												189	
CampGTIII41F	GTTTTCTCATATTAATCTGTGC	AJ865355	$(GT)_6CT(GT)_4CT(GT)_7$	55	13	4	177–195	0.48	0.31	185	173	185	173	199
CampGTIII41R	GTAGGTTAGCAGTGTCTTATCA										175			
CampGTI19F	GTCGGCTGAATGGAGTGACACT	AJ865356	$(GT)_{12}$	55	13	6	188–202	0.56	0.46	186	228	150	196	198
CampGTI19R	GTCAAGTTGAGGGATATGCTGC											186		
CampGTII2aF	GTTCATGTGTTAAATCAAGGCG	AJ865357	$(GT)_9$	55	13	11	193–249	0.85	0.69	227	227	219	—	249
CampGTII2aR	GGGAAATCAAAATGGTGACAG													255
CampGAII26F	GTGAGGCTGATGAGCACAACAT	AJ865358	$(GT)_6GGCA(GA)_{14}$	55	13	6	208–228	0.72	0.46	250	—	—	—	—
CampGAII26R	GCCTGGCTCTCAACAACCTGTG													
CampGTII27F	GATCAGTGTTCTTTTTCAAAT	AJ865359	$(GT)_5GC(GT)_3TT(GT)_7$	50	13	1	279	0.00	0.00	267	—	—	—	—
CampGTII27R	GGGAACTGTAGTAAAATAAAG													
CampGAIII8F	GTCTTCTCTGATTAACTGCTCT	AJ865360	$(GA)_3AAGC(GA)_{20}$	55	13	13	379–433	0.82	0.69	405	—	—	—	—
CampGAIII8R	GTAGAACAAATGTTCCTTCGT													
CampGTI18aF	GCCAGAAAGGATGTCCCAGCTC	AJ865361	$(GT)_5GC(GT)_3$	55	13	4	150–158	0.46	0.38	192	–	158	140	170
CampGTI18aR	GGACCCCCCACTGACAAATTT									202				
CampGTI39F	GTCACTCAATGTATAAAATGCA	AJ865362	$(GT)_{16}$	55	13	13	171–213	0.92	0.85	201	—	—	—	—
CampGTI39R	GAGATGTGAAAACTGAGCAACT									215				
CampGTIII4bF	GTTCCACTTCTATGCTTCCCTG	AJ865363	$(GT)_{12}$	55	13	14	194–236	0.94	1.00	190	—	—	190	182
CampGTIII4bR	GTGTCACAATTTCTTAACGCCA									202				
CampGAI8F	GACATTGTCTGAACTGAAACGA	AJ865364	$(GA)_{26}$	55	13	13	177–215	0.92	0.92	193	—	—	—	—
CampGAI8R	GCTTCTGTTGTTGAGGGCTTAA													
CampGAI14F	GGTTCCCCGTTGGTTCTGTCT	AJ865365	$(GA)_{22}$	55	13	15	189–237	0.94	0.92	181	—	—	—	183
CampGAI14R	GCATCCAGGGCTTTCACTTTCC													
CampGAII17F	GCAAGGTGCATTATTGCGATTT	AJ865366	$(GA)_{12}$	55	13	9	222–244	0.86	0.69	214	214	236	228	216
CampGAII17R	GCCTGTCAAGTCCCGACTCACG													
CampGTII6aF	GTTGGACCTGAAATGTAAGAT	AJ865367	$(GATG)_{12}$	48	13	10	188–236	0.87	0.85	188	—	184	200	180
CampGTII6aR	GACTTGCACATCTGTCACCTAT											188	204	
CampGAII42F	GTCATGAGGTCAGTAGGAAGAA	AJ865368	$(GA)_5AG(GA)_{16}$	55	13	10	345–376	0.86	0.92	360	360	414	356	348
CampGAII42R	GATGGAGAGTTGTTTTGAAGAA											430		368
CampGAIII4F	GATGAATGAACTGGCGAGATGC	AJ865369	$(GA)_{16}$	52	13	11	450–494	0.81	0.54	470	—	—	—	—
CampGAIII4R	GTCACTTCCACCTTCCCTTAGC													

T_a (°C), PCR annealing temperature; *n*, number of *Campylomormyrus* specimens analysed; H_E, expected heterozygosity; H_O, observed heterozygosity; Gp, *Gnathonemus petersii*; Ps, *Petrocephalus soudanensis*; Bn, *Brienomyrus niger*; Mr, *Mormyrus rume proboscirostris*; and Hp, *Hippopotamyrus pictus*.

blotted onto a nylon membrane, and again hybridized with the microsatellite probes. Positive clones were detected using the Phototope-Star chemiluminescent detection kit (New England Biolabs), sequenced with the BigDye version 1.1 Terminator Cycle Sequencing Kit (Applied Biosystems), and analysed on an AB 3100 multicapillary automatic sequencer (Applied Biosystems). Primers were constructed from flanking regions of microsatellite loci (Table 1). To facilitate cloning of PCR products for allele-specific DNA sequencing using the TOPO TA Cloning Kit (Invitrogen), we designed primers starting on guanin (G) at the 5′-end.

About 100 ng of genomic DNA were used as template. The PCR was performed in a total volume of 37.5 μL, containing 1 mM Tris-HCl, pH 9.0, 5 mM KCl, 0.15 mM $MgCl_2$, 0.2 mM of each dNTP, 0.13 μM of both forward and reverse primers (forward primer was 5′-fluorescence-labelled), and 0.75 U *Taq* polymerase (Qbiogene). Amplifications were performed in a Biometra TGradient thermocycler according to the following reaction profile: one cycle at 95 °C for 5 min, 40 cycles at 94 °C for 1 min 30 s, at the locus-specific annealing temperature (T_a in Table 1) for 1 min 15 s, 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min. Fragment size was determined on an AB 3100 automatic sequencer, using the GENEMAPPER version 3.5 software and an internal size standard (LIZ500, Applied Biosystems).

We identified 18 new microsatellite loci for *Campylomormyrus numenius*, of which 17 are polymorphic. No linkage disequilibrium among the loci was detected, except for two pairs of loci. Data analysis using the ARLEQUIN software (Schneider *et al*. 2000) indicates linkage disequilibrium between *CampGAII26* and *CampGAIII4* as well as between *CampGTI39* and *CampGAIII4*. The polymorphic loci had four to 15 different alleles, an expected heterozygosity (H_E) from 0.46 to 0.94, and an observed heterozygosity (H_O) from 0.31 to 1.00 (Table 1). A few loci exhibited heterozygote deficits. Because of small sample size, however, we were unable to discriminate, whether this was caused by null alleles, population subdivision, or stochastic effects. These first microsatellite loci specifically developed for African weakly electric fish of the genus *Campylomormyrus* comprise a set of molecular markers for various applications, from moderately polymorphic loci suitable for population studies to highly polymorphic loci for pedigree analysis. We also investigated the cross-species amplification with our primers in species of several other mormyrid genera. Most primers yield reproducible results in several mormyrid species (Table 1), indicating their potentially wide applicability.

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References

Alves-Gomes J, Hopkins CD (1997) Molecular insights into the phylogeny of mormyriform fishes and the evolution of their electric organs. *Brain, Behavior and Evolution*, **49**, 324–351.

Hamilton MB, Pincus EL, DiFiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.

Lauder GV, Liem KF (1983) The evolution and interrelationships of the actinopterygian fishes. *Bulletin of the Museum of Comparative Zoology*, **150**, 95–197.

Lavoué S, Bigorne R, Lecointre G, Agnèse J-F (2000) Phylogenetic relationships of mormyrid electric fishes (Mormyridae; Teleostei) inferred from cytochrome *b* sequences. *Molecular Phylogenetics and Evolution*, **14**, 1–10.

Lavoué S, Sullivan JP, Hopkins CD (2003) Phylogenetic utility of the first two introns of the *S7* ribosomal protein gene in African electric fishes (Mormyroidea; Teleostei) and congruence with other molecular markers. *Biological Journal of the Linnean Society*, **78**, 273–292.

Paulus KB, Tiedemann R (2003) Ten polymorphic autosomal microsatellite loci for the eider duck *Somateria mollissima* and their cross-species applicability among waterfowl species (Anatidae). *Molecular Ecology Notes*, **3**, 250–252.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: a software for population genetics data version 2.000*. Department of Anthropology, University of Geneva, Switzerland.

Sullivan JP, Lavoué S, Hopkins CD (2000) Molecular systematics of the African electric fishes (Mormyroidea; Teleostei) and a model for the evolution of their electric organs. *Journal of Experimental Biology*, **203**, 665–683.

Taverne L (1972) *Ostéologie des Genres* Mormyrus *Linné,* Mormyrops *Müller,* Hyperopisus *Gill,* Isichthys *Gill,* Myomyrus *Boulenger,* Stomatorhinus *Boulenger et* Gymnarchus *Cuvier Considérations Générales sur la Systématique des Poissons de l'Ordre des Mormyriformes*. Musee Royal de L'Afrique Central, Tervuren.

7.2 Article II:

Feulner, P. G. D., Kirschbaum, F., Schugardt C., Ketmaier V. & Tiedemann, R. 2006. Electrophysiological and molecular genetic evidence for sympatrically occuring cryptic species in African weakly electric fish (Teleostei: Mormyridae: *Campylomormyrus*). Molecular Phylogenetics and Evolution **39**: 198-208.

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Electrophysiological and molecular genetic evidence for sympatrically occuring cryptic species in African weakly electric fishes (Teleostei: Mormyridae: *Campylomormyrus*)

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Abstract

For two sympatric species of African weakly electric fish, *Campylomormyrus tamandua* and *Campylomormyrus numenius*, we monitored ontogenetic differentiation in electric organ discharge (EOD) and established a molecular phylogeny, based on 2222 bp from cytochrome *b*, the S7 ribosomal protein gene, and four flanking regions of unlinked microsatellite loci. In *C. tamandua*, there is one common EOD type, regardless of age and sex, whereas in *C. numenius* we were able to identify three different male adult EOD waveform types, which emerged from a single common EOD observed in juveniles. Two of these EOD types formed well supported clades in our phylogenetic analysis. In an independent line of evidence, we were able to affirm the classification into three groups by microsatellite data. The correct assignment and the high pairwise F_{ST} values support our hypothesis that these groups are reproductively isolated. We propose that in *C. numenius* there are cryptic species, hidden behind similar and, at least as juveniles, identical morphs.

Keywords: Cryptic species; Electric fish; Mormyridae; *Campylomormyrus*; Phylogeny; Microsatellite; EOD polymorphism; Ontogeny

1. Introduction

With at least 188 described species (Gosse, 1984), the mormyrid weakly electric fishes (Mormyridae) comprise one of the most diverse clades of freshwater fishes from Africa and the single largest known group of electric fishes (Alves-Gomes and Hopkins, 1997). Together with their sister taxon, the monotypic Gymnarchidae, they form the Mormyroidea. Mormyroidea are endemic to Africa and belong to the Osteoglossomorpha, considered one of the phylogenetically basal groups of extant teleosts (Lauder and Liem, 1983). The monophyly of Mormyroidea as well as the sister relationship between Gymnarchidae and Mormyridae is supported by morphological data and molecular analysis (Alves-Gomes and Hopkins, 1997; Nelson, 1994; Sullivan et al., 2000; Taverne, 1972). However, at and near the species level, the existing morphological and molecular data sets support conflicting phylogenies (Lavoué et al., 2000; Sullivan et al., 2000). Very few molecular data are available for the mormyrid genus *Campylomormyrus*, the main object of the current study. According to Lavoué et al. (2000, 2003) and to Sullivan et al. (2000), *Campylomormyrus* is considered monophyletic with *Campylomormyrus numenius* and *Campylomormyrus tamandua* placed as each other's closest relatives, i.e., sister taxa. However, such a phylogenetic hypothesis awaits support by the analysis of a higher number of taxa. Indeed, the number of species currently described for this genus varies between 3 and 16 (Poll et al., 1982; Roberts and Stewart, 1976; Taverne, 1972); according to the list of the freshwater fishes of Africa (Gosse, 1984), *Campylomormyrus* includes 14 species. *C. numenius*, as most of the species included in the genus, is

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endemic to a single river system, the Congo and its tributary streams (Fig. 1): Its putative sister taxon, *C. tamandua* occurs sympatrically in the Congo river, but also in the river systems of Niger, Tchad/Shari, and Volta (Fig. 1). Interestingly, *Campylomormyrus* exhibits a wide diversity of electric organ discharge (EOD) waveform types, which may be used as an important character for discrimination among species that are otherwise cryptic (Hopkins, 1999).

Weakly electric fishes detect objects and analyse their electrical properties by measuring distortions in a self-produced electrical field, a process called active electrolocation (Bastian, 1994; Lissman and Machin, 1958; von der Emde, 1999). In addition, the EOD of weakly electric fish plays an essential role in social communication (Hopkins and Bass, 1981; Kramer and Kuhn, 1994; Werneyer and Kramer, 2002). The EOD is species-specific (Bass, 1986; Crawford and Huang, 1999; Kramer and Kuhn, 1994), and species recognition depends on a two-spike code characterizing the EOD (Hopkins and Bass, 1981). There is also evidence that the EOD may allow for individual identification and discrimination (Crawford, 1992; Crawford and Huang, 1999; Friedman and Hopkins, 1996). Many mormyrid species show sex differences in EOD at least during the breeding season (Crawford, 1991; Crawford and Huang, 1999; Friedman and Hopkins, 1996; Hopkins and Bass, 1981; Landsman, 1993; Westby and Kirschbaum, 1982). Mormyrids discriminate between the EODs of conspecifics and heterospecifics and between those of males and females, based on the temporal pattern of the EOD, i.e., the duration and shape of the EOD waveform (Hopkins and Bass, 1981). Therefore, EOD plays a key role in pair formation, mating, and social attraction (Bratton and Kramer, 1989; Crawford, 1991; Kramer and Kuhn, 1994). As an effective prezygotic isolation mechanism, the EOD might have been of paramount importance for speciation during the diversification of mormyrid fish (Sullivan et al., 2002). Previous studies have aimed at correlating the EOD mode and the phylogeny throughout the entire Mormyridae (Lavoué et al., 2000, 2003; Sullivan et al., 2000). However, the importance of EOD as a factor during speciation per se has not been addressed specifically.

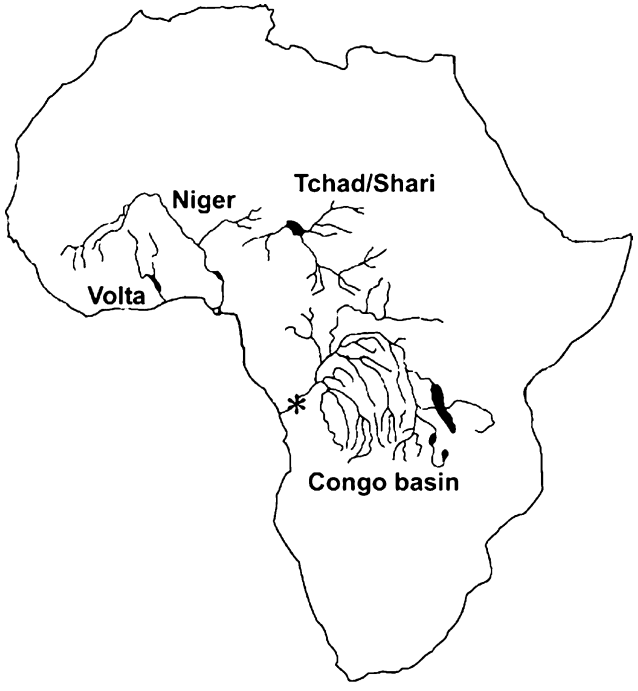

Fig. 1. Geographic location of the African river systems in which *Campylomormyrus* occurs. Most species are endemic to the Congo basin. *depicts the sampling location at Kinshasa/Brazzaville.

We hypothesize here that—as a feature for species recognition—EOD potentially provides an important isolation mechanism, which can promote sympatric speciation. Under our hypothesis, we predict that (1) differences in the EOD are indeed confined to reproductively isolated groups of specimens and (2) closely related species should be expected to exhibit significantly different EODs. In this context, it is crucial to be aware of the EOD changes during ontogeny of single individual specimens: Larvae of those mormyrids investigated so far possess a larval electric organ with an EOD very different from the adults' EOD (Denizot et al., 1982; Kirschbaum, 1977; Westby and Kirschbaum, 1977, 1978). Later in ontogeny, the larval electric organ degenerates and is substituted by the adult's electric organ, located in the caudal peduncle (Kirschbaum, 1981). Importantly, the EOD of the adult's electric organ can exhibit a dramatic shift, from a juvenile EOD during adolescence to an adult EOD confined to specimens above a threshold body size and presumably associated with sexual maturity (Schugardt and Kirschbaum, 2002). We argue that neglecting these ontogenetic changes in EOD potentially compromises some previous studies on species-specific EODs, as these EODs might comprise an erratic mixture of juvenile vs. adult EODs, depending on the body size of the specimens collected.

To better understand the role of adult EOD during speciation, we specifically investigated *C. numenius*. For the genus *Campylomormyrus*, Lovell et al. (1997) demonstrated the existence of striking different EODs among morphologically indistinguishable specimens. In addition, *C. numenius* shows identical juvenile EODs while individuals develop different adult EODs during ontogeny, a phenomenon not reported from any other mormyrid species so far. This evidence suggests that *C. numenius* could be comprised of a complex of cryptic distinct species, rather than a single species. To evaluate this hypothesis, we intensively observed 21 specimens of *C. numenius*—sampled in the same geographic area (Congo River near Kinshasa)—during their ontogeny and compared them to a group of 19 individuals of its putative sister species, *C. tamandua*. These two species occur in sympatry and show remarkable morphological differences. We designed this study to compare ontogenetic observations of EOD development to a molecular phylogeny, based on a selection of mitochondrial and nuclear genes (cyt *b*: the complete mitochondrial cytochrome *b* gene, 1142 bp; S7: the first and second introns as well as the second exon of the nuclear gene coding for the S7 ribosomal

protein, 895 bp; flr: four flanking regions of unlinked microsatellite loci, 185 bp; for a total of 2222 bp). As an independent line of evidence, we analysed length polymorphisms at 16 microsatellite loci for all the specimens of *C. numenius*. The ultimate goal of this study is to better understand the relevance of weak electricity in the speciation processes of the *C. numenius* group.

2. Materials and methods

2.1. Sampling location and maintenance of Mormyrids

For this study, we examined 21 specimens of *C. numenius* obtained as juveniles (total length less than 14 cm) from the Congo River near Kinshasa (Fig. 1). At the date of capture, they showed uniform dark colouration and no morphological differences. We compared this group to 19 *C. tamandua* captured at the same site, a clearly distinct species characterised by a peculiar colour pattern comprised of striking yellow bands. We maintained these fishes during an extended period of time (from two to ten years), such that we were able to monitor morphological as well as electrophysiological changes during ontogeny. General keeping conditions are described elsewhere (Kirschbaum and Schugardt, 2002; Schugardt and Kirschbaum, 1998). As an outgroup for the phylogenetic analysis, we obtained one *Gnathonemus petersii* specimen; *Gnathonemus* is considered to be the sister genus of *Campylomormyrus* (Lavoué et al., 2000, 2003; Sullivan et al., 2002).

2.2. Measurements of EOD

To record a fish's EOD, we placed each specimen individually into a plastic container, where its mobility was restricted. The container was filled with water from the respective fish tank (water temperature 27 ± 1 °C). An electrode was positioned at each end of the fish, the positive electrode near its head and oriented parallel to its body axis. After a short period of acclimation, the preamplified EOD was displayed and stored on an oscilloscope (TDS 3100B series digital phosphor oscilloscope, ADA 400 A differential preamplifier, Tektronix, Beaverton, USA). Because we regularly measured the EOD of each fish over the course of years, we were able to document ontogenetic changes and determine groups with identical adult EOD signal forms. We examined EOD variation among types of signal forms by overlaying amplitude-normalized plot of electric recordings. These were centred on the major head-positive peak and plotted on the same time base (described in Arnegard et al., 2005).

2.3. DNA sequence analysis

For tissue samples, we anesthetized the fishes with the fish anaesthetic MS222 (tricaine methane sulfonate) and cut off a small piece of the dorsal fin (which healed within a few weeks). The fin clip was stored in 1 ml of tissue buffer (Seutin et al., 1991). Genomic DNA was extracted using the DNeasy DNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

We chose to analyse the complete mitochondrial cytochrome *b* gene (cyt *b*: 1142 bp) because earlier studies using this marker in mormyrids (Lavoué et al., 2000, 2003; Sullivan et al., 2000, 2002) indicated its utility as a species-specific marker. We additionally selected several unlinked nuclear markers, which showed informative polymorphisms in a preliminary screening of a few individuals: These are an 895 bp portion of the nuclear gene coding for S7 ribosomal protein gene and short flanking regions (flr: between 31 and 71 bp, in total 185 bp) of four different microsatellite loci. All PCRs were performed according to Feulner et al. (2005), with the following locus-specific conditions. (1) cyt *b*: primers L14724 and H15930 (Palumbi, 1996), annealing temperature $T_a = 48$ °C; (2) S7: primers S7RPEX1F and S7Ex3Ralt (Chow and Hazama, 1998; Lavoué et al., 2003), $T_a = 55.7$ °C; (3) flr: primers CampGAIII8F/R, CampGTI19F/R, CampGTII2aF/R, and CampGTII27F/R (Feulner et al., 2005), $T_a = 50$ or 55 °C, respectively. We purified our PCR products with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). We sequenced cyt *b* and S7 in both directions with the primers used for amplification. The following primers were used for sequencing of flanking regions of four microsatellite loci: CampGAIII8R, CampGTI19F, CampGTII2aF, and CampGTII27R (Feulner et al., 2005). For sequencing, we used the BigDye v3.1 Terminator Cyclesequencing Kit (Applied Biosystems, Foster City, USA). The Multiscreen-HV (Millipore, Bedford, USA) purified products were analysed on an AB 3100 multicapillary automatic sequencer (Applied Biosystems, Foster City, USA). All sequences were submitted to GenBank (Accession No. DQ231063–DQ231135).

2.4. Phylogenetic analysis

We aligned sequences using BioEdit version 7.0.0 (Hall, 1999) and reconstructed a phylogeny for the cyt *b* gene as well as for the combined dataset. We used PAUP 4.0b10 (Sinauer, Sunderland, USA) to calculate variability estimates, the number of transition (Ti) and transversion (Tv), and to perform χ^2 test for homogeneity of base frequencies. Saturation of sequences was investigated by plotting the absolute number of Ti and Tv against the percentage of sequence divergence. This was done for each gene separately and for all genes combined. For the cyt *b* gene, this analysis was performed at all codon positions and at 3rd codon position only. Aligned sequences were analysed by Maximum Parsimony (Farris et al., 1970), Neighbor-Joining (Saitou and Nei, 1987), and Bayesian methods (Huelsenbeck et al., 2000; Larget and Simon, 1999; Mau and Newton, 1997; Mau et al., 1999; Rannala and Yang, 1996). We used Modeltest version 3.06 (Posada and Crandall, 1998) to identify the best model of sequence evolution for each dataset. These models were then

applied to calculate genetic distances and to construct trees via NJ in PAUP version 4.0b10. To gain statistical support, we performed 1000 bootstrap replicates. Complex models of nucleotide substitution for estimating evolutionary distances and the application of neighbour joining methods are recommended for large datasets, like in the case of our combined dataset (Tamura et al., 2004). We also carried out a MP analysis: For the cyt *b* dataset, we performed a full heuristic MP search; robustness of this phylogenetic hypothesis was tested by 100 bootstrap replicates with PAUP version 4.0b10. For the combined dataset, this analysis was performed as a fast stepwise search (100 bootstrap replicates), as computational effort was prohibitive for a heuristic search here. Intraindividual indels among homologous alleles (nuclear markers only) were defined as new characters (macros), taking advantage of the format equate command in PAUP. For the Bayesian approach, we employed the same models of sequence evolution used in the NJ analyses, allowing for site-specific rate variation partitioned by gene and for cyt *b* by codon position. We ran one cold and three heated Markov chains for two million generation using MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003). We saved trees every 100 generations for a total sample size of 20,000. We discharged the first 2000 sampled trees as burn-in and used the remaining to calculate a 50% majority rule consensus tree. Representing intraindividual indels by new characters is not implemented in MrBayes, such that we counted a deletion as a single transversion and used the IUPAC code in case of intraindividual polymorphism at homologous alleles. Phylogenetic tree topologies generated with different phylogenetic methods and competing phylogenetic hypotheses were statistically evaluated with the approximately unbiased tree selection test (AU) (Shimodaira, 2002), as implemented in the software package CONSEL (Shimodaira and Hasegawa, 2001). For comparison, we also performed the more conservative Shimodaira & Hasegawa test (SH; Shimodaira and Hasegawa, 1999) as implemented in PAUP version 4.0b10 with the resampling estimate log-likelihood (RELL) technique. We always compared tree topologies simultaneously (Shimodaira and Hasegawa, 1999).

A network analysis was also performed to estimate gene genealogies using the TCS program (Clement et al., 2000), which implements the Templeton et al. (1992) statistical parsimony. Input data were cyt *b* sequences of all the *C. numenius* individuals. TCS collapses sequences into haplotypes and produces a network linking different haplotypes only if they have a 95% probability of being justified by the parsimony criterion.

2.5. Microsatellite analysis

As an independent line of molecular evidence, all 21 *C. numenius* specimens were genotyped at 16 microsatellite loci (listed in Table 1) as described in Feulner et al. (2005). We calculated observed and expected heterozygosity using Arlequin version 2.000 (Schneider et al., 2000) and tested for linkage disequilibrium and deviation from Hardy–Weinberg equilibrium using Genepop on the web. Significance was tested after correction for multiple comparisons via sequential Bonferroni correction at an experiment-wise error rate of $\alpha = 0.05$. In addition, analyses of microsatellite data were conducted at different hierarchical level. In this way, we were able to assess the accuracy of classifications based on EOD, morphology, and molecular phylogenetic results. To summarise the degree of genetic differentiation, we calculated pairwise F_{ST} values using *F* statistics (Weir and Cockerham, 1984). The significance of F_{ST} was tested by permutation analyses and an analysis of molecular variance (AMOVA; Excoffier et al., 1992) was conducted as implemented in Arlequin version 2.000 (Schneider et al., 2000). By means of pairwise *T* tests, we compared mean differences between expected heterozygosities to contrast genetic variability within groups. Assignment tests were performed with GeneClass version 2.0 (Piry et al., 2004). Two Bayesian-based tests (Baudouin and Lebrun, 2001; Rannala and Mountain, 1997) and one frequence- based test (Paetkau et al., 1995) were used to calculate the probability of each individual's assignment to a particular clade. Genetic subdivision was further evaluated using the STRUCTURE software, estimating the likelihood and sample composition of different numbers of subgroups ($k = 2$; $k = 3$; and $k = 4$; Pritchard et al., 2000).

3. Results

3.1. Electric organ discharge

All EODs of the *C. tamandua* specimens are identical in shape, course, and duration (Fig. 2), such that this species is characterised both by its coloration and its distinct EOD. We did not detect any change from juvenile to adult EOD during ontogeny in *C. tamandua* (Fig. 4). In *C. numenius* species, all specimens show the same juvenile EOD (see Fig. 2 with 5 examples shown), but their EOD changed when they reached a total length between 14 and 20 cm (cf. also Schugardt and Kirschbaum, 2002). Among our 21 specimens, we were able to identify four strikingly different adult EOD types (Fig. 2). Apart from the differences in shape and course, there is also a large difference in the duration of the altered EOD types. One EOD type, recorded from female and male specimens, just differs slightly in duration from the juvenile EOD (EOD of morph C, Fig. 2). By contrast, other male specimens extend their EOD by 15 times or more (i.e., from 1 to 15 or 25 m). We were able to identify two different waveforms of these elongated male EOD types (male EOD of morphs A and B, Fig. 2). In morph A, we demonstrate that this shift to very long EOD is restricted to the males, as the analyzed female showed a much shorter EOD, with a length of about 1 m similar to the juvenile EOD (female EOD of morph A, Fig. 2).

Table 1
Genetic diversity at 16 microsatellite loci in morphs of *C. numenius*

Locus	Number of alleles	Range of allele size (bp)	Test	Morph A	Morph B	Morph C	Mean
CampGAI14	14	189–237	H_O	1.00	0.67	0.83	0.86
			H_E	0.96	0.80	0.87	0.93
CampGAI28	7	165–181	H_O	0.66	0.67	0.50	0.57
			H_E	0.64	0.87	0.88	0.85
CampGAI8	15	173–215	H_O	1.00	1.00	0.75	0.86
			H_E	0.91	0.73	0.94	0.92
CampGAII17	8	222–244	H_O	0.83	0.33	0.75	0.71
			H_E	0.83	1.00	0.90	0.89
CampGAII26	8	208–224	H_O	0.33	0.33	0.75	0.57
			H_E	0.58	0.80	0.80	0.81
CampGAII42	12	345–376	H_O	1.00	0.67	0.90	0.90
			H_E	0.88	0.87	0.90	0.88
CampGAIII8	15	377–433	H_O	0.83	0.33	0.63	0.65
			H_E	0.85	0.80	0.95[a]	0.87
CampGTI18a	9	136–166	H_O	0.67	0.00	0.50	0.48
			H_E	0.67	0.00	0.78	0.62
CampGTI19	7	188–202	H_O	0.33	0.67	0.42	0.43
			H_E	0.45	0.80	0.37	0.52
CampGTI39	17	167–215	H_O	0.67	0.67	0.75	0.71
			H_E	0.76	0.80	0.93	0.93
CampGTII27	2	279–281	H_O	0.00	0.00	0.17	0.10
			H_E	0.00	0.00	0.24	0.14
CampGTII2a	17	193–259	H_O	0.67	0.67	0.75	0.71
			H_E	0.74	0.60	0.96	0.92
CampGTII6a	10	188–224	H_O	0.83	0.67	0.83	0.81
			H_E	0.74	0.60	0.90	0.88
CampGTII6b	16	144–188	H_O	0.83	0.33	0.67	0.67
			H_E	0.88	0.93	0.73	0.90
CampGTIII41	7	152–195	H_O	0.00	0.00	0.67	0.38
			H_E	0.00	0.00	0.85	0.67
CampGTIII4b	20	194–244	H_O	1.00	1.00	0.83	0.90
			H_E	0.92	1.00	0.96	0.96
All loci			H_O	0.67	0.50	0.67	0.64
			H_E	0.68	0.66	0.80	0.79

For each morph and each microsatellite locus, expected (H_E) and observed (H_O) heterozygosity is given. The range of allele sizes is given for the entire data set.
[a] A single significant heterozygote deficiency after Bonferroni correction at an experiment-wise error rate of $\alpha = 0.05$.

3.2. Phylogenetic and network analysis

We sequenced a total of 2222 bp for each individual included in the study. We found eight indels in the alignment of the nuclear genes, while no indels were found in the alignment of the cytochrome *b* (cyt *b*) gene; cyt *b* has a low frequency of G's, especially in 3rd codon position (G = 0.033). This finding is typical of mitochondrial (mt) genome. Fig. 3 shows the level of sequence variability for each gene and, for cyt *b*, for each codon position. Most of the variation in the combined dataset is due to variation in the 3rd codon positions of the cyt *b* gene. Inspection of the saturation plots (not shown) revealed that saturation is not apparent in our data set. The χ^2 test for base homogeneity indicates that base frequency distribution is always homogeneous among taxa, both when genes are analysed separately (tests were also performed on each codon position for cyt *b*) and when genes are combined in a single data set. Analyses of all genes combined or of cyt *b* alone yielded trees with almost identical topologies. Fig. 4 shows the Bayesian tree obtained on the complete data set using the Hasegawa, Kishino and Yano (HKY) model (Hasegawa et al., 1985) of evolution (model chosen with Modeltest) and summarises the results of the other phylogenetic methods employed in the study. MP and NJ trees were statistically indistinguishable from the Bayesian tree with the AU and the SH test ($0.125 \leqslant p \leqslant 0.996$). MP, NJ, and Bayesian analyses place the *C. numenius* and the *C. tamandua* in two reciprocally strongly supported monophyletic clades. The two species are separated by a mean genetic divergence of 0.0401 ± 0.0001 (Maximum Likelihood ML distances $\pm$ standard error *SE*). Within *C. tamandua*, there are four individuals that are placed sister in a strongly supported clade, which shows a remarkable amount of genetic divergence (0.0089 ± 0.0001) from all other conspecific individuals. The topology of Fig. 4 suggests the existence of a considerable degree of genetic heterogeneity within *C. numenius*. Males showing explicitly elongated but obviously different EODs are placed in two supported clades (morphs A and B in Fig. 4). In addition, clade morph A also contains one female (Cn10), with a strikingly different EOD compared to the males of the

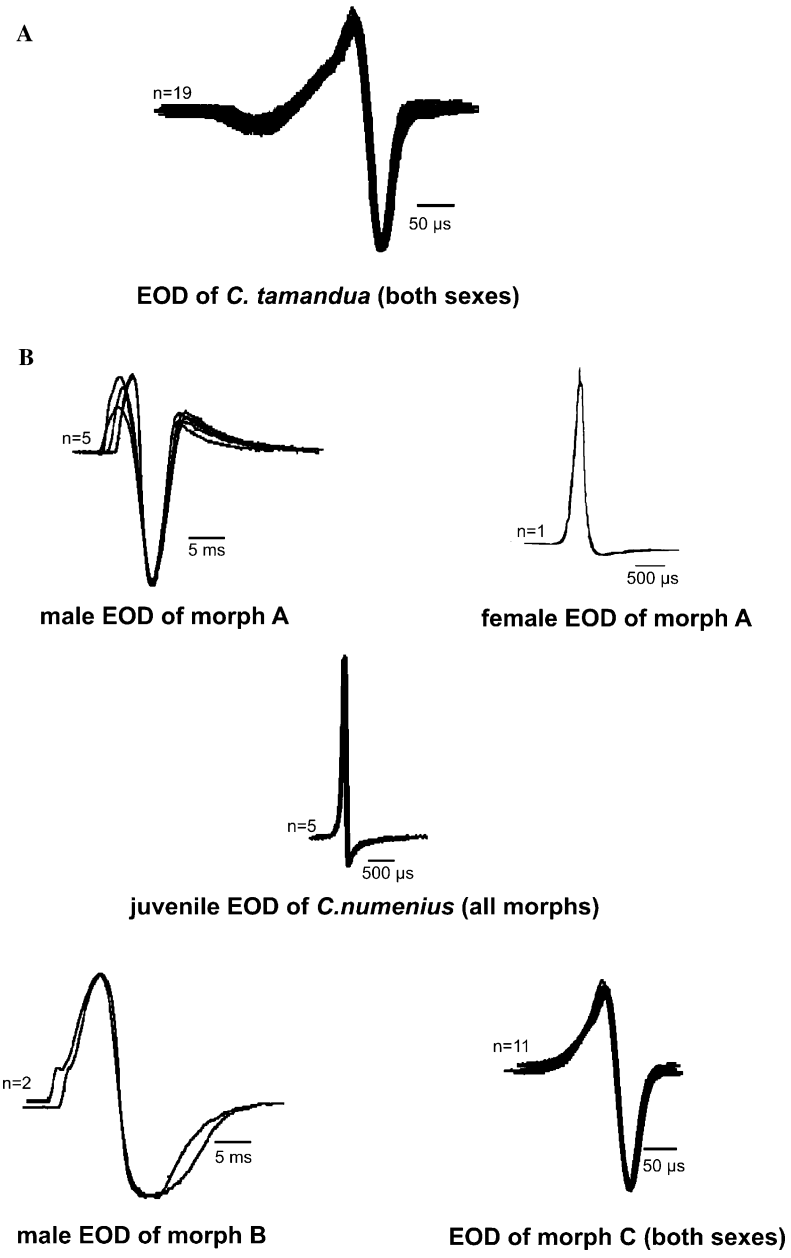

Fig. 2. Overlays of amplitude-normalized EODs (n = number of individuals per overlay). (A) Common EOD type of *C. tamandua*. (B) Common juvenile and different adult EOD types of three different morphs (A, B, and C) of *C. numenius*. Note the different time scales.

same clade. We found a genetic divergence of 0.0060 ± 0.0002 between morphs A and C, and 0.0102 ± 0.0003 between morph B and C. The mean genetic divergence between the morphs A and B is 0.0080 ± 0.0002. The group with the slightest ontogenetic EOD change and no sexual EOD polymorphism (morph C) does not form a single monophyletic clade. Rather, it is possible to individuate two supported clusters (clade C1 and C2). Clade C1 is placed sister to all the other *C. numenius* individuals, while the position of the clade C2 is not supported. Similarly, there are three individuals whose position in the tree is not resolved. On the average, there is a sequence divergence of 0.0044 ± 0.0003 (all genes, ML distances $\pm$ *SE*) within this group of individuals.

Because of some lack of resolution within clade C, we used the AU and SH tests to evaluate three alternative hypotheses. First, we constrained Cn25, Cn29, and Cn35 to be nested alternatively within clade C1 or clade C2. Second, we forced clade C1 and C2 to collapse in a single monophyletic clade. The AU test rejected all these alternative hypotheses ($0.005 \leqslant p \leqslant 0.001$). The more conservative SH test rejected the placement of Cn25, Cn29, and Cn35 within C2 ($p = 0.023$) as well as C1 and C2 forming a single monophyletic clade ($p = 0.01$). The same test did not reject the placement of the above three individuals within clade C1 ($p = 0.161$).

Fig. 5 shows the result of the network analysis obtained from the cyt *b C. numenius* data set. There are four groups of

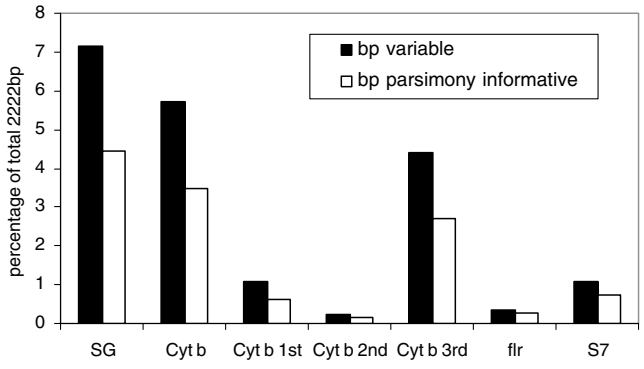

Fig. 3. Percentage of variable and parsimony informative sites in DNA sequence analysis. SG: super gene, the combination of all genes (2222 bp); Cyt *b*: mitochondrial cytochrome *b* gene, total and subdivided into the three codon positions (1142 bp); flr: flanking regions of four unlinked microsatellite loci (185 bp); S7: part of the nuclear S7 ribosomal protein gene (895 bp).

haplotypes in the network. Three of these groups correspond to clades A, B, and C2 in the Bayesian tree of Fig. 4. Individuals placed in the clade C1 by the phylogenetic analyses do not cluster together in the network. Rather, individuals Cn25, Cn29, and Cn35, whose position was not resolved in the phylogenetic tree, are linked to them. This group of haplotypes is the only one to show ambiguities (i.e., alternative most parsimonious connections among haplotypes), while the other clades (A, B, C2) are supported by the network analysis.

3.3. Microsatellite analysis

Summary statistics for microsatellite variation are shown in Table 1. Between 2 and 20 alleles were found per locus. The loci are inherited independently, as no significant linkage disequilibrium was detected among any pair of loci. The mean observed heterozygosity within morphs ranged from 0.50 to 0.67, the mean expected heterozygosity ranged from 0.66 to 0.80. Only one significant deviation from Hardy–Weinberg expectations was detected, i.e., for morph C at locus CampGAIII8. We performed microsatellite analysis as an independent line of molecular evidence to further evaluate the degree of genetic divergence among clades identified by EOD and phylogenetic analysis. Indeed, microsatellite analysis further corroborates our phylogenetic results: Our three different morphs (A, B, and C) were also significantly differentiated from one another at microsatellite loci, as pairwise F_{ST} values were between 0.10 and 0.16 and the overall fixation index was 0.12, all values highly significant (Table 2). The F_{ST} value comparing the two subclades of C (C1 and C2) was, however, not significant ($F_{ST} = 0.05$, $p = 0.252$). The assignment test based on three different methods assigned all individuals correctly to the three different morphs with their characteristic EODs, with very few exceptions: The two Bayesian-based methods were not able to correctly assign the female of morph A (Cn10), whereas the frequency-based method assigned it to morph A with a probability of 0.31. Furthermore, one individual of morph C (Cn26) failed to be assigned with a significant probability in one method, but was correctly assigned by the other methods. The STRUCTURE analysis yielded the highest likelihood for $k = 3$ subgroups and all but one specimen (Cn10) were unambiguously assigned to their correct respective morph A, B, or C (data not shown). By statistically comparing the mean expected heterozygosities, we were able to show that genetic variability (at microsatellite loci) is significantly higher within morph C than within morphs A and B (Table 2).

Table 2
Genetic variation and pairwise divergence at microsatellite loci in morphs of *C. numenius*

Clade	A	B	C
A	0.68	0.16 ($p < 0.001$)	0.10 ($p < 0.001$)
B	0.02 ($p = 0.313$)	0.66	0.15 ($p < 0.001$)
C	0.12 ($p < 0.001$)	0.14 ($p = 0.001$)	0.80

Diagonal, mean expected heterozygosity (H_E); Above, pairwise F_{ST}; Below, pairwise difference in H_E.

4. Discussion

4.1. Genetic heterogeneity in C. tamandua and C. numenius

The predefinition of *C. tamandua* and *C. numenius* by morphological characteristics is strongly supported by our phylogenetic analyses. Individuals of both species form monophyletic groups, which, however, differ considerably from each other in several aspects: (1) Despite exhibiting considerable genetic variation, all our *C. tamandua* individuals are morphologically uniform, both as juveniles and as adults; in *C. numenius*, juveniles are uniform, while adults develop into three morphs, differentiated from one another by slight, but consistent morphological differences, especially regarding trunk (tube like snout) shape and body shape (height vs. length; cf. Fig. 4). (2) Apart from the larval stage (see above), *C. tamandua* displays only one waveform of EOD, regardless of age, body size, sex, and genotype. In contrast, there is one common juvenile EOD waveform in all *C. numenius*, which develops into different adult EOD waveforms. In at least one morph, EODs are also sex-specific. Within both species, there is a great amount of genetic heterogeneity. In *C. numenius*, this variation might be due to cryptic species, indicated by the congruence between morphology, adult male EOD, and several unlinked genetic markers (see below). As morphology and EOD were uniform within *C. tamandua*, we could not associate any other factor with a particular genetic lineage here. So far, we have no evidence to reject the hypothesis that *C. tamandua*, albeit genetically very variable, comprises a single interbreeding species. One possible explanation for the high within-species genetic variation is a potentially large effective population size, as *C. tamandua* is the *Campylomormyrus* species with the widest geographic distribution (Gosse, 1984). However, to clarify this point, additional samples over the entire distribution range of *C. tamandua* would be necessary, which was outside the scope of this study.

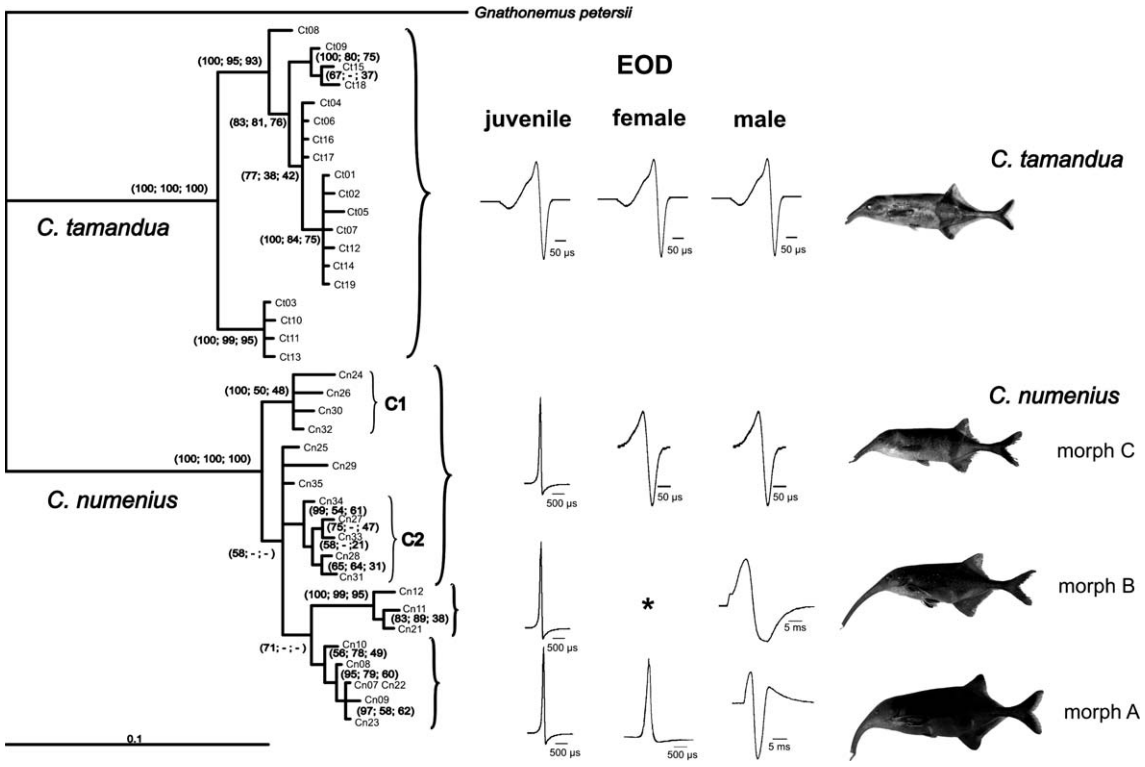

Fig. 4. Bayesian phylogeny based on the combined dataset of mitochondrial cytochrome *b*, nuclear S7 gene and flanking regions of four unlinked microsatellite loci (2222 bp). Branch length is proportional to the amount of character change. Statistical supports from Bayesian, Neighbour Joining and Parsimony analyses are given in brackets. Associated EOD types and morphs are shown within the tree. All specimens of the *C. numenius* complex produce a common juvenile EOD and change their EOD with maturity. Missing data are indicated by *. Note the different time scales of EOD duration.

4.2. Electric organ discharge as indicator of reproductive isolation

Due to our electrophysiological and phylogenetic data, we were able to determine at least three different clades within *C. numenius* (morphs A, B, and C). As juveniles, all these fishes were morphologically indistinguishable and showed identical EODs. The fact that these fishes change their EOD dramatically at maturity points towards the importance of this mechanism for species recognition in the course of mating. This is particularly likely when looking at morph A. This is the phylogenetic group with strikingly different EOD among the two sexes (cf. Fig. 2), where females show 15 times shorter EOD than males, together with large difference in shape and course. An important role of the EOD during mate choice has been previously suggested in other mormyrids, either triggered by the sequence of pulse intervals (Bratton and Kramer, 1989; Crawford, 1991) or by the EOD waveform (Hopkins and Bass, 1981). A single previous study on *Campylomormyrus* (Kramer and Kuhn, 1994) stressed the importance of the sequence of discharge intervals for mate recognition. This study, however, might be partially compromised by investigating mainly small fishes (under 12 cm), some of which—according to our findings—might not have displayed adult EODs.

Direct evidence from behavioural experiments is still lacking to prove our hypothesis that it is indeed the EOD's waveform and duration which triggers species-specific mate recognition in the genus *Campylomormyrus*. Nevertheless, our findings strongly support that, in *C. numenius*, different adult male EODs are indeed confined to sympatrically occurring, yet most likely reproductively isolated groups of fishes (i.e., biological species), as they are associated with genetic sequence divergence at a series of unlinked loci. Furthermore, our microsatellite data independently proved our classification into three different morphs. This is particularly shown by the correct assignment of all individuals to the three different morphs. In theory, reproductive isolation should be shown on multiple independent loci, as is the case here. Our F_{ST} estimates are at the upper level found in studies on closely related fish that exhibit behavioural evidence of reproductive isolation ranging from at least moderate assortative mating to rather complete premating reproductive isolation (Barluenga and Meyer, 2004; Schliewen et al., 2001; Taylor and McPhail, 2000; van Oppen et al., 1998; Wilson et al., 2000). Another study about morphological cryptic sympatric mormyrids of the genus *Brienomyrus*, which produce alternate EOD types, yielded considerably lower values (highest five-locus $F_{ST} = 0.007$, highest single locus $F_{ST} = 0.015$) (Arnegard et al., 2005). We therefore conclude that our genetic evidence supports the hypothesis of reproductive isolation between identical juvenile morphs with dramatic adult EOD differences.

If we accept that *C. numenius* consists of several species, it remains to be evaluated whether EOD divergence is the *cause* or the *effect* of speciation, i.e., whether EOD, as a reproduc-

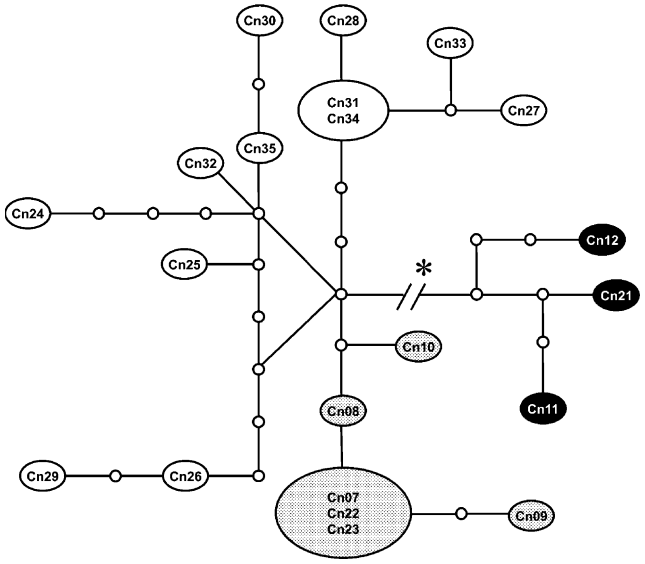

Fig. 5. Network analysis based on statistical parsimony (95% criterion) of Templeton et al. (1992) showing the relationships among the cyt *b* haplotypes found within *C. numenius*. Each haplotype is represented by a circle; the size of each circle is proportional to the number of individuals bearing the haplotype. Empty circles indicate intermediate haplotypes not present in our sample. * Bracketed connection indicates nine additional nucleotide substitutions.

tive isolation mechanism, possesses the ability to play a key role during sympatric speciation, or whether it just diverges, after speciation driven by other factors has occurred. We are unable to present direct evidence to distinguish between these alternative scenarios. However, we hypothesize that, if EOD divergence plays a key role in speciation, divergence should be particularly prominent among closely related clades. This is indeed supported by our data (see Fig. 4) as most variation in EOD (especially in EOD duration) occurs among males of closely related groups (morphs A, B, and C in *C. numenius*), while EOD difference among more distantly related groups can be much less pronounced (see male EOD in *C. tamandua* vs. *C. numenius* morph C), as other isolation mechanisms might have evolved here. We also argue that the specific ontogenetic shift in EOD observed especially in adult males (Fig. 4) is a further indicator of the importance of this character for mate recognition.

4.3. Cryptic sympatric species within C. numenius

Like other authors (Hopkins, 1999), we hypothesized beforehand that, especially in the genus *Campylomormyrus*, cryptic species might be hidden behind similar and, at least as juvenile, identical morphs. Indeed, our data support the existence of several cryptic species with considerably different characteristic adult EOD waveform types. Throughout our sampling, we were able to identify three different EOD waveform types (morphs A, B, and C). Two of them show radical EOD changes towards clearly distinct EOD types at maturity and form well supported phylogenetic clades (morphs A and B).

Morph C, however, does not perfectly fit into this picture. These fishes show little change in their morphological characteristic during growth compared to the other types and their ontogenetic change in EOD waveform is only slight. Clade C specimens exhibited significantly more genetic variation than the others EOD types, both with regard to the sequenced genes (cytochrome *b*, S7, and flanking regions of microsatellite loci) and at 16 microsatellite loci. In theory, this could reflect a larger effective population size of a single "morph C" species. This view is however challenged, as none of our phylogenetic analyses assigned all morph C specimens to a single supported clade. On the base of our DNA sequence data, phylogenetic searches identified two supported clades within the morph C specimens (C1 and C2). We used our microsatellite data as unlinked markers to test the hypothesis of genetic divergence among these clades. The F_{ST} was not significant among these two clades, and the assignment test with the same microsatellite data support a correct assignment of all morph C specimens into a single clade. This finding is indicative of a single "morph C" species. The genetic heterogeneity among "morph C" specimens could then suggest a large effective population size of this species. Our most recent catch campaign at the study site at Congo River indeed revealed morph C specimens to be most numerous there (pers. obs.).

Our observations of the EOD development during growth, the EOD differences between morphs, and the correct assignment of the different morphs based on microsatellites to clades identified by unlinked sequence data support the idea that mormyrid fishes of the genus *Campylomormyrus* comprise a set of cryptic species living in sympatry. Evidently, that does not necessarily imply that they have evolved in sympatry from a common ancestor. Whether the speciation has occurred in sympatry or whether allopatric speciation was followed by secondary contact, is hard to evaluate by direct evidence. However, our data would also be consistent with a scenario of sympatric speciation in *C. numenius*. There is theoretical evidence for sympatric speciation driven by sexual selection (Doebeli and Dieckmann, 2000; Kirkpatrick and Ravigne, 2002; van Doorn et al., 2004; van Doorn et al., 1998), as well as an increasing number of case studies, especially in fishes (Lande et al., 2001; Mendelson, 2003; Seehausen and van Alphen, 1999). Additionally, the importance of sexual selection for maintaining reproductive barriers between species has been demonstrated as well (Seehausen et al., 1997). In our case, assuming strong assortative mating based upon the EOD characteristics (which still has to be proven by mate choice experiments), sexual selection could have forced speciation. The variation of the EOD waveform might not be the only responsible factor, but at least could have played an important role during speciation. Interestingly, the small morphological differences among our adult morphs in *C. numenius* are mainly in their trunk morphology (trophic apparatus). This might indicate small differences in feeding ecology of these morphs. Possibly, the numerous sympatric mormyrid species are highly adapted

to a particular ecological niche and the development of EOD differences as a prezygotic isolation mechanism could have triggered the enormous radiation of mormyrid fishes in Africa.

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References

Alves-Gomes, J., Hopkins, C.D., 1997. Molecular insights into the phylogeny of Mormyriform fishes and the evolution of their electric organs. Brain Behav. Evol. 49, 324–350.

Arnegard, M.E., Bogdanowicz, S.M., Hopkins, C.D., 2005. Multiple cases of striking genetic similarity between alternate electric fish signal morphs in sympatry. Evolution 59, 324–343.

Barluenga, M., Meyer, A., 2004. The Midas cichlid species complex: incipient sympatric speciation in Nicaraguan cichlid fishes? Mol. Ecol. 13, 2061–2076.

Bass, A.H., 1986. Species differences in electric organs of mormyrids: substrates for species-typical electric organ discharge waveforms. J. Comp. Neurobiol. 244, 313–330.

Bastian, J., 1994. Electrosensory organisms. Phys. Today 47, 30–37.

Baudouin, L., Lebrun, P., 2001. An operational Bayesian approach for the identification of sexually reproduced cross-fertilized populations using molecular markers. Acta Horticultura 546, 81–94.

Bratton, B.O., Kramer, B., 1989. Patterns of the electric organ discharge during courtship and spawning in the mormyrid fish, *Pollimyrus isidori*. Behav. Ecol. Sociobiol. 24, 349–368.

Chow, S., Hazama, K., 1998. Universal PCR primers for S7 ribosomal protein gene introns in fish. Mol. Ecol. 7, 1255–1256.

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1659.

Crawford, J.D., 1991. Sex recognition by electric cues in a sound-producing mormyrid fish, *Pollimyrus isidori*. Brain Behav. Evol. 38, 20–38.

Crawford, J.D., 1992. Individual and sex specificity in the electric organ discharges of breeding mormyrid fish (*Pollimyrus isidori*). J. Exp. Biol. 164, 79–102.

Crawford, J.D., Huang, X.F., 1999. Communication signals and sound production mechanisms of mormyrid electric fish. J. Exp. Biol. 202, 1417–1426.

Denizot, J.P., Kirschbaum, F., Westby, G.W.M., Tsuji, S., 1982. On the development of the adult electric organ in the Mormyrid fish *Pollimyrus isidori* (with special focus on the innervation). J. Neurocytol. 11, 913–934.

Doebeli, M., Dieckmann, U., 2000. Evolutionary branching and sympatric speciation caused by different types of ecological interactions. Am. Nat. 156, S77–S101.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes—application to human mitochondrial-DNA restriction data. Genetics 131, 479–491.

Farris, J.S., Kluge, A.G., Eckardt, M.J., 1970. A numerical approach to phylogenetic systematics. Syst. Zool. 19, 172.

Feulner, P.G.D., Kirschbaum, F., Tiedemann, R., 2005. Eighteen microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa. Mol. Ecol. Notes. 5, 446–448.

Friedman, M.A., Hopkins, C.D., 1996. Tracking individual mormyrid electric fish in the field using electric organ discharge waveforms. Anim. Behav. 51, 391–407.

Gosse, J.-P., 1984. Mormyriformes. In: Daget, J., Gosse, J.-P., Thys van den Audenaerde, D.F.E. (Eds.), Check-List of The Freshwater Fishes of Africa, vol. 1. ORSTOM MRAC Tervuren, Paris, pp. 63–124.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symp. ser. 41, 95–98.

Hasegawa, M., Kishino, H., Yano, T.A., 1985. Dating of the human ape splitting by a molecular clock of mitochondrial-DNA. J. Mol. Evol. 22, 160–174.

Hopkins, C.D., Bass, A.H., 1981. Temporal coding of species recognition signals in an electric fish. Science 212, 85–87.

Hopkins, C.D., 1999. Design features for electric communication. J. Exp. Biol. 202, 1217–1228.

Huelsenbeck, J.P., Rannala, B., Masly, J.P., 2000. Accommodating phylogenetic uncertainty in evolutionary studies. Science 288, 2349–2350.

Kirkpatrick, M., Ravigne, V., 2002. Speciation by natural and sexual selection: models and experiments. Am. Nat. 159, S22–S35.

Kirschbaum, F., 1977. Electric-organ ontogeny—distinct larval organ precedes adult organ in weakly electric fish. Naturwissenschaften 64, 387–388.

Kirschbaum, F., 1981. Ontogeny of both larval electric organ and electromotoneurones in *Pollimyrus isidori* (Mormyridae, Teleostei). In: Szabo, T., Czeh, G. (Eds.), Advances in Physiological Sciences. Sensory Physiology of Aquatic Lower Vertebrates, vol. 31. Pergamon Press, Oxford and New York, pp. 129–157.

Kirschbaum, F., Schugardt, C., 2002. Reproductive strategies and developmental aspects in mormyrid and gymnotiform fishes. J. Physiol.-Paris 96, 557–566.

Kramer, B., Kuhn, B., 1994. Species recognition by the sequence of discharge intervals in weakly electric fishes of the genus *Campylomormyrus* (Mormyridae, Teleostei). Anim. Behav. 48, 435–445.

Lande, R., Seehausen, O., van Alphen, J.J.M., 2001. Mechanisms of rapid sympatric speciation by sex reversal and sexual selection in cichlid fish. Genetica 112, 435–443.

Landsman, R.E., 1993. Sex-differences in external morphology and electric organ discharges in imported *Gnathonemus petersii* (Mormyriformes). Anim. Behav. 46, 417–429.

Larget, B., Simon, D.L., 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Mol. Biol. Evol. 16, 750–759.

Lauder, G.V., Liem, K.F., 1983. The evolution and interrelationship of the actinopterygian fishes. Bull. Mus. Comp. Zool. 150, 95–197.

Lavoué, S., Bigorne, R., Lecointre, G., Agnese, J.F., 2000. Phylogenetic relationships of mormyrid electric fishes (Mormyridae; Teleostei) inferred from cytochrome *b* sequences. Mol. Phylogenet. Evol. 14, 1–10.

Lavoué, S., Sullivan, J.P., Hopkins, C.D., 2003. Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. Biol. J. Linn. Soc. 78, 273–292.

Lissman, H.W., Machin, K.E., 1958. The mechanism of object location in *Gymnarchus niloticus* and similar fish. J. Exp. Biol. 35, 451–486.

Lovell, P.V., Hopkins, C.D., Harned, G.D., 1997. The diversity of electric organ discharges found in eight species of *Campylomormyrus* electric fish (Mormyridae). In: Abstracts of the Annual Meeting of the Society of Neurosciences vol. 23, p.2.

Mau, B., Newton, M.A., 1997. Phylogenetic inference for binary data on dendograms using Markov chain Monte Carlo. J. Comput. Graph. Stat. 6, 122–131.

Mau, B., Newton, M.A., Larget, B., 1999. Bayesian phylogenetic inference via Markov chain Monte Carlo methods. Biometrics 55, 1–12.

Mendelson, T.C., 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: Etheostoma). Evolution 57, 317–327.

Nelson, J.S., 1994. Fishes of The World. John wiley, New York.

Paetkau, D., Calvert, W., Stirling, I., Strobeck, C., 1995. Microsatellite analysis of population-structure in Canadian polar bears. Mol. Ecol. 4, 347–354.

Palumbi, S.R., 1996. Nucleic acids. II. The polymerase chain reaction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics. Sinauer Associates, Sunderland, MA, pp. 205–248.

Piry, S., Alapetite, A., Cornuet, J.M., Paetkau, D., Baudouin, L., Estoup, A., 2004. GENECLASS2: a software for genetic assignment and first-generation migrant detection. J. Hered. 95, 536–539.

Poll, M., Gosse, J.-P., Orts, S., 1982. Le genre *Campylomormyrus* Bleeker, 1874, étude systématique et description d'une espéce nouvelle (Pisces Mormyridae). Bulletin Institut Royal des Sciences Naturelles de Belgique Biologie 54, 1–44.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Rannala, B., Yang, Z.H., 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol. 43, 304–311.

Rannala, B., Mountain, J.L., 1997. Detecting immigration by using multilocus genotypes. Proc. Natl. Acad. Sci. USA 94, 9197–9201.

Roberts, T.R., Stewart, D.J., 1976. An ecological and systematic survey of fishes in the rapids of the lower Zaire or Congo river. Bull. Mus. Comp. Zool. 147, 239–317.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Saitou, N., Nei, M., 1987. The neighbor-joining method—a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T., Tautz, D., 2001. Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. Mol. Ecol. 10, 1471–1488.

Schneider, S., Roessli, D., Excoffier, L., 2000. ARLEQUIN: a software for population genetics data version 2.000. Department of Anthropology, University of Geneva, Switzerland.

Schugardt, C., Kirschbaum, F., 1998. Sozial- und Fortpflanzungsverhalten von Mormyriden (Nilhechten). In: Greven, H., Riehl, R. (Eds.) Verhalten der Aquarienfische. Birgit Schmettkamp Verlag, Bornheim, vol Symposiumband, pp. 87–98.

Schugardt, C., Kirschbaum, F., 2002. Nilhechte des *Campylomormyrus numenius*-Formenkreises: Bemerkungen zur Ontogenese des Habitus, der elektrischen Entladung sowie zum Fortpflanzungsverhalten. In: Greven, H., Riehl, R. (Eds.), Verhalten der Aquarienfische, Vol. 2. Birgit Schmettkamp Verlag, Bornheim, pp. 137–146.

Seehausen, O., van Alphen, J.J.M., Witte, F., 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. Science 277, 1808–1811.

Seehausen, O., van Alphen, J.M., 1999. Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? Ecol. Lett. 2, 262–271.

Seutin, G., White, B.N., Boag, P.T., 1991. Preservation of avian blood and tissue samples for DNA analyses. Can. J. Zool. 69, 82–90.

Shimodaira, H., 2002. An approximately unbiased test of phylogenetic tree selection. Syst. Biol. 51, 492–508.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Shimodaira, H., Hasegawa, M., 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics Application Notes 17, 1246–1247.

Sullivan, J.P., Lavoué, S., Hopkins, C.D., 2000. Molecular systematics of the African electric fishes (Mormyroidea: Teleostei) and a model for the evolution of their electric organs. J. Exp. Biol. 203, 665–683.

Sullivan, J.P., Lavoué, S., Hopkins, C.D., 2002. Discovery and phylogenetic analysis of a riverine species flock of African electric fishes (Mormyridae: Teleostei). Evolution 56, 597–616.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. USA 101, 11030–11035.

Taverne, L., 1972. Ostéologie des genres *Mormyrus* Linné, *Mormyrops* Müller, *Hyperopisus* Gill, *Isichthys* Gill, *Myomyrus* Boulenger, *Stomatorhinus* Boulenger et *Gymnarchus* Cuvier considérations générales sur la systématique des poissons de l'ordre des Mormyriformes. MRAC, Tervuren.

Taylor, E.B., McPhail, J.D., 2000. Historical contingency and ecological determinism interact to prime speciation in sticklebacks, *Gasterosteus*. Proc. R. Soc. Lond. B 267, 2375–2384.

Templeton, A.R., Crandall, K.A., Sing, C.F., 1992. A cladistic inference of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132, 619–633.

van Doorn, G.S., Noest, A.J., Hogeweg, P., 1998. Sympatric speciation and extinction driven by environment dependent sexual selection. Proc. R. Soc. Lond. B 265, 1915–1919.

van Doorn, G.S., Dieckmann, U., Weissing, F.J., 2004. Sympatric speciation by sexual selection: A critical reevaluation. Am. Nat. 163, 709–725.

van Oppen, M.J.H., Turner, G.F., Rico, C., Robinson, R.L., Deutsch, J.C., Genner, M.J., Hewitt, G.M., 1998. Assortative mating among rock-dwelling cichlid fishes supports high estimates of species richness from Lake Malawi. Mol. Ecol. 7, 991–1001.

von der Emde, G., 1999. Active electrolocation of objects in weakly electric fish. J. Exp. Biol. 202, 1205–1215.

Weir, B.S., Cockerham, C.C., 1984. Estimating F-statistics for the analysis of population structure. Evolution 38, 1358–1370.

Werneyer, M., Kramer, B., 2002. Intraspecific agonistic interactions in freely swimming mormyrid fish, *Marcusenius macrolepidotus* (South African form). J. Ethol. 20, 107–121.

Westby, G.W.M., Kirschbaum, F., 1977. Emergence and development of electric organ discharge in Mormyrid fish, *Pollimyrus isidori* 1. Larval discharge. J. Comp. Physiol. 122, 251–271.

Westby, G.W.M., Kirschbaum, F., 1978. Emergence and development of electric organ discharge in Mormyrid fish, *Pollimyrus isidori* 2. Replacement of larval by adult discharge. J. Comp. Physiol. 127, 45–59.

Westby, G.W.M., Kirschbaum, F., 1982. Sex-differences in the waveform of the pulse-type electric fish, *Pollimyrus isidori* (Mormyridae). J. Comp. Physiol. 145, 399–403.

Wilson, A.B., Noack-Kunnmann, K., Meyer, A., 2000. Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: sexual selection versus ecological diversification. Proc. R. Soc. Lond. B Biol. Sci. 267, 2133–2141.

7.3 Article III:

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Adaptive radiation in African weakly electric fish (Teleostei: Mormyridae: *Campylomormyrus*): a combined molecular and morphological approach

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Running title: Adaptive radiation in *Campylomormyrus*

Abstract

We combined multiple molecular markers and geometric morphometrics to revise the current taxonomy and to build a phylogenetic hypothesis for the African weakly electric fish genus *Campylomormyrus*. Genetic data (2039bp DNA sequence of mitochondrial cytochrome *b* and nuclear S7 genes) on 106 specimens support the existence of at least six species occurring in sympatry. We were able to further confirm these species by microsatellite analysis at 16 unlinked nuclear loci and landmark-based morphometrics. We assigned them to nominal taxa by comparisons to type specimens of all *Campylomormyrus* species recognised so far. Additionally, we showed that the shape of the elongated trunk-like snout is the major source of morphological differentiation among them. This finding suggests that the radiation of this speciose genus might have been driven by adaptation to different food sources. The divergence in adult electric organ discharge (EOD) among *Campylomormyrus* species might comprise an effective prezygotic isolation mechanism.

Keywords: Electric fish; Mormyridae; *Campylomormyrus*; Phylogeny; S7; Cytochrome *b*; Microsatellites; Geometric morphometrics; Morphology.

Introduction

Africa hosts a great number of so-called fish species flocks (i.e. speciose monophyletic groups with restricted distributions). Among these, lacustrine cichlid species flocks are probably the most renowned and well studied. However, such explosive speciation phenomena are not limited to this taxonomic group. Specifically, Sullivan *et al.* (2002; 2004) proposed weakly electric fish of the family Mormyridae as potential model organisms to study species flock evolution in rivers. Mormyrids comprise one of the most diverse clades of freshwater fish from Africa and the single largest known group of electric fish (AlvesGomes & Hopkins, 1997). Being nocturnal, these fishes use electric cues to actively locate objects in darkness (Lissman & Machin, 1958; Bastian, 1994; von der Emde, 1999). More importantly from an evolutionary point of view, the electric organ discharge (EOD) plays a key role in pair formation, mating, and social attraction (Bratton & Kramer, 1989; Crawford, 1991; Kramer & Kuhn, 1994). This is particularly evident in *Campylomormyrus*, the main object of the study presented here. This genus exhibits a wide diversity of EOD waveform types, which are almost invariably species-specific. Therefore, EOD can be used as an important character to discriminate species that are otherwise cryptic (Hopkins, 1999).

By monitoring ontogenetic changes in EOD and by combining these observations with the analysis of multiple molecular markers, we recently demonstrated the existence of cryptic species hidden under the formal name *C. numenius* (Feulner *et al.*, 2006). Our findings suggest that *Campylomormyrus* could potentially be another compelling system to investigate speciation in the light of competing models of diversification of ecological, morphological and behavioural traits. While morphological and genetic analysis strongly support the monophyly of mormyroids as well as the sister taxa relationship between gymnarchids and mormyrids (Lauder & Liem, 1983), the existing molecular data sets suggest conflicting phylogenies at and near the species level. In addition, very few studies considered the genus

Campylomormyrus, whose systematics are extremely puzzling. Based on the analysis of morphological characters, the number of described species fluctuated through the years from 16 (Taverne, 1972) to three (Roberts & Stewart, 1976) and again to 14 (Poll *et al.*, 1982) (Table 1). Most of the species considered nowadays valid are endemic to a single river system, the Congo and its tributary streams (Table 1 and Fig. 1). Some of them can be found throughout the entire Congo basin, others are restricted to certain areas (Luapula River/Lake Moero or Kasai River). *C. phantasticus* is the only *Campylomormyrus* species not present in the Congo Basin, being limited to the Sanaga River (Cameroon). *C. tamandua* is the most widely distributed species and the only one whose range extends across different river systems, including Congo, Volta, Niger, and Tchad/Shari (Gosse, 1984). So far, only two species (*C. numenius and C. tamandua*) have been included in molecular phylogenies (Sullivan *et al.*, 2000; Lavoué *et al.*, 2003).

While this genus may be a unique system to study general evolutionary phenomena of speciation and adaptation, neither a robust phylogenetic hypothesis nor a thoroughly tested taxonomic arrangement have been proposed so far. Our study aims at addressing both these essential pre-requisites towards a proper understanding of the relationship between speciation and ecological/phenotypic diversification in this still enigmatic group. To this end, we rely on two lines of evidence: (1) we evaluate morphology by quantitative morphometric assessment based on eleven landmarks. We analysed type specimens representing all the 17 *Campylomormyrus* species recognized to date, deposited at the Royal Museum for Central Africa (MRAC) Tervuren (Belgium), also including three type specimens not available there (Harder, 2000). We compared these museum specimens to 106 individuals caught in the wild in the course of our project, in order to match our samples to the typed representatives of the genus *Campylomormyrus*. Such an approach should ensure a cross-check of correct identification of the wild samples as well as reveal patterns of morphological variation among them. (2) We produced for the first time a comprehensive molecular phylogeny for the genus

Campylomormyrus by using sequence polymorphisms of one mitochondrial (cyt *b*: the complete cytochrome *b* gene, 1142 bp) and one nuclear gene (S7: the first and second introns as well as the second exon of the gene coding for the S7 ribosomal protein, 897 bp). These genes proved useful in studies on mormyrids at a comparable level of taxonomic separation (Sullivan *et al.*, 2002; Lavoué *et al.*, 2003; Feulner *et al.*, 2006). Furthermore, we also screened all individuals included in the study for length polymorphisms at 16 microsatellite loci. We previously demonstrated for *C. numenius* that combining such different and unlinked molecular markers is a powerful method to correctly identify distinct evolutionary lineages and cryptic species (Feulner *et al.*, 2006). Ultimately, by relating genetic distinctness to morphological divergence, we aim at identifying those morphological traits which might relate to adaptation in the radiation of the genus *Campylomormyrus*.

Materials and methods

Field sampling

We sampled 66 *Campylomormyrus* specimens during an expedition to Brazzaville in August 2004 (Fig. 1). All but seven specimens were sampled at the same location (rapids south of Brazzaville). Four specimens (K01, K66, K67, K68) were sampled a few kilometers southwards at the inflow of the Foulakari River, while three specimens (K69, K70, K71) are from Kintele, just north of Brazzaville. Fin clips of the right pectoral fin were taken and stored in 1ml of tissue buffer (Seutin *et al.*, 1991).To expand the sample size, we included the data on 40 *Campylomormyrus* specimens from Brazzaville/Kinshasa from our previous study (Feulner *et al.*, 2006) in the statistical analyses. As outgroup species we included one *Hippopotamyrus wilverthi* and one *Marcusenius sp.* specimen.

Phylogenetic analysis

DNA extraction, polymerase chain reaction (PCR) amplifications, and sequencing was performed as described in Feulner *et al.* (2006). All obtained sequences for cyt *b* and S7 were submitted to GenBank (Accession No. XXxxxxxx-XXxxxxxx). We aligned sequences using BioEdit version 7.0.0 (Hall, 1999) and reconstructed a phylogeny for the two genes (cyt *b* and S7) separately, for the combined dataset and for the 3rd codon postitions of cyt *b* alone. We used PAUP 4.0b10 (Sinauer, Inc., Sunderland, USA) to calculate variability estimates, the number of transition (Ti) and transversion (Tv) and to perform χ^2-test for homogeneity of base frequencies. Saturation of sequences was investigated by plotting the absolute number of Ti and Tv against the percentage of sequence divergence. This was done for the cyt *b* gene at 3rd codon position only. Aligned sequences were analysed by Neighbor-Joining (Saitou & Nei, 1987) and Bayesian methods (Rannala & Yang, 1996; Mau & Newton, 1997; Larget & Simon, 1999; Mau *et al.*, 1999; Huelsenbeck *et al.*, 2000). We used Modeltest version 3.06

(Posada & Crandall, 1998) to identify the best model of sequence evolution for each dataset. These models were then applied to calculate genetic distances and to construct trees via NJ in PAUP version 4.0b10. To gain statistical support, we performed 1000 bootstrap replicates. Complex models of nucleotide substitution for estimating evolutionary distances and the application of neighbour joining methods are recommended for large datasets, like in the case of our combined dataset (Tamura *et al.*, 2004). For the Bayesian approach, we employed the same models of sequence evolution used in the NJ analyses, allowing for site-specific rate variation partitioned by gene and for cyt *b* by codon position. We ran one cold and three heated Markov chains for two million generation using MrBayes version 3.0b4 (Ronquist & Huelsenbeck, 2003). We saved trees every 100 generations for a total sample size of 20,000. We discharged the first 2,000 sampled trees as burn-in and used the remaining to calculate a 50% majority rule consensus tree. Phylogenetic tree topologies generated with different phylogenetic methods and competing phylogenetic hypotheses were statistically evaluated with the approximately unbiased tree selection test (AU; Shimodaira, 2002), as implemented in the software package CONSEL (Shimodaira & Hasegawa, 2001). For comparison, we also performed the more conservative Shimodaira & Hasegawa test (SH; Shimodaira & Hasegawa, 1999) as implemented in PAUP version 4.0b10 with the resampling estimate log-likelihood (RELL) technique. We always compared tree topologies simultaneously (Shimodaira & Hasegawa, 1999).

Microsatellite analysis

For genotyping we used microsatellites specifically developed for *Campylomormyrus* (Feulner *et al.*, 2005). Specifically, we used the same 16 microsatellite loci and the same experimental conditions as in Feulner *et al.* (2006). We calculated observed and expected heterozygosity using Arlequin version 2.000 (Schneider *et al.*, 2000) and tested for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium using Genepop on the web

(Raymond & Rousset, 1995). Significance was tested after correction for multiple comparisons via sequential Bonferroni correction at an experiment-wise error rate of $\alpha = 0.05$. In addition, we used microsatellites to assess the accuracy of classifications based on morphology and sequence data. To summarise the degree of genetic differentiation, we calculated pairwise F_{ST}-values using F statistics (Weir & Cockerham, 1984). The significance of F_{ST} was tested by permutation analyses and an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was conducted as implemented in Arlequin version 2.000 (Schneider et al., 2000). By means of pairwise t-tests, we compared mean pairwise differences between expected heterozygosities to contrast genetic variability within groups. Assignment tests were performed with GeneClass version 2.0 (Piry *et al.*, 2004). Two Bayesian-based tests (Rannala & Mountain, 1997; Baudouin & Lebrun, 2001) and one frequence- based test (Paetkau *et al.*, 1995) were used to calculate the probability of each individual's assignment to a particular clade. Genetic subdivision was further evaluated using the STRUCTURE software, estimating the likelihood and sample composition of different numbers of subgroups (k=4; k=5; k=6; Pritchard *et al.*, 2000).

Morphometric analysis

In addition to the specimens we sampled in the field, we performed morphometric analysis on the type material of all the 17 recognized taxa in *Campylomormyrus* (Table 1). Digital images of the types were taken at the Royal Museum for Central Africa (MRAC) Tervuren (Belgium). Images of the three types not available at MRAC were taken from Harder (2000). Digital photographs were taken with a scale bare forthright beside the animal. Landmark-based geometric morphometric methods were used to record x, y coordinates of 11 homologous landmarks and capture information of body shape using TPSdig (Rohlf, 2003). Landmark configuration is shown in Fig. 4a. All the following morphometric analyses were conducted using the IMP package (Sheets, 2002). First, differences due to size, orientation,

and position were removed by Generalized Procrustes Analysis (Rohlf, 1999; Slice, 2001). After superimposition, the data were converted into Principal Warps using the thin-plate spline model (Bookstein, 1989). These variables can then be used in conventional multivariate analyses because they posses the same number of variables as degrees of freedom (Zeldith *et al.*, 2004). We used canonical variates analysis (CVA) of morphological variables to demonstrate the discrimination among predefined groups identified by the screening for genetic polymorphisms. In addition, we also performed a principal component analysis for subsets of samples whose placement was not resolved in the phylogenetic tree. Shape based assignments were performed with CVAgen6N (part of IMP) following the method outline by Nolte & Sheets (2005) which includes a jackknifing procedure as a test of performance of the assignment. In 500 replicates 10% of the data were left out and assigned to groups in the remaining data set. In this way, we were able to test the distinctiveness of the different groups. We used the same approach to assign the samples screened for genetic variation to the type specimens. To this purpose, we calculated mean Mahalanobis distances between each type specimen and each clade recovered in the phylogenetic analyses.

Results

Phylogenetic analysis of sequence data

We sequenced a total of 2039 bp for each individual included in the study. The alignment of the S7 gene included ten indels, while no indels were found in the alignment of cytochrome *b* (cyt *b*) gene. As typical for mitochondrial genomes, there was a low frequency of G`s in cyt *b*, especially in 3rd codon position (G = 0.033). Most of the variation in the combined data set is due to variation in 3rd codon position of the cyt *b* gene. Inspection of the saturation plots (not shown) revealed that saturation is not apparent in our data set. The χ^2 test for base homogeneity indicates that base frequency distribution is always homogenous among taxa, both when genes are analysed separately (test were also performed on each codon position on cyt *b*) and when genes are combined in a single data set. Fig. 2 shows the Bayesian tree obtained on the complete data set using the unequal-frequency Kimura 3-parameter plus Gamma (K81uf+I+G) model (Rodriguez *et al.*, 1990) of evolution (model chosen with Modeltest) and summarises the results of the NJ analysis. Trees obtained with different methods (Bayesian or NJ) and based on different data sets (cyt *b*, S7 or both combined) were statistically indistinguishable with the AU and the SH tests ($0.137 \leq p \leq 0.991$). We consistently recovered five strongly supported monophyletic clades (A, B, D, E, and F). Clade F is clearly separated from the rest, while clade D forms a monophylum with A, B, and C. The node grouping A, B, C *vs.* D is strongly supported. Within C there are two weakly supported clades (C_a and C_b) plus three specimens (K13; K16; Cn29) whose placement could not be resolved by the data. In the case of Cn34 the two homologous alleles we detected at the S7 gene (Cn34a and Cn34b; differing by a single indel) do not cluster together. Rather, Cn34a is placed within the C_a clade while Cn34b is embedded within the C_b clade. To further evaluate resolution among clades A, B, and C as well as within clade C, we used the AU and SH test to evaluate two alternative hypotheses. First, we forced clade C to be monophyletic

with A and B basal to it. Second, we took advantage of the morphometric results to constrain four individuals (K13; K14; K16; K52, see below) to form a monophyletic clade within C. Both the AU and SH tests rejected these two alternative hypotheses ($0.003 \leq p \leq 0.027$).

Microsatellite analysis

Summary statistics for microsatellite variation are shown in Table 2. Between seven and 33 alleles were found per locus. The loci are inherited independently, as just one significant linkage was detected between locus GAII26 and locus GAII42 in a single clade (clade C). The mean observed heterozygosity within morphs ranged from 0.51 to 0.70, the mean expected heterozyosity ranged from 0.61 to 0.78. Only five significant deviations from Hardy-Weinberg expectations were detected, but they are scattered among loci and populations. We performed microsatellite analysis as an independent line of molecular evidence to further evaluate the degree of genetic divergence among clades identified by analyses of sequence and morphometric data. Indeed, microsatelitte analysis further corroborates our phylogenetic analysis: All the five identified clades (A, B, C, D, and F) were also significantly differentiated from one another at microsatellite loci, as pairwise F_{ST} values were between 0.08 and 0.33 and the overall fixation index was 0.19, all values highly significant (Table 3). We did not consider clade E in the microsatellite analysis due to its small sample size. The F_{ST} value comparing the two poorly supported subclades of C (C_a and C_b) was lower but still significant ($F_{ST} = 0.01$, $p = 0.007$). However, within C the four samples identified morphologically (K13; K14; K16; K52) were clearly differentiated from the remaining individuals ($F_{ST} = 0.08$, $p < 0.001$). The assignment test based on three different methods assigned all individuals correctly to the five phylogenetic clades, with very few exceptions: All methods were unable to assign Cn10 to clade A, and one method failed in assigning K16 and K17 to clade C. The sample composition by the STRUCTURE analysis yielding the highest likelihood ($k = 6$) showed similar results (Fig. 3). All five clades were clearly distinct.

Again, Cn10 was either placed in A or D. Clade C was separated from the other clades and a large amount of genetic heterogeneity was evident within this clade.

Morphometric analysis

All the six distinct clades (A, B, C, D, E, F) identified by our phylogenetic analysis are clearly differentiated also on morphometric grounds. All of them formed discrete clusters in a canonical variates analysis (CVA) along the first two axes, which displayed the greatest separation between groups relative to within group variance (Fig. 5a). The differentiation in shape as captured by CV axes could be visualized as displacement vectors for each landmark on a deformation grid relative to a reference (Fig. 4). The first axis (Lambda=0.0007 chisq.=619.1097 df=108 p<0.001) described variation in trunk length, whereas the second axis (Lambda=0.0076 chisq.=417.0081 df=85 p<0.001) was mainly related to body height. There were four more significant but less differentiating CV axes (Lambda>0.04). By means of a distance-based assignment test, all the individuals could be assigned correctly to their source cluster. This proved the utility of the derived axes to discriminate among groups and to determine a given specimens group affinity. The robustness of the CV axes and the assignment test were evaluated by a jackknife test. In this way, 89.8% of the left out data were assigned significantly and correctly into their source groups. PCA analysis identified four specimens within C (K13; K14; K16; K52), which differed clearly from the other individuals in the clade. These individuals formed a distinct cluster in CVA with one significant CV axis (Lambda=0.1068 chisq.=87.2181 df=18 p<0.001) and could be assigned to an additional morphological group (Fig. 5b). Jackknifing-based tests (500 replicates, 10% unknowns) resulted in 90.6% of correct and significant assignments. Table 4 reports the shortest Mahalonobis distances between each cluster of the morphometric analyses (clades recovered by phylogenetic analyses of sequence data) and the assigned type specimen. Clade A was assigned to *C. rhynchophorus*, clade B to *C. numenius*, clade C-I to *C. compressirostris*, clade

C-II to *C. curvirostris*, clade D to *C. tshokwe*, clade E to *C. elephas* and clade F to *C. tamandua*. Specimen K15 achieved the shortest distance to *C. bredoi*.

Discussion

Phylogeny and taxonomy within the genus *Campylomormyrus*

To our knowledge, we were the first to prove that there are genetically distinct clades within *Campylomormyrus* which correspond to characteristic waveform types of the electric organ discharge (EOD) (Feulner *et al.*, 2006). In the study presented here, we were able to substantially expand our sample set to 106 *Campylomormyrus* specimens, as well as we associated phylogenetic clades with taxonomic units by morphometric means. Morphometric analysis has already proven to possess the ability to investigate complex taxonomic problems at the species level (Fink & Zelditch, 1997; Fadda & Corti, 2001; Dobigny *et al.*, 2002; Baylac *et al.*, 2003). Here we used a distance-based method already successfully used in teleosts for identifying hybrids and assigning species into their source population (Nolte & Sheets, 2005). By this approach, distinct phylogenetic clades could be associated with type specimens to obtain a classification. This classification is generally in line with the currently accepted taxonomy proposed by Poll *et al.* (1982), with a few exceptions: The genetically supported clade C-I was assigned to *C. compressirostris*. *C. compressirostris* was first described by Pellegrin (1928) and later also denominated in the classification of Taverne (1972), but was considered synonymous to *C. rhynchophorus* by Poll *et al.* (1982). Both genetically and morphologically, our analysis significantly supported the distinct clades A and C-I, assigned to *C. rhynchophorus* and *C. compressirostris* types, respectively. Genetically, this distinctiveness is not only proven by our phylogenetic result based on two unlinked genes, but further independently confirmed with our microsatellite data. Hence, we consider *C. compressirostris* a valid species.

Generally, the resolution within clade C was complicated by its high variation, both in morphology and genetics. The genetic heterogeneity is particularly well reflected in the results of our microsatellite analysis: Clade C has the highest mean expected heterozygosity

(Table 3) and STRUCTURE reveals specimens with diverse genotype composition (Fig. 3). By means of our morphometric analysis we could identify four specimens within C (C-II) which are differentiated from the rest of C (C-I) (see Fig. 5b) and could be assigned to *C. curvirostris*. While this separation is not visible in the phylogenetic tree, it is supported by the multilocus microsatellite data, which yield a highly significant pairwise F_{ST}. Our molecular data obviously reject the assumption that *Campylomormyrus* includes only three species, as suggested by Roberts & Steward (1976). Nevertheless, all taxa called "species" by these authors form well supported clades in our molecular phylogeny, some of them consisting however of several valid species (Fig. 2, Table 4): *C. tamandua*, considered a separate species by all authors (Table 1), is confirmed as such (clade F; Fig. 2). *C. mirus* (sensu Roberts & Stewart, 1976) is reflected by clade E which we assigned to *C. elephas*, a taxon incorporated in the "*C. mirus*" summation (Table 1). In fact, the number of species within clade E could not be resolved here, because of small sample size and the large differentiation between these specimens within this robust clade. At least, the three specimens analyzed here show slight but pronounced morphological differences. Finally, *C. rhynchophorus* sensu Roberts & Steward (1976) formed a large and strongly supported monophyletic group in our phylogenetic analyses, consisting of clades A to D (Fig. 2). By combining genetics and morphology, we could however unambiguously demonstrate that these clades (A, B, C-I, C-II, and D) represent five sympatrically occurring reproductively isolated groups (i.e., biological species) hidden under the name "*rhynchophorus*".

Basal in the phylogenetic tree, we found a single specimen (K15), not significantly associated with any other clade. In our morphological assignment to type material, K15 appeared most similar to *C. bredoi* (Table 4). We are however in doubt about this assignment, for two reasons: (1) K15 shows special morphological features, e.g. a relatively narrow elongated caudal peduncle, which are not shared by the holotype of *C. bredoi*. (2) *C. bredoi* is so far only known from the headwater region of the Congo and has never been detected in our

sampling region, the Lower Congo. Unfortunately, genetic comparison to the type material is precluded by the fact that all type specimens have been stored in concentrated formalin for prolonged periods of time. Based on its distinct position in our phylogenetic tree, the peculiar morphology, and the lack of a morphologically similar specimen among the *Campylomormyrus* types, it appears possible that K15 might comprise a so far undescribed new species. This has to be verified by further extensive examination of this specimen, beyond the scope of the study presented here.

Adaptive radiation within the genus *Campylomormyrus*

Beside the taxonomic clarification, the major aim of this study was to better understand the adaptive radiation of this group of weakly electric fish. According to Schluter (2000), an adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage. Adaptive radiations can be identified by certain criteria, i.e., common ancestry, rapid speciation, phenotype/environment correlation and trait utility: Common ancestry has been proven multiple times for *Campylomormyrus*, a single genus clearly characterised by its morphology. *Campylomormyrus* formed a well-supported monophyletic taxon in our phylogeny with two different outgroup genera, as well as in various other phylogenies which included a higher number of mormyrid genera (Alves-Gomes & Hopkins, 1997; Lavoué *et al.*, 2000; Sullivan *et al.*, 2000; Lavoué *et al.*, 2003). Rapid speciation is apparent both generally in the entire group of mormyrids and specifically in the genus *Campylomormyrus*. This can be shown by a comparison of numbers of species among contempory clades: While the mormyrids comprise almost 200 described species, their sister taxon, the gymnarchids, are monotypic (Nelson, 1994). Moreover the species-rich mormyrids belong to the osteoglossomorpha, an otherwise species-poor group (Lavoué & Sullivan, 2004). In our study on *Campylomormyrus*, particularly rapid speciation has apparently occurred in the monophyletic group consisting of clades A to D (see Fig. 2; "*C.*

rhynchophorus" sensu Roberts and Steward, 1976), as we could detect 5 species, some of which genetically similar, yet significantly different. This is in particular true for the Clades C-I and C-II, which were significantly separated in the morphological and multilocus microsatellite analysis, but had not acquired reciprocal monophyly at the cytochrome *b* and S7 locus.

Finally, our morphometric analysis allows us to discuss this speciation in the light of phenotype-environment correlation and trait utility. We were able to show that our distinct phylogenetic clades are associated with significant morphological differences (Fig. 5a); therefore the variation at neutral genetic markers is consistent with phenotypic traits. Visualization of the morphological changes captured by the CV axis reveals that shape changes are mainly caused by differences in the trunk like snout (Fig.4b). *Campylomormyrus* feed on insect larvae that burrow into, or hide within, interstitial spaces and holes in clay sediment or river channels (Marrero & Winemiller, 1993). It is therefore reasonable to hypothesise that different trunk shapes are associated with different diets, as the accessibility of certain food items might depend on the morphology of the trophic apparatus. At present, we cannot verify our hypothesis of a correlation between trophic apparatus and feeding ecology by direct evidence, as neither feeding behaviour nor stomach content has been analyzed so far in these nocturnal tropical fishes. Nevertheless, as all species identified on genetic grounds significantly differed in an important morphological trait, i.e., their trophic apparatus, we consider this a first indication for a correlation with the substrate structure (criterion of "phenotype-environment correlation"), as well as the shape of the snout presumably impacts the accessibility of specific food resources (criterion of "trait utility"). We could previously demonstrate that reproductive isolation in *Campylomormyrus* (as detected by phylogenetic analysis and further confirmed by multilocus microsatellite genotyping) is strongly correlated with divergence in waveform types of the electric organ discharge (EOD, Feulner *et al.*, 2006). Here we could demonstrate that these reproductively

isolated groups, i.e., biological species, have significantly diverged in their feeding apparatus. Therefore we are arguing for a diversification of *Campylomormyrus* caused by an adaptation to different food sources and triggered by EOD differences as prezygotic isolation mechanism. A few other studies have also postulated speciation due to disruptive natural selection as adaptation to different food sources and sexual selection via assortative mating as the isolation mechanism (Schliewen *et al.*, 2001; Salzburger *et al.*, 2005; Barluenga *et al.*, 2006). While theoretical models show that speciation by sexual selection alone is unlikely, because of the lack of ecological differentiation to stabilize coexistence of incipient species (Arnegard & Kondrashov, 2004; Kirkpatrick & Nuismer, 2004; van Doorn *et al.*, 2004), sexual selection can promote speciation during an adaptive radiation. While our findings on *Campylomormyrus* are fully consistent with such a hypothesis of sexual selection as a trigger for speciation, we have so far no observational data confirming mate choice based on EOD. We nevertheless argue that, if the species-specific EODs are subject to sexual selection, they should be particularly diverse in adult males. This is indeed confirmed by experimental data, as morphologically indistinguishable juveniles with a common EOD develop into morphologically (slightly) distinct adults with very diverse male EOD (Feulner *et al.*, 2006). In summary, we conclude that *Campylomormyrus* has undergone a rapid speciation with disruptive selection for diverse feeding apparatus and promoted by sexual selection based on strikingly different adult male electric signals (EODs), serving as an effective prezygotic isolation mechanism.

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References

Alves-Gomes, J. & Hopkins, C. D. 1997. Molecular insights into the phylogeny of Mormyriform fishes and the evolution of their electric organs. *Brain Behav. Evol.* **49**: 324-350.

Arnegard, M. E. & Kondrashov, A. S. 2004. Sympatric speciation by sexual selection alone is unlikely. *Evolution* **58**: 222-237.

Barluenga, M., Stolting, K. N., Salzburger, W., Muschick, M. & Meyer, A. 2006. Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature* **439**: 719-723.

Bastian, J. 1994. Electrosensory organisms. *Physics Today* **47**: 30-37.

Baudouin, L. & Lebrun, P. 2001. An operational Bayesian approach for the identification of sexually reproduced cross-fertilizied populations using molecular markers. *Acta Horticultura* **546**: 81-94.

Baylac, M., Villemant, C. & Simbolotti, G. 2003. Combining geometric morphometrics with pattern recognition for the investigation of species complexes. *Biol. J. Linn. Soc.* **80**: 89-98.

Bookstein, F. L. 1989. Principal warps - thin-plate splines and the decomposition of deformations. *Ieee Transactions On Pattern Analysis And Machine Intelligence* **11**: 567-585.

Bratton, B. O. & Kramer, B. 1989. Patterns of the electric organ discharge during courtship and spawning in the mormyrid fish, *Pollimyrus isidori*. *Behav. Ecol. Sociobiol.* **24**: 349-368.

Crawford, J. D. 1991. Sex recognition by electric cues in a sound-producing mormyrid fish, *Pollimyrus isidori*. *Brain Behav. Evol.* **38**: 20-38.

Dobigny, G., Baylac, M. & Denys, C. 2002. Geometric morphometrics, neural networks and diagnosis of sibling Taterillus species (Rodentia, Gerbillinae). *Biol. J. Linn. Soc.* **77**: 319-327.

Excoffier, L., Smouse, P. E. & Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial-DNA restriction data. *Genetics* **131**: 479-491.

Fadda, C. & Corti, M. 2001. Three-dimensional geometric morphometrics of Arvicanthis: implications for systematics and taxonomy. *J. Zool. Syst. Evol. Res.* **39**: 235-245.

Feulner, P. G. D., Kirschbaum, F. & Tiedemann, R. 2005. Eighteen microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa. *Mol. Ecol. Notes* **5**: 446-448.

Feulner, P. G. D., Kirschbaum, F., Schugardt, C., Ketmaier, V. & Tiedemann, R. 2006. Electrophysiological and molecular genetic evidence for sympatric speciation in African weakly electric fishes (Teleostei: Mormyridae: *Campylomormyrus*). *Mol. Phylogenet. Evol.* **39**: 198-208.

Fink, W. L. & Zelditch, M. L. 1997. Shape analysis and taxonomic status of Pygocentrus piranhas (Ostariophysi, Characiformes) from the Paraguay and Parana river basins of South America. *Copeia* 179-182.

Gosse, J.-P. (1984) Mormyriformes. In: *Check-list of the Freshwater Fishes of Africa* (eds. Daget, J., Gosse, J.-P. & Thys van den Audenaerde, D. F. E.), pp. 63-124. ORSTOM, Paris, MRAC, Tervuren.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series* **41**: 95-98.

Harder, W. 2000. *Mormyridae and other Osteoglossomorpha*. Expert Center for Taxonomic Identification, ETI, University of Amsterdam, Amsterdam.

Hopkins, C. D. 1999. Design features for electric communication. *J. Exp. Biol.* **202**: 1217-1228.

Huelsenbeck, J. P., Rannala, B. & Masly, J. P. 2000. Accommodating phylogenetic uncertainty in evolutionary studies. *Science* **288**: 2349-2350.

Kirkpatrick, M. & Nuismer, S. L. 2004. Sexual selection can constrain sympatric speciation. *Proc. R. Soc. Lond. B Biol. Sci.* **271**: 687-693.

Kramer, B. & Kuhn, B. 1994. Species recognition by the sequence of discharge intervals in weakly electric fishes of the genus *Campylomormyrus* (Mormyridae, Teleostei). *Anim. Behav.* **48**: 435-445.

Larget, B. & Simon, D. L. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Mol. Biol. Evol.* **16**: 750-759.

Lauder, G. V. & Liem, K. F. 1983. The evolution and interrelationship of the actinopterygian fishes. *Bull. Mus. Comp. Zool.* **150**: 95-197.

Lavoué, S., Bigorne, R., Lecointre, G. & Agnèse, J. F. 2000. Phylogenetic relationships of mormyrid electric fishes (Mormyridae; Teleostei) inferred from cytochrome b sequences. *Mol. Phylogenet. Evol.* **14**: 1-10.

Lavoué, S., Sullivan, J. P. & Hopkins, C. D. 2003. Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. *Biol. J. Linn. Soc.* **78**: 273-292.

Lavoué, S. & Sullivan, J. P. 2004. Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). *Mol. Phylogenet. Evol.* **33**: 171-185.

Lissman, H. W. & Machin, K. E. 1958. The mechanism of object location in *Gymnarchus niloticus* and similar fish. *J. Exp. Biol.* **35**: 451-486.

Marrero, C. & Winemiller, K. O. 1993. Tube-snouted gymnotiform and mormyriform fishes - convergence of a specialized foraging mode in teleosts. *Environ. Biol. Fishes* **38**: 299-309.

Mau, B. & Newton, M. A. 1997. Phylogenetic inference for binary data on dendograms using Markov chain Monte Carlo. *Journal Of Computational And Graphical Statistics* **6**: 122-131.

Mau, B., Newton, M. A. & Larget, B. 1999. Bayesian phylogenetic inference via Markov chain Monte Carlo methods. *Biometrics* **55**: 1-12.

Nelson, J. S. 1994. *Fishes of the World*. John wiley & Sons, Inc., New York.

Nolte, A. W. & Sheets, H. D. 2005. Shape based assignment tests suggest transgressive phenotypes in natural sculpin hybrids (Teleostei, Scorpaeniformes, Cottidae). *Front. Zool.* **2**: 11.

Paetkau, D., Calvert, W., Stirling, I. & Strobeck, C. 1995. Microsatellite analysis of population-structure in Canadian polar bears. *Mol. Ecol.* **4**: 347-354.

Pellegrin, J. 1928. Poisson du Chiloango et du Congo recueillis par l`expédition du Dr. H. Schouteden (1920-22). *Annales du Musée du Congo Belge, Zoologie, Série I* **3**: 1-51.

Piry, S., Alapetite, A., Cornuet, J. M., Paetkau, D., Baudouin, L. & Estoup, A. 2004. GENECLASS2: A software for genetic assignment and first-generation migrant detection. *J. Hered.* **95**: 536-539.

Poll, M., Gosse, J.-P. & Orts, S. 1982. Le genre *Campylomormyrus* Bleeker, 1874, étude systématique et description d`une espèce nouvelle (Pices, Mormyridae). *Bulletin Institut Royal des Sciences Naturelles de Belgique Biologie* **54**: 1-44.

Posada, D. & Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

Pritchard, J. K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945-959.

Rannala, B. & Yang, Z. H. 1996. Probability distribution of molecular evolutionary trees: A new method of phylogenetic inference. *J. Mol. Evol.* **43**: 304-311.

Rannala, B. & Mountain, J. L. 1997. Detecting immigration by using multilocus genotypes. *Proc. Natl. Acad. Sci. U. S. A.* **94**: 9197-9201.

Raymond, M. & Rousset, F. 1995. Genepop (version-1.2) - population-genetics software for exact tests and ecumenicism. *J. Hered.* **86**: 248-249.

Roberts, T. R. & Stewart, D. J. 1976. An ecological and systematic survey of fishes in the rapids of the lower Zaire or Congo river. *Bull. Mus. Comp. Zool.* **147**: 239-317.

Rodriguez, F., Oliver, J. L., Marin, A. & Medina, J. R. 1990. The general stochastic-model of nucleotide substitution. *J. Theor. Biol.* **142**: 485-501.

Rohlf, F. J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. *J. Classif.* **16**: 197-223.

Rohlf, J. F. 2003. *TpsDig, Digitize Landmarks and Outlines*. Department of Ecology and Evolution, State University of New York at Stony Brook.

Ronquist, F. & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.

Saitou, N. & Nei, M. 1987. The Neighbor-Joining method - a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.

Salzburger, W., Mack, T., Verheyen, E. & Meyer, A. 2005. Out of Tanganyika: Genesis, explosive speciation, key-innovations and phylogeography of the haplochromine cichlid fishes. *BMC Evol. Biol.* **5**:

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T. & Tautz, D. 2001. Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Mol. Ecol.* **10**: 1471-1488.

Schluter, D. 2000. *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.

Schneider, S., Roessli, D. & Excoffier, L. 2000. *ARLEQUIN: A Software for Population Genetics Data Version 2.000*. Department of Anthropology, University of Geneva, Switzerland.

Seutin, G., White, B. N. & Boag, P. T. 1991. Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal Of Zoology-Revue Canadienne De Zoologie* **69**: 82-90.

Sheets, H. D. 2002. *IMP - Integrated Morphometrics Package*. Department of Physics, Canisius College, Buffalo, New York.

Shimodaira, H. & Hasegawa, M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* **16**: 1114-1116.

Shimodaira, H. & Hasegawa, M. 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* **17**: 1246-1247.

Shimodaira, H. 2002. An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.* **51**: 492-508.

Slice, D. E. 2001. Landmark coordinates aligned by procrustes analysis do not lie in Kendall's shape space. *Syst. Biol.* **50**: 141-149.

Sullivan, J. P., Lavoué, S. & Hopkins, C. D. 2000. Molecular systematics of the African electric fishes (Mormyroidea: Teleostei) and a model for the evolution of their electric organs. *J. Exp. Biol.* **203**: 665-683.

Sullivan, J. P., Lavoué, S. & Hopkins, C. D. 2002. Discovery and phylogenetic analysis of a riverine species flock of African electric fishes (Mormyridae: Teleostei). *Evolution* **56**: 597-616.

Sullivan, J. P., Lavoué, S., Arnegard, M. E. & Hopkins, C. D. 2004. AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea: Teleostei). *Evolution* **58**: 825-841.

Tamura, K., Nei, M. & Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 11030-11035.

Taverne, L. 1972. *Ostéologie des genres Mormyrus Linné, Mormyrops Müller, Hyperopisus Gill, Isichthys Gill, Myomyrus Boulenger, Stomatorhinus Boulenger et Gymnarchus Cuvier considérations générales sur la systématique des poissons de l`ordre des Mormyriformes*. MRAC, Tervuren.

van Doorn, G. S., Dieckmann, U. & Weissing, F. J. 2004. Sympatric speciation by sexual selection: A critical reevaluation. *Am. Nat.* **163**: 709-725.

von der Emde, G. 1999. Active electrolocation of objects in weakly electric fish. *J. Exp. Biol.* **202**: 1205-1215.

Weir, B. S. & Cockerham, C. C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358-1370.

Zeldith, M. L., Swiderski, D. L., Sheets, H. D. & Fink, W. L. 2004. *Geometric Morphometrics for Biologists: A Primer*. Elsevier Academic Press, London.

Table 1

Described species according to three different authors, their distribution, and type museum material used for morphometric comparison. H, S, P refers to inclusion of holotypus, syntypus, or paratypus in the analysis.

Taverne 1972	Roberts & Steward 1976	Poll *et al.*, 1982	distribution (Gosse 1984)	type
C. alces	*C. mirus*	*C. alces*	Congo Basin	S
C. bredoi	*C. rhynchophorus*	*C. bredoi*	Lake Moero and Luapula River	H
C. cassaicus	*C. mirus*	*C. cassaicus*	Afflux Kasai River	P
C. christyi	*C. mirus*	*C. christyi*	Congo Basin	S
C. curvirostris	*C. rhynchophorus*	*C. curvirostris*	Congo Basin	H
C. elephas	*C. mirus*	*C. elephas*	Congo Basin	S
C. luapulaensis	*C. rhynchophorus*	*C. luapulaensis*	Upper Luapula	H
C. mirus	*C. mirus*	*C. mirus*	Congo Basin	H
C. numenius	*C. rhynchophorus*	*C. numenius*	Congo Basin	S
C. ibis	*C. rhynchophorus*	*C. numenius*	Congo Basin	S
		C. orycteropus	Lake Moero	H*
C. phantasticus	*C. rhynchophorus*	*C. phantasticus*	Sanaga River	H*
C. rhynchophorus	*C. rhynchophorus*	*C. rhynchophorus*	Congo Basin	S
C. compressirostris	*C. rhynchophorus*	*C. rhynchophorus*	Congo Basin	H
C. lualabaensis	*C. rhynchophorus*	*C. rhynchophorus*	Congo Basin	H
C. tamandua	*C. tamandua*	*C. tamandua*	Volta, Niger, Tchad/Shari and Congo Basin	H*
C. tshokwe	*C. rhynchophorus*	*C. tshokwe*	Kasai River and afflux	H

Type museum specimens were analyzed at the Royal Museum for Central Africa (MRAC), Tervuren (Belgium), except for * for which type specimen information was taken from Harder (2000).

Table 2

Genetic diversity at 16 microsatellite loci in clades of *Campylomormyrus.* For each clade and each microsatellite locus, expected (H_E) and observed (H_O) heterozygosity is given. The range of allele sizes is given for the entire data set.

Locus	Number of alleles	Range of allele size	Test	Clade A	Clade B	Clade C	Clade D	Clade F	Mean
CampGAI14	23	189-243	H_O	1.00	0.60	0.78	0.71	0.43	0.71
			H_E	0.97	0.67	0.82	0.92	0.37	0.90
CampGAI28	22	165-233	H_O	0.57	0.60	0.69	0.36	0.62	0.62
			H_E	0.57	0.73	0.86	0.71	0.88[a]	0.84
CampGAI8	19	173-215	H_O	1.00	0.80	0.85	0.86	0.71	0.83
			H_E	0.90	0.64	0.92	0.84	0.66	0.92
CampGAII17	15	216-246	H_O	0.71	0.40	0.89	0.21	0.57	0.70
			H_E	0.76	0.87	0.89	0.27	0.68	0.88
CampGAII26	24	204-284	H_O	0.43	0.60	0.78	0.71	0.86	0.75
			H_E	0.60	0.87	0.80	0.71	0.84	0.88
CampGAII42	13	346-376	H_O	1.00	0.60	0.89	0.42	0.05	0.65
			H_E	0.86	0.80	0.91	0.61	0.09	0.88
CampGAIII8	27	377-451	H_O	0.86	0.40	0.33	0.29	0.95	0.48
			H_E	0.88	0.67	0.93*	0.74*	0.92	0.94
CampGTI18a	18	136-192	H_O	0.71	0.00	0.43	0.64	0.45	0.47
			H_E	0.66	0.00	0.52	0.69	0.91*	0.69
CampGTI19	8	188-204	H_O	0.29	0.60	0.53	0.79	0.62	0.57
			H_E	0.40	0.67	0.50	0.69	0.64	0.69
CampGTI39	31	159-227	H_O	0.71	0.80	0.93	0.93	1.00	0.92
			H_E	0.74	0.80	0.91	0.80	0.96	0.94
CampGTII27	7	257-281	H_O	0.00	0.00	0.16	0.07	0.33	0.17
			H_E	0.00	0.00	0.20	0.37	0.48	0.47
CampGTII2a	28	193-273	H_O	0.57	0.60	0.82	0.71	0.95	0.80
			H_E	0.69	0.53	0.93	0.80	0.90	0.93
CampGTII6a	18	184-228	H_O	0.86	0.60	0.84	0.64	0.05	0.65
			H_E	0.75	0.53	0.92	0.69	0.15	0.89
CampGTII6b	33	136-204	H_O	0.86	0.60	0.87	0.86	0.90	0.86
			H_E	0.90	0.98	0.94*	0.90	0.95	0.96
CampGTIII41	12	177-217	H_O	0.00	0.00	0.40	0.00	0.62	0.34
			H_E	0.00	0.00	0.44	0.00	0.87	0.58
CampGTIII4b	30	188-248	H_O	1.00	1.00	0.93	1.00	0.76	0.91
			H_E	0.95	0.93	0.95	0.94	0.88	0.96
All loci			H_O	0.66	0.51	0.70	0.58	0.62	0.65
			H_E	0.66	0.61	0.78	0.68	0.70	0.83

* Significant heterozygote deficiency after Bonferroni correction at an experiment-wise error rate of $\acute{\alpha} = 0.05$.

Table 3

Genetic variation and pairwise divergence at 16 microsatellite loci in clades of *Campylomormyrus*. Diagonal, mean expected heterozygosity (H_E); Above, pairwise difference in H_E; Below, pairwise F_{ST}.

Clade	A	B	C	D	F
A	0.66	0.06 (p = 0.345)	0.11 (p = 0.008)	0.00 (p = 0.924)	0.04 (p = 0.741)
B	0.20 (p < 0.001)	0.61	0.17 (p = 0.003)	0.06 (p = 0.385)	0.09 (p = 0.391)
C	0.08 (p < 0.001)	0.13 (p < 0.001)	0.78	0.11 (p = 0.070)	0.08 (p = 0.391)
D	0.17 (p < 0.001)	0.23 (p < 0.001)	0.12 (p < 0.001)	0.68	0.03 (p = 0.739)
F	0.29 (p < 0.001)	0.33 (p < 0.001)	0.23 (p < 0.001)	0.29 (p < 0.001)	0.70

Table 4

Assignment of type specimens to phylogenetic clades based on morphometric analysis of eleven landmarks. Clade C-II consists of K13, K14, K16, and K52, Clade C-I of the remaining specimens of clade C. Typus with the nearest Mahalanobis distances was assigned to each clade.

	n	least distance species	value
clade A	6	*C. rhynchophorus*	4.672
clade B	5	*C. numenius*	5.122
clade C-I	46	*C. compressirostris**	4.789
clade C-II	4	*C. curvirostris*	7.272
clade D	14	*C. tshokwe*	6.080
clade E	3	*C. elephas*	5.396
specimen K15	1	*C. bredoi*	6.984
clade F	20	*C. tamandua*	3.663

* Clade C-I yielded a lower distance (3.595) to *C. tamandua* in this analysis. However, based on a unique coloration pattern not captured in the landmarks, *C. tamandua* can unambiguously be assigned to clade F.

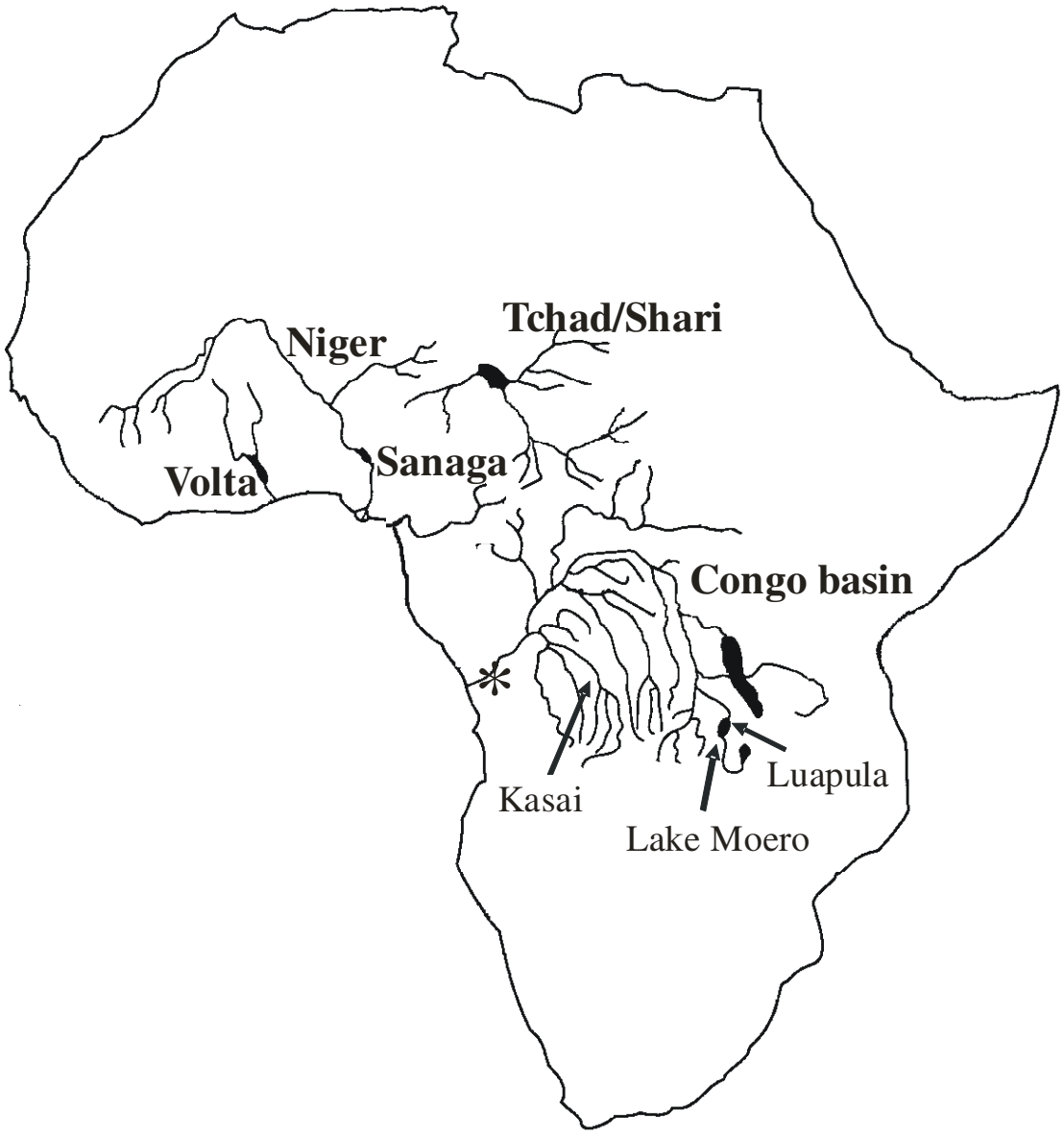
Fig. 1 Geographic location of African river systems in which *Campylomormyrus* occurs. Most species are endemic to the Congo Basin. * indicates the sampling location at Brazzaville/Kinshasa.

Fig. 2 Bayesian phylogeny based on the combined dataset of mitochondrial cytochrome *b* and nuclear S7 gene (2039 bp). Branch length is proportional to the amount of character changes. Numbers in brackets give statistical support (Bayesian and Neighbour Joining analysis) for the respective clade.

Fig. 3 Sample composition (clade A, B, C, D, and F) by the STRUCTURE analysis yielding the highest likelihood (k = 6). Clear structuring into five clades is visible as well as heterogeneity within clade C.

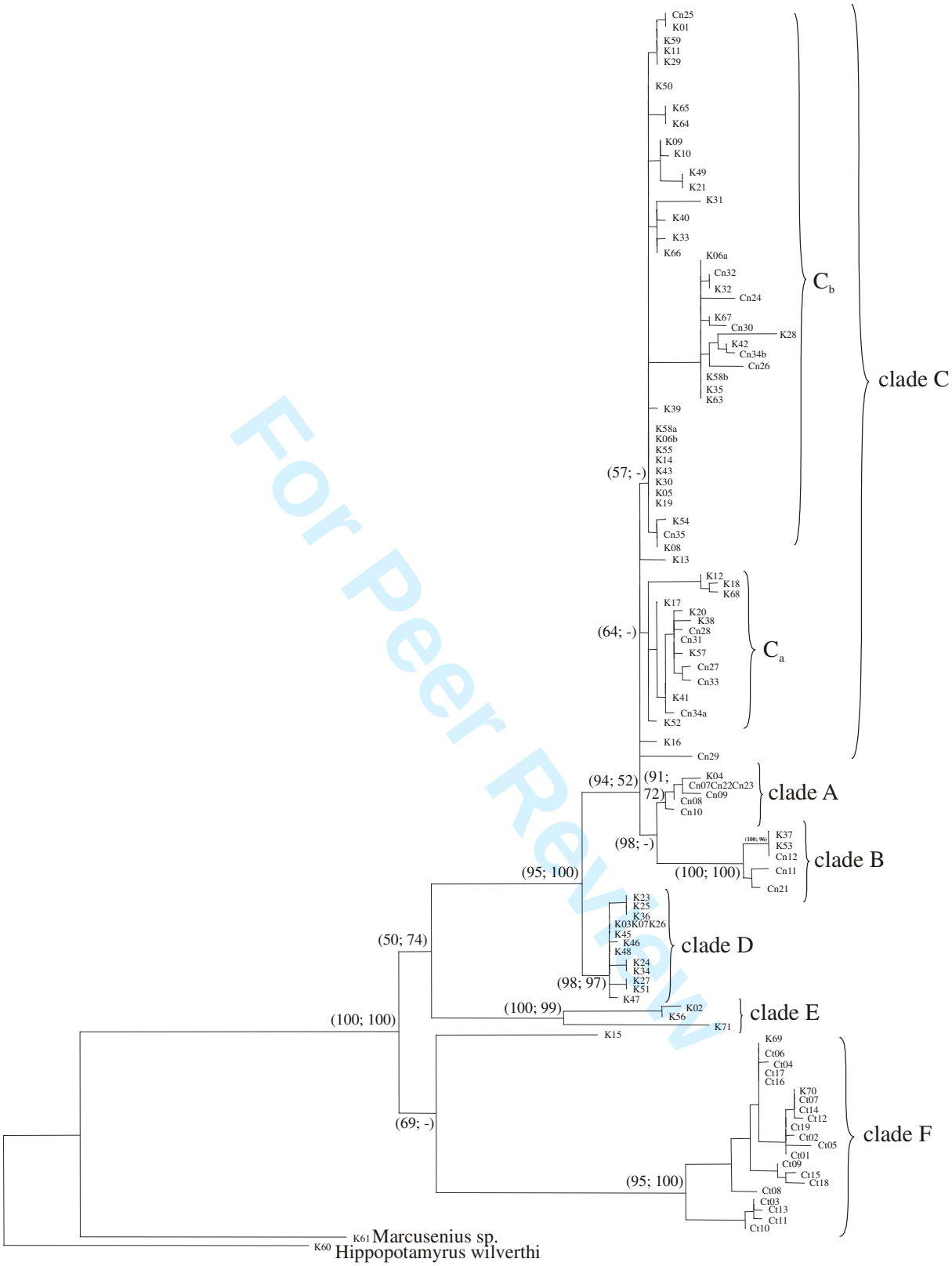
Fig. 4 Landmark configuration and displacement vectors that distinguish clades of *Campylomormyrus*. (a) The eleven landmarks chosen to analyse variability in *Campylomormyrus* body shape. (b-c) Deformation grid with relative displacement vectors visualizing for each landmark the shape changes captured by CVA axes, due to which different groups can be discriminated. The first two of six significant axes are shown. The two shown axes possess by far the smallest Lambda values (Wilks` Lambda 0.0007, respectively 0.0076), indicating the greatest differentiation between the groups along these axes.

Fig. 5 (a) Results of the CVA analysis conducted on morphological variables for all *Campylomormyrus* samples analysed genetically. Letters (A to F) match coding of clades in the tree of Fig. 2. The main six different clades identified on genetic grounds are clearly separated due to the first two CVA axes. (b) CVA analysis restricted to individuals of clade C in the tree of Fig. 2. Four individuals (K13-14-16-52) are clearly differentiated.



1

2 Fig. 1



1 – 0.001 substitutions/site
2 Fig. 2

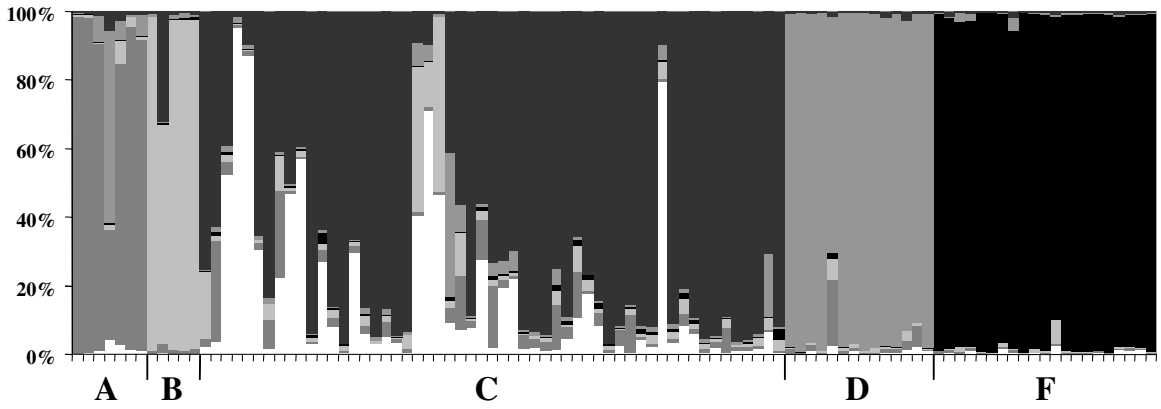


Fig. 3

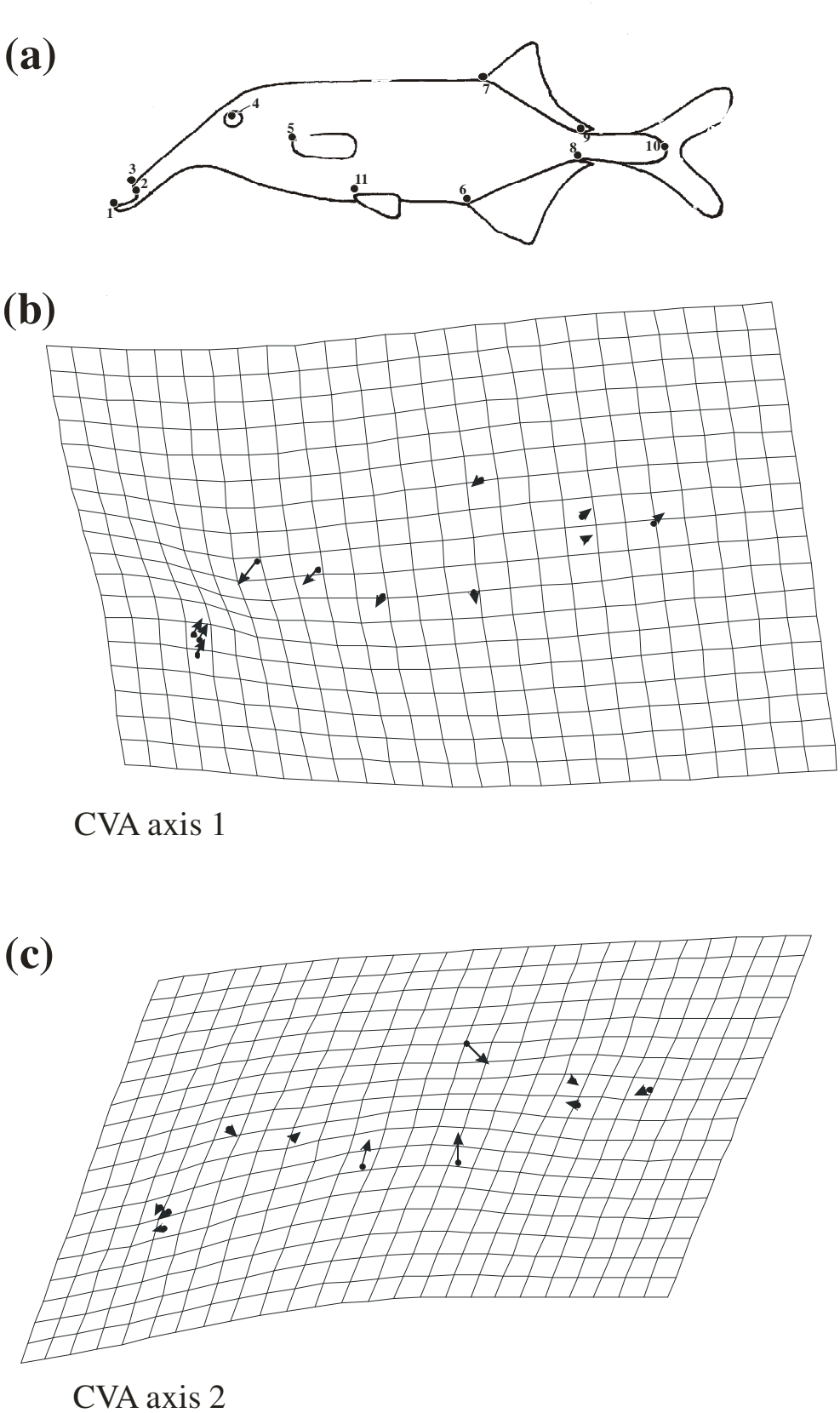


Fig. 4

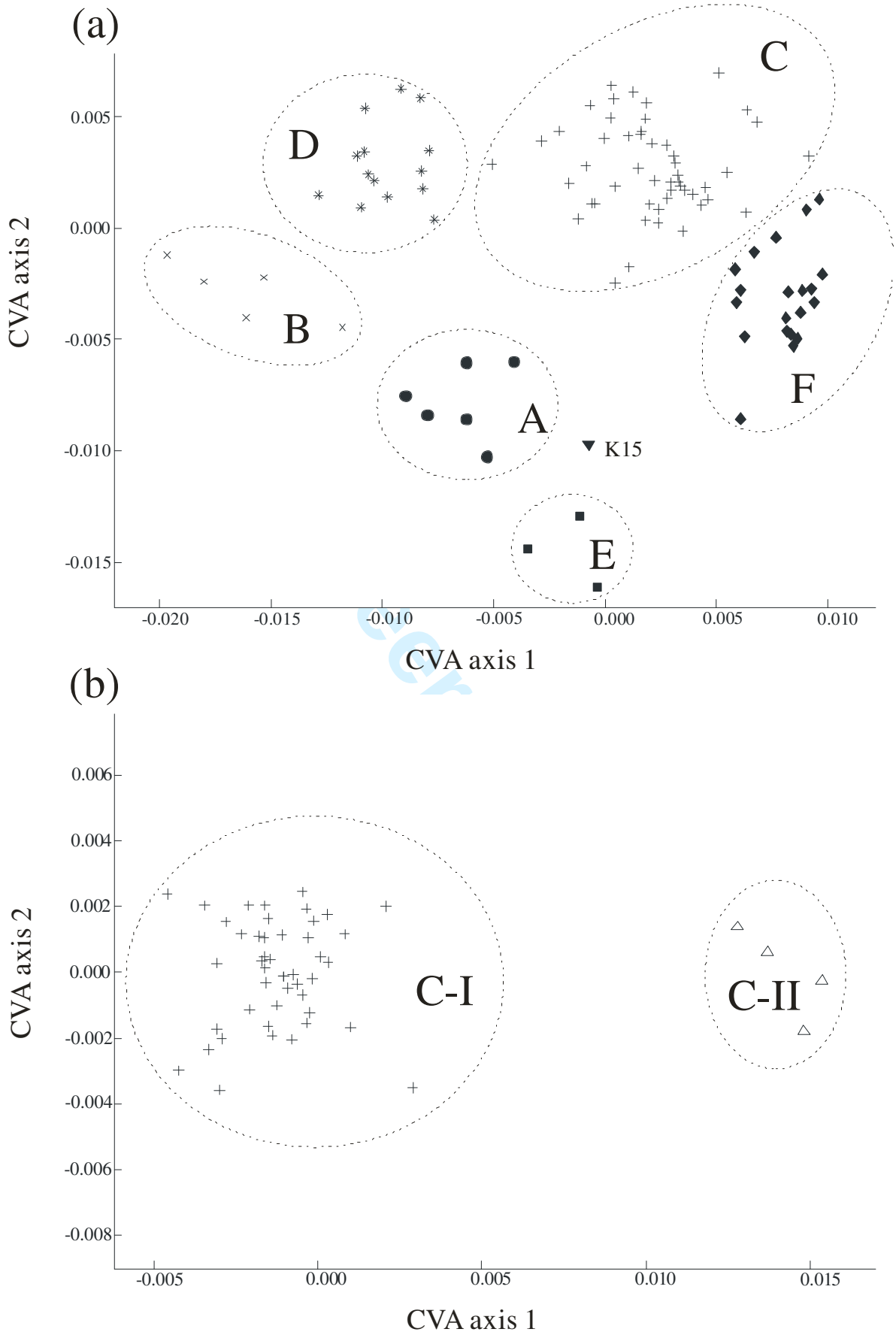


Fig. 5