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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.

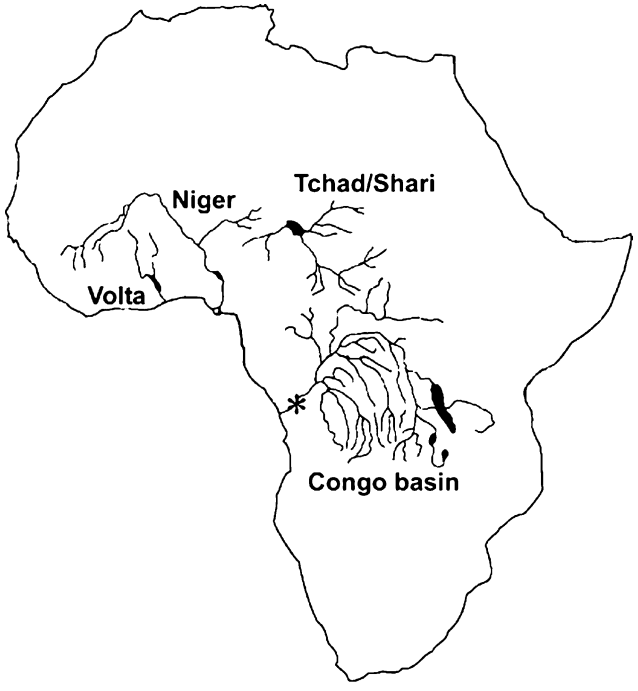

Fig. 1. Geographic location of the African river systems in which *Campylomormyrus* occurs. Most species are endemic to the Congo basin. *depicts the sampling location at Kinshasa/Brazzaville.

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

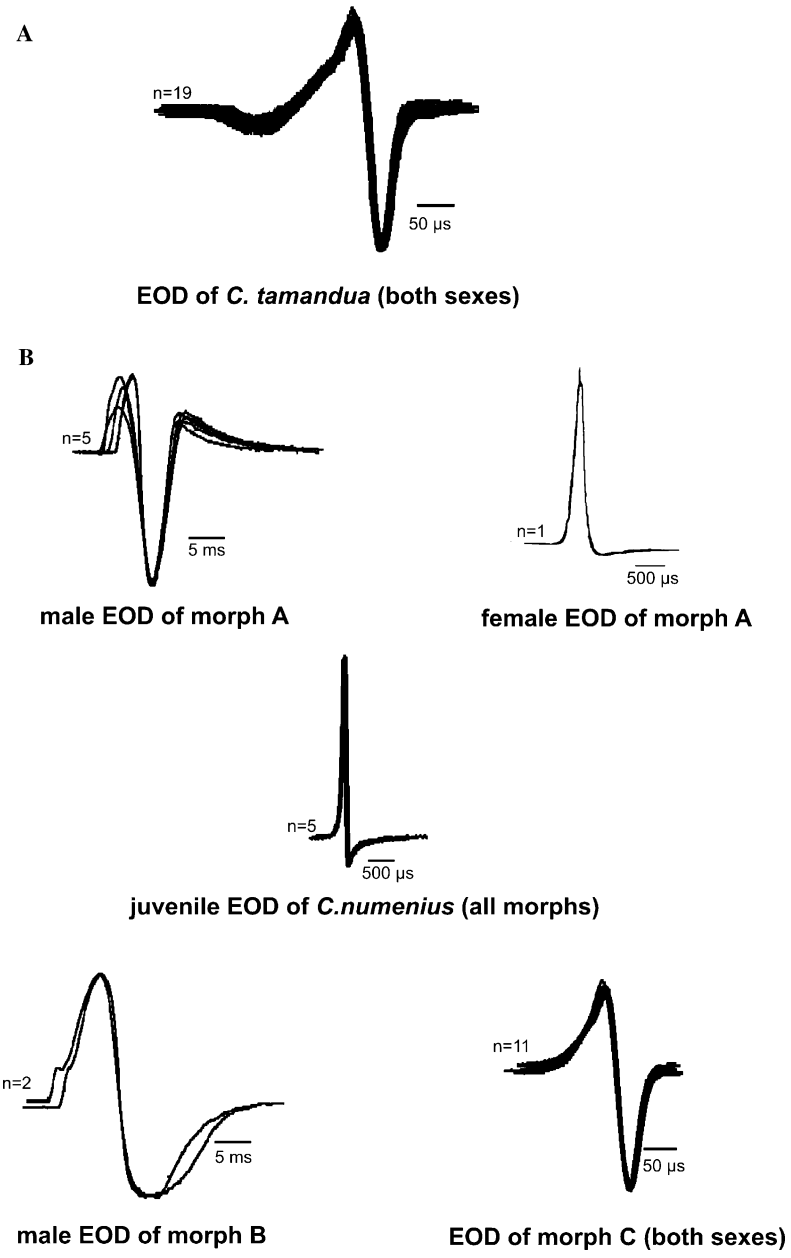

Fig. 2. Overlays of amplitude-normalized EODs (n = number of individuals per overlay). (A) Common EOD type of *C. tamandua*. (B) Common juvenile and different adult EOD types of three different morphs (A, B, and C) of *C. numenius*. Note the different time scales.

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

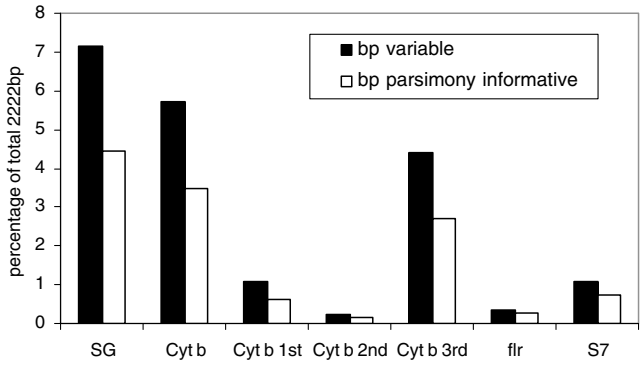

Fig. 3. Percentage of variable and parsimony informative sites in DNA sequence analysis. SG: super gene, the combination of all genes (2222 bp); Cyt *b*: mitochondrial cytochrome *b* gene, total and subdivided into the three codon positions (1142 bp); flr: flanking regions of four unlinked microsatellite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

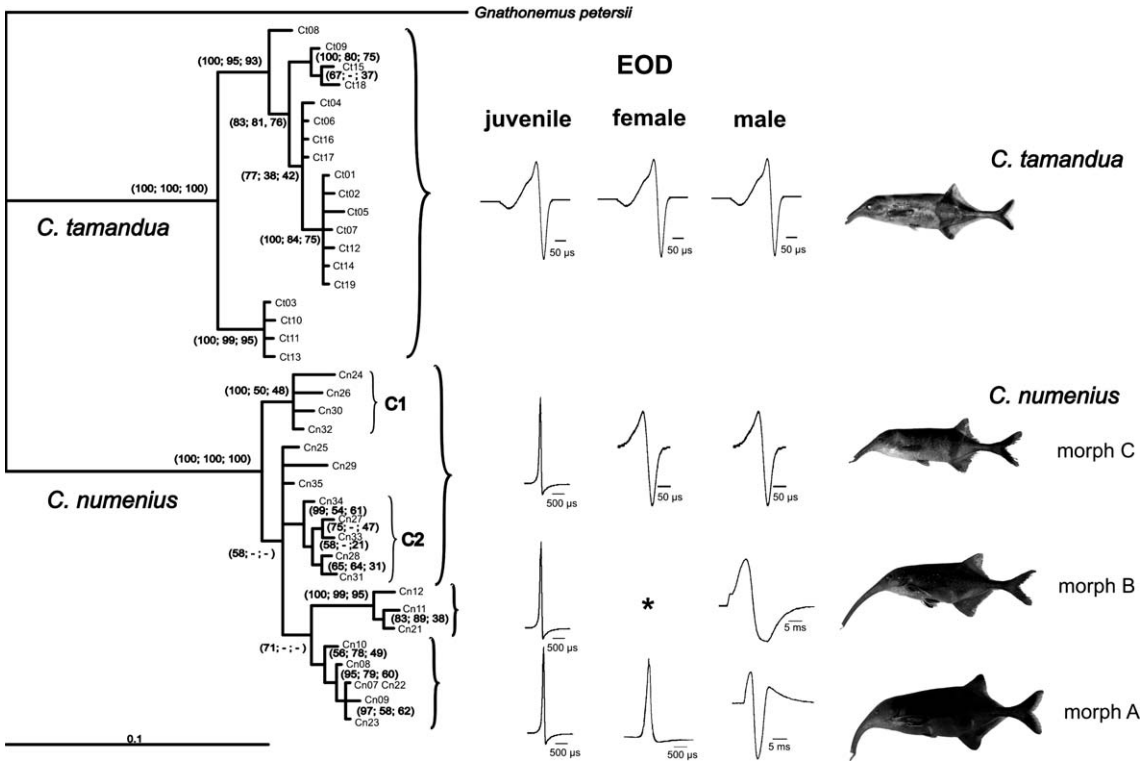

Fig. 4. Bayesian phylogeny based on the combined dataset of mitochondrial cytochrome *b*, nuclear S7 gene and flanking regions of four unlinked microsatellite loci (2222 bp). Branch length is proportional to the amount of character change. Statistical supports from Bayesian, Neighbour Joining and Parsimony 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-

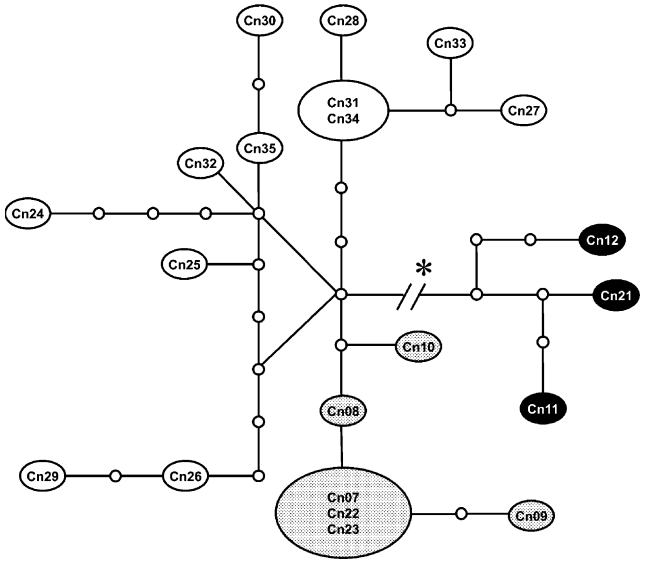

Fig. 5. Network analysis based on statistical parsimony (95% criterion) of Templeton et al. (1992) showing the relationships among the cyt *b* haplotypes found within *C. numenius*. Each haplotype is represented by a circle; the size of each circle is proportional to the number of individuals bearing 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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