doi: 10.1111/j.1420-9101.2008.01544.x

Differentiation of morphology, genetics and electric signals in a region of sympatry between sister species of African electric fish (Mormyridae)

S. LAVOUÉ, J. P. SULLIVAN[1], M. E. ARNEGARD[2] & C. D. HOPKINS

Department of Neurobiology and Behavior, W263 Seeley G. Mudd Hall, Cornell University, Ithaca, NY, USA

Keywords:

electric fish;
electric organ discharge;
introgression;
reproductive isolation;
speciation.

Abstract

Mormyrid fishes produce and sense weak electric organ discharges (EODs) for object detection and communication, and they have been increasingly recognized as useful model organisms for studying signal evolution and speciation. EOD waveform variation can provide important clues to sympatric species boundaries between otherwise similar or morphologically cryptic forms. Endemic to the watersheds of Gabon (Central Africa), *Ivindomyrus marchei* and *Ivindomyrus opdenboschi* are morphologically similar to one another. Using morphometric, electrophysiological and molecular characters [cytochrome *b* sequences and amplified fragment length polymorphism (AFLP) genotypes], we investigated to what extent these nominal mormyrid species have diverged into biological species. Our sampling covered the known distribution of each species with a focus on the Ivindo River, where the two taxa co-occur. An overall pattern of congruence among datasets suggests that *I. opdenboschi* and *I. marchei* are mostly distinct. Electric signal analysis showed that EODs of *I. opdenboschi* tend to have a smaller initial head-positive peak than those of *I. marchei*, and they often possess a small third waveform peak that is typically absent in EODs of *I. marchei*. Analysis of sympatric *I. opdenboschi* and *I. marchei* populations revealed slight, but significant, genetic partitioning between populations based on AFLP data ($F_{ST} \approx 0.04$). Taken separately, however, none of the characters we evaluated allowed us to discriminate two completely distinct or monophyletic groups. Lack of robust separation on the basis of any single character set may be a consequence of incomplete lineage sorting due to recent ancestry and/or introgressive hybridization. Incongruence between genetic datasets in one individual, which exhibited a mitochondrial haplotype characteristic of *I. marchei* but nevertheless fell within a genetic cluster of *I. opdenboschi* based on AFLP genotypes, suggests that a low level of recent hybridization may also be contributing to patterns of character variation in sympatry. Nevertheless, despite less than perfect separability based on any one dataset and inconclusive evidence for complete reproductive isolation between them in the Ivindo River, we find sufficient evidence to support the existence of two distinctive species, *I. opdenboschi* and *I. marchei*, even if not 'biological species' in the Mayrian sense.

Correspondence: Sébastien Lavoué, Department of Marine Bioscience, Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164-8639, Japan.
Tel.: (81) 3 5357 6396; fax: (81) 3 5351 6488;
e-mail: lavoue@ori.u-tokyo.ac.jp
[1]Present address: Department of Ichthyology, The Academy of Natural Sciences, 1900 Benjamin Franklin Parkway, Philadelphia, PA 19103, USA.
[2]Present address: Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, BC, Canada V6T 1Z4.

Introduction

Areas of sympatry between otherwise allopatrically distributed taxa afford valuable natural tests of species validity under Mayr's (1942) widely utilized biological species concept (Hey, 2001; de Queiroz, 2005). Here, species are defined on the basis of reproductively isolating barriers between groups of potentially interbreeding

individuals (Avise, 1994; Nelson, 1999). Contact zones and regions of sympatry also have importance when species are defined on the basis of distinct genotypic or phenotypic clusters (Mallet, 1995), which can sometimes be maintained by selection even in the face of low levels of ongoing gene flow between species. *Ivindomyrus marchei* and *Ivindomyrus opdenboschi* are allopatrically distributed over most of their combined range in west Central Africa. However, their co-occurrence in the Ivindo River (Gabon) makes possible a test of how differentiated they are in sympatry using phenotypic and genetic characters. In this study, we test whether these two nominal species of mormyrid electric fish are distinct 'biological species'.

As weakly electrogenic fishes, mormyrids provide an additional set of characters describing their electric organ discharges (EODs), which function in communication and object detection (von der Emde, 2004; Bullock *et al.*, 2005). Many sympatric mormyrid assemblages are characterized by stereotyped EOD waveform differences among species (Hopkins, 1999). EODs have proven to be extremely useful in the delimitation of mormyrid species boundaries (Arnegard & Hopkins, 2003; Kramer *et al.*, 2004; Lavoué *et al.*, 2004; Arnegard *et al.*, 2005; Sullivan & Hopkins, 2005; Feulner *et al.*, 2006). Such interspecific signal variation in the electrosensory modality likely functions in species recognition during mate choice in a number of mormyrid lineages (Hopkins & Bass, 1981; Graff & Kramer, 1992).

Taverne & Géry (1975) described the genus *Ivindomyrus* for the single species, *I. opdenboschi*, which they thought to be restricted to the Ivindo River (Fig. 1). Kamdem Toham (1998) later found that its present distribution also includes the Ntem River, which marks the border between Gabon and Cameroon. The other species, *I. marchei*, was originally described by Sauvage (1879) as *Petrocephalus marchei* from the Upper Ogooué River (Fig. 1). During the course of our fieldwork in Gabon, we encountered *Ivindomyrus* specimens exhibiting a range of morphological variation which suggested to us the presence of two morphotypes in the Ivindo River, rather than just the typical *I. opdenboschi*. One morphotype resembled *I. marchei* more closely. However, the distinction between morphotypes was slight, and a few specimens proved to be difficult for us to assign to one morphotype or the other. In their description of *I. opdenboschi*, Taverne & Géry (1975) made no comparison between this species and *I. marchei*, because the latter species was then misclassified within the genus *Pollimyrus*. Hopkins *et al.* (2008) discuss the complicated taxonomic history of *I. marchei* and provide justification for their reassignment of this species to the genus *Ivindomyrus*.

In the region of sympatry, our field identifications were based on visual assessment of a suite of morphological features that appeared to be divergent between type specimens as well as allopatric populations of the two *Ivindomyrus* species. Two characters were emphasized in particular: head profile and caudal peduncle length. Head profile appears straight to concave in *I. opdenboschi* vs. more convexly rounded in *I. marchei*, while relative caudal peduncle length is generally longer in *I. marchei* than in *I. opdenboschi* (see illustrations in Fig. 2a). In a recent molecular study of the family Mormyridae, Lavoué *et al.* (2003) found *I. marchei* and *I. opdenboschi* to be sister groups. The observed genetic divergence between these nominal species of *Ivindomyrus* is

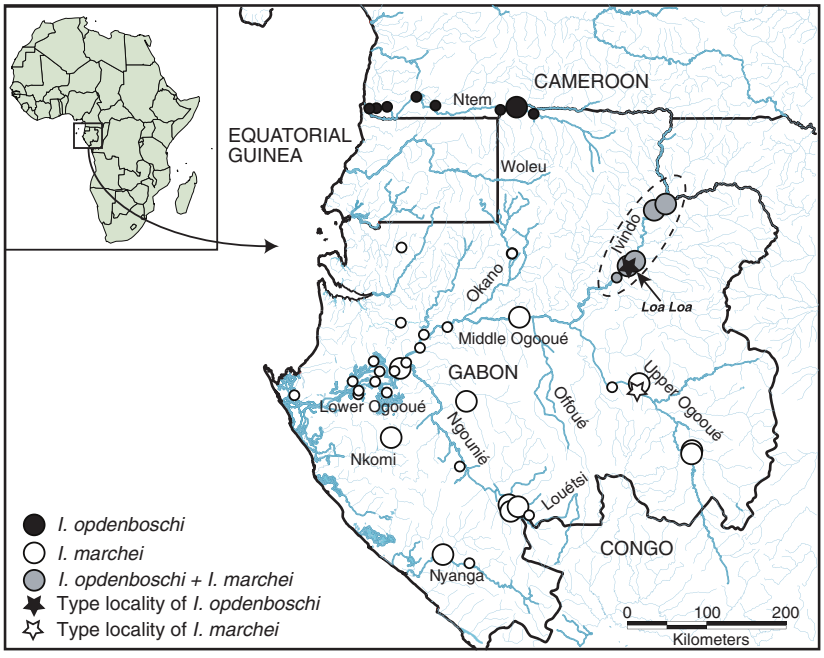

Fig. 1 Map of west Central Africa showing the known distributions of *Ivindomyrus opdenboschi* and *Ivindomyrus marchei*. Large circles mark locations where specimens were collected for this study; small circles and stars mark collection localities for other specimens obtained from museum collections. A dashed oval highlights the region of sympatry between the two species.

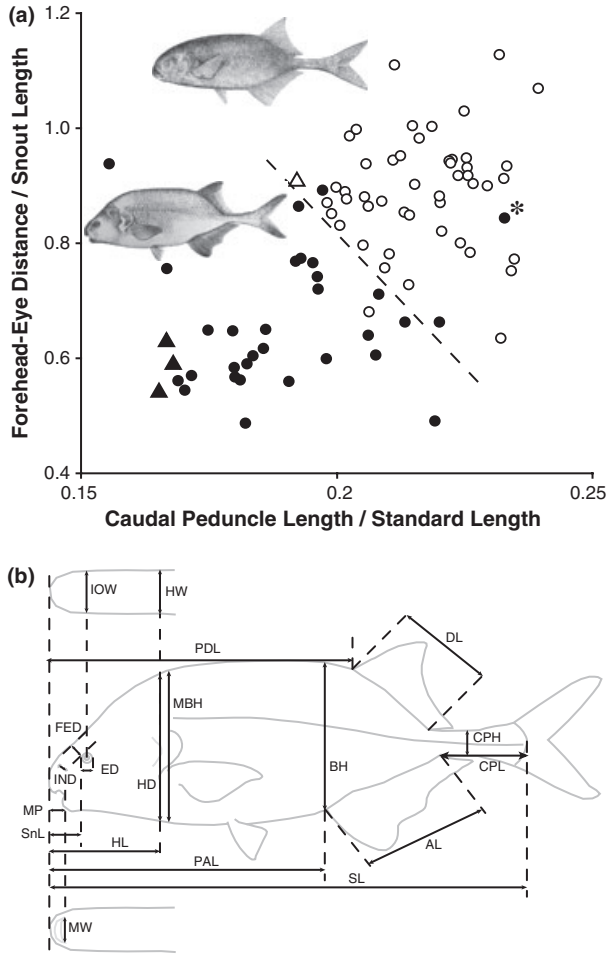

Fig. 2 (a) Co-varying expression of two traits measured in preserved Ivindo River specimens that were identified in the field as *Ivindomyrus opdenboschi* (filled circles) or *Ivindomyrus marchei* (open circles). The horizontal axis shows caudal peduncle length scaled by standard length, and the vertical axis shows forehead-eye distance scaled by snout length (a measure of head curvature). Filled and open triangles indicate type specimens of *I. opdenboschi* and *I. marchei* respectively (asterisk: outlier discussed in Materials and methods). Inset drawings taken from Taverne & Géry (1975) and Boulenger (1909–1916). (b) Nineteen point-to-point measurements used in this study: SL, standard length; AL, anal fin length; DL, dorsal fin length; PDL, predorsal fin length; PAL, pre-anal fin length; CPL, caudal peduncle length; CPH, caudal peduncle height; BH, body height; MBH, mid-body height; HL, head length; HD, head depth; SnL, snout length; MP, mouth position; HW, head width; IOW, inter-orbital width; ED, eye diameter; IND, inter-nostril distance; FED, forehead-eye distance; MW, mouth width.

extremely small based on DNA sequences determined for cytochrome *b*, 12S/16S rRNA and the first two introns of the S7 gene (Lavoué *et al.*, 2003).

Here, we characterize variation in multiple character sets to test for the presence of two *Ivindomyrus* species in the Ivindo River and to gain insights into their evolutionary history. Our approaches include morphometrics, mitochondrial sequence analysis and estimation of genetic variation across the nuclear genome using amplified fragment length polymorphism (AFLP) markers. In addition, we quantify divergence of EOD waveforms between the morphotypes as a first step in understanding the potential scope of electric signal variation for possibly providing recognition cues in *Ivindomyrus*. If complete or partial reproductive isolation exists between sympatric morphotypes, our expectation is a pattern of significant discontinuities in genetic and/or electrophysiological variation that is congruent with our morphological assignments.

Materials and methods

Specimen collection

We collected most of the *Ivindomyrus* specimens included in this study over the course of several years of fieldwork in Gabon. Currently housed in the Cornell University Museum of Vertebrates, specimens were sampled across almost the entire distribution of each species (Fig. 1). We acquired additional specimens for morphometric study from the National Museum of Natural History (Paris, France) and the Royal Museum for Central Africa (Tervuren, Belgium), including the holotype of *I. marchei* and three paratypes of *I. opdenboschi*. Because *I. opdenboschi* and *I. marchei* co-occur in the Ivindo River basin, we surveyed this area particularly extensively. Once all individuals were collected, we constructed an exploratory plot of measurements describing caudal peduncle length and head profile for preserved specimens from this region (Fig. 2a). The plot failed to identify two completely distinct groups along these two morphological axes, but it confirmed the overall reliability of our field identifications for describing quantitative differences among sympatric specimens when evaluated again in the laboratory. Field identifications of sympatric morphotypes based on morphology were retained throughout all subsequent analyses for consistency in referring to the same individuals. The only noticeable outlier in the exploratory plot (marked by an asterisk in Fig. 2a) was identified as *I. opdenboschi* in the field based on several morphological features characteristic of this species (e.g. mouth width, head width and depth) other than head profile and caudal peduncle length. This individual was not used in any genetic analyses (described below), as we happened not to collect a suitable tissue sample from this specimen in the field. Table 1 lists sample sizes of *Ivindomyrus* specimens for each of our analytical approaches. Museum catalogue numbers and field collection information for every specimen used in this study are provided in 'Supplementary material'. All of our fish collection and handling methods conformed to protocols approved by Cornell's Center for Research Animal Resources.

Table 1 Number of specimens of *Ivindomyrus opdenboschi* and *Ivindomyrus marchei* (by locality), which were analysed using each method employed in this study.

Species	Localities	Morphology	Signal analysis	Cyt *b* sequencing	AFLP genotyping
Ivindomyrus opdenboschi	Ivindo River	34	24	9	7
	Ntem River	5	5	5	3
Ivindomyrus marchei	Ivindo River	48	20	18	7
	Upper Ogooué River	11	6	6	2
	Middle Ogooué River	7	5	4	1
	Lower Ogooué River	20	16	5	2
	Ngounié River	14	4	7	3
	Nkomi River	2	0*	2	1
	Nyanga River	10	0*	2	2
	Total	151	80	58	28

All specimens used for EOD recording and signal analysis, cyt *b* sequencing or AFLP genotyping were first examined morphologically for assignment to one of the two nominal species. These initial field identifications were consistently retained during all of the subsequent analyses. Detailed specimen data for all individuals are provided in 'Supplementary material'.

*Specimens from these localities were collected using gillnets, which did not allow EODs to be recorded.

Recording and analysis of electric signals

Electric organ discharge recording methods have been described in detail elsewhere (Sullivan *et al.*, 2002; Lavoué *et al.*, 2004; Arnegard *et al.*, 2005). Here, bio-amplifier frequency response was flat from 0.1 Hz to 50 kHz. High sampling rates (100–500 kHz) were used to ensure capture of fine waveform details. Water temperature and conductivity measured during the recordings ranged from 23 to 26 °C and from 25 to 70 μS cm^{-1} respectively.

We investigated EOD waveform variation among 80 specimens using a single EOD recorded from each individual. Although the range of recording temperatures was small, we attempted to remove any trace of temperature-induced waveform variation by normalizing the time bases of all EODs using the temperature coefficient formula for rate functions (e.g. see Kramer & Westby, 1985) and an empirically established value of Q_{10} = 1.6. We then quantified waveform variation using a custom program written in MATLAB (Mathworks, Inc., Natick, MA, USA), which we adapted from Arnegard & Hopkins (2003). In this study, the beginning (*T*1) and end (*T*2) of the EOD waveform were taken as the first and last points that deviated by more than 1.5% of peak-to-peak height. The following four measures proved most useful for quantifying EOD variation between species: relative amplitude of the first head-positive peak (P1); relative amplitude of the second head-positive peak (P3); frequency of maximum energy in the EOD's power spectrum (FFT peak frequency, in kHz); and total EOD duration (calculated as *T*2–*T*1, in ms).

We tested for differences in each waveform measurement between species and sexes using two-way analysis of variance (ANOVA), which included a species × sex interaction term. Because we found no statistical evidence of geographic variation in our EOD measurements for either sex within either species, data were pooled across regions for ANOVA, as well as for all other analyses of EOD features. For example, we performed two-sided *t*-tests on the geographically pooled samples to examine differences between sexes within each species. EOD duration was transformed by calculating $\log_{10}$(duration in μs) to improve homoscedasticity for this character. Relative P1 and P3 voltages were measured as percentages of overall peak-to-peak amplitude of the EOD. Arcsine transformations improved the fit of these ratio data to normal distributions. Unless otherwise noted, all reported descriptive statistics (i.e. mean values, standard deviations and ranges) were calculated from temperature-normalized EODs, but they did not involve these other kinds of transformations in any instance. Despite arcsine transformation, we deemed variation in P3 amplitude to be inappropriate for parametric analysis due, among other things, to the presence of excessive zeros in some groups. Instead of two-way ANOVA, we evaluated variation in P3 amplitude among the four groups of individuals defined by species and sex using the Kruskal–Wallis test (Sokal & Rohlf, 1998). *Post hoc* Mann–Whitney *U*-tests (two-sided) were then used to evaluate differences in P3 amplitude between sexes within each species, as well as among individuals of the same sex between species. All statistical analyses of EOD measurements were performed using STATISTICA v.6.1 (StatSoft, Inc., Tulsa, OK, USA).

Morphometrics and meristics

Except as noted below, methods for making counts and measurements follow Bigorne & Paugy (1991) and Boden *et al.* (1997). The sex of each specimen (male, female or indeterminate) was evaluated by examining

the base of its anal fin (Pezzanite & Moller, 1998). We then made seven counts and 19 measurements on each specimen (Fig. 2b). The former were made with the aid of a dissecting microscope, and the latter were made with a digital calliper (0.1 mm precision). In the case of standard length, body height, number of dorsal fin branched rays and number of anal fin branched rays, we adhered to the modifications of Lavoué *et al.* (2004). In addition, 'forehead-eye distance' is here defined as the minimum point-to-point distance between the edge of the orbit and the mid-sagittal plane of the forehead. 'Mid-body height' is the vertical distance between the dorsum and ventrum of the body measured mid-sagittally at the level of the pectoral fin origin. 'Mouth position' is the point-to-point distance from the anterior extremity of the snout (at the midline) to the corner of the mouth. 'Mouth width' is the point-to-point distance between the right and left corners of the mouth. Morphometric measurements were scaled by dividing them either by standard length or head length (of the same specimen) depending on the particular measurement being normalized. We analysed morphological variation among specimens by principal components analysis (PCA) using STATISTICA v.6.1 (StatSoft Inc.). Separate analyses were conducted on morphometric and meristic datasets.

Cytochrome *b* amplification and sequencing

We generated complete sequences of the mitochondrial cytochrome *b* (cyt *b*) gene for 12 specimens of *I. opdenboschi* and 44 specimens of *I. marchei* (Table 1) (Genbank accession numbers: DQ166635–90). To this dataset, we added sequences already published for two specimens of *I. opdenboschi* and for the following three out-groups: *Boulengeromyrus knoepffleri*, *Marcusenius ntemensis* and *Pollimyrus* sp. 'Nyanga' (Sullivan *et al.*, 2000, 2004; Lavoué *et al.*, 2003).

We employed methods described by Sullivan *et al.* (2000) for DNA extraction from muscle (preserved in 90% ethanol), PCR amplification and DNA sequencing. We amplified and sequenced the entire cyt *b* gene (1140 bp) using the following primer pair: forward L14724 (5′-TGA TAT GAA AAA CCA TCG TTG-3′); and reverse H15930 (5′-CTC CAG TCT TCG RCT TAC AAG-3′). Alignment of DNA by eye using PAUP* version 4.1.10 (Swofford, 1999) was trivial and did not require any insertions or deletions. Phylogenetic relationships among cyt *b* haplotypes were then reconstructed by maximum parsimony (MP) and maximum likelihood (ML) using PAUP * and GARLI ver. 0.951 (Zwickl, 2006) respectively. Inferred trees were rooted using the aforementioned out-groups. To find the MP trees, we conducted heuristic searches with initial trees obtained via stepwise addition with 100 iterations of the random addition sequence using the 'Tree-Bisection-Reconnection' branch-swapping option. All characters were weighted equally and treated as unordered. To perform ML analyses, we conducted heuristic phylogenetic searches under a GTR + I + G model, which is the default model implemented by GARLI. Twenty individual runs were performed using the default search settings (5 000 000 generations) and termination criteria with random starting topologies. To evaluate the robustness of the internal branches of the MP and ML trees, 100 bootstrap replications were calculated using PAUP* and GARLI respectively. In an attempt to distinguish population structure and population history, we also performed a network analysis on the subset of cyt *b* sequences within *Ivindomyrus* 'clade 3' (see below) using the software package, TCS ver. 1.2.1 (Clement *et al.*, 2000).

Amplified fragment length polymorphism analysis

We investigated overall patterns of nuclear genetic variation among populations of *I. opdenboschi* and *I. marchei* using a fluorescent AFLP procedure, which we previously applied to the *Paramormyrops* species flock of Gabon (Sullivan *et al.*, 2004). Conditions for DNA restriction, ligation of oligonucleotide adapters and preselective and selective PCR are described in detail by Sullivan *et al.* (2004). We digested total genomic DNA with the restriction enzymes *Bfa*I and *Eco*RI, and we used a total of eight selective primers pairs. Sequences for all

Table 2 Oligonucleotide adapters and primers used for the AFLP method.

Primer/adapter names	Sequences
Adapters	
*Eco*RI	5′-CTC-GTA-GAC-TGC-GTA-CC-3′
	5′-AAT-TGG-TAC-GCA-GTC-TAC-3′
*Bfa*I	5′-GAC-GAT-GAG-TCC-TGA-G-3′
	5′-TAC-TCA-GGA-CTC-AT-3′
Preselective primers	
*Eco*RI + G	5′-GAC-TGC-GTA-CCA-ATT-CG-3′
*Bfa*I + T	5′-GAT-GAG-TCC-TGA-GTA-GT-3′
Selective primers pairs	
*Eco*RI G-AT	5′-GAC-TGC-GTA-CCA-ATT-CGA-T-3′
*Bfa*I T-TA	5′-GAT-GAG-TCC-TGA-GTA-GTT-A-3′
*Eco*RI G-A	5′-GAC-TGC-GTA-CCA-ATT-CGA-3′
*Bfa*I T-CT	5′-GAT-GAG-TCC-TGA-GTA-GTC-T-3′
*Eco*RI G-AT	5′-GAC-TGC-GTA-CCA-ATT-CGA-T-3′
*Bfa*I T-CT	5′-GAT-GAG-TCC-TGA-GTA-GTC-T-3′
*Eco*RI G-A	5′-GAC-TGC-GTA-CCA-ATT-CGA-3′
*Bfa*I T-CA	5′-GAT-GAG-TCC-TGA-GTA-GTC-A-3′
*Eco*RI G-A	5′-GAC-TGC-GTA-CCA-ATT-CGA-3′
*Bfa*I T-TG	5′-GAT-GAG-TCC-TGA-GTA-GTT-G-3′
*Eco*RI G-C	5′-GAC-TGC-GTA-CCA-ATT-CGC-3′
*Bfa*I T-TA	5′-GAT-GAG-TCC-TGA-GTA-GTT-A-3′
*Eco*RI G-A	5′-GAC-TGC-GTA-CCA-ATT-CGA-3′
*Bfa*I T-TC	5′-GAT-GAG-TCC-TGA-GTA-GTT-C-3′
*Eco*RI G-C	5′-GAC-TGC-GTA-CCA-ATT-CGC-3′
*Bfa*I T-AC	5′-GAT-GAG-TCC-TGA-GTA-GTA-C-3′

adapters, preselective primers and selective primers are provided in Table 2.

We purified final amplification products following Sullivan *et al.* (2004) and electrophoresed them on 5% polyacrylamide gels using an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Resulting electropherograms were analysed using GeneScan software (Applied Biosystems). We scored each gel using peak detection thresholds of 40, 80 and 120 relative fluorescence units (r.f.u.) to assess the effect of threshold on results of subsequent analyses. Using the program 'BinThere' (Garnhart, 2001), we scored fragments between 50 and 625 bp as either present (= 1) or absent (= 0) in the ABI trace files. The 'expert' binning algorithm and a bin width of 1 bp were selected for this purpose. Three nexus files for phylogenetic analysis (corresponding to the 3 r.f.u. thresholds) were created from the complete set of 'BinThere' output files.

At each detection threshold, we reconstructed phylogenetic relationships using three different methods implemented in PAUP*: MP assuming Dollo parsimony; MP assuming Wagner parsimony; and Neighbour-Joining (NJ) based on corrected pairwise genetic distances (Nei & Li, 1979). We explored results of all nine approaches to identify emergent phylogenetic patterns that were the most robust across methods. Under both parsimony assumptions, we conducted heuristic searches with initial trees obtained via stepwise addition with 100 iterations of the random addition sequence and the 'Tree Bisection-Reconnection' branch-swapping option. Rooting was attempted with the same three out-groups mentioned above. Doing so resulted in differences in rooting point among the nine approaches. Rooting an intraspecific phylogeny is known to be problematic when using outgroups that are too divergent from the in-group populations (Wheeler, 1990; Sanderson & Shaffer, 2002). Due to the instability in rooting point and the extremely close apparent relationship between *Ivindomyrus* species, we only present results based on unrooted trees.

The magnitude of genetic differentiation between populations can be difficult to judge from tree topologies estimated on the basis of large numbers of arbitrary, dominant markers like AFLPs (Hollingsworth & Ennos, 2004). Therefore, we also quantified genetic partitioning between sympatric *I. opdenboschi* and *I. marchei* by estimating Wright's fixation index (F_{ST}) between them on the basis of AFLP genotypes. This *F*-statistic measures the degree to which substructuring of a total population reduces overall heterozygosity relative to an expectation under random mating among subpopulations (Nei, 1977). A value of $F_{ST} = 0$ indicates no substructuring within the total population. A value of $F_{ST} = 1$ corresponds to subpopulations being fixed for different alleles that do not pair as heterozygotes due to complete partitioning between subpopulations.

We used the program AFLP-SURV 1.0 (Vekemans, 2002) to calculate F_{ST} based on the approach that Lynch & Milligan (1994) developed for dominant markers. Our analysis was restricted to the single collection site from which the largest numbers of individuals (six adults of each species) were sampled: a 200-m stretch of the Ivindo River just downstream of Loa-Loa rapids (Fig. 1). We only included AFLP bands that were polymorphic among these twelve specimens (detection threshold = 80 r.f.u.). A Bayesian approach with nonuniform prior distribution (assuming Hardy–Weinberg equilibria for both morphotypes) was used to make the necessary estimates of allele frequencies (Zhivotovsky, 1999). Following the recommendation of Lynch & Milligan (1994), we minimized bias in our parameter estimates by removing loci for which 'recessive alleles' (i.e. absent bands) were genotyped in (i) only one specimen or (ii) up to two of the 12 specimens. We estimated F_{ST} for the unaltered data set (539 loci total), as well as both of the pruned data sets (469 and 437 loci respectively). Statistical significance of each F_{ST} estimate was determined using 50 000 random permutations.

Results

Electric signal variation

Ivindomyrus marchei and *I. opdenboschi* produce qualitatively similar EOD waveforms possessing two main phases (Fig. 3). The first phase (P1) is head-positive, and the second phase (P2) is head-negative. Some individuals exhibit a small, positive peak (P3) after P2. EOD duration is relatively short in both *I. marchei* and *I. opdenboschi*. Ranges of EOD duration among all measured individuals (males and females) of both species are 0.190–1.862 and 0.175–1.933 ms before and after temperature correction respectively. Both species exhibit sex differences in their EODs, with identifiable males producing longer duration waveforms having correspondingly lower FFT peak frequencies (Table 3). As revealed by a significant species × sex interaction term ($P < 0.0001$) in the two-way ANOVA of FFT peak frequency, the degree of sex difference appears to vary between the two species (see Table 3). Possible influences of season, individual size and/or male reproductive status on the EOD sex differences remain unclear at present, largely due to our limited sampling of *I. opdenboschi* males and the lack of sampling at certain times of year.

Despite the sex difference in EOD duration within each species, we did not detect a robust statistical difference in waveform duration between *I. marchei* and *I. opdenboschi* (Table 3). The three longest-duration EODs detected in this study were recorded in *I. marchei* males (total range of temperature-corrected EOD durations in all males of this species = 0.268–1.933 ms), yet comparatively few identifiable males of *I. opdenboschi* were recorded (total range of EOD durations = 0.295–0.845 ms). We did find a significant difference in FFT peak frequencies between

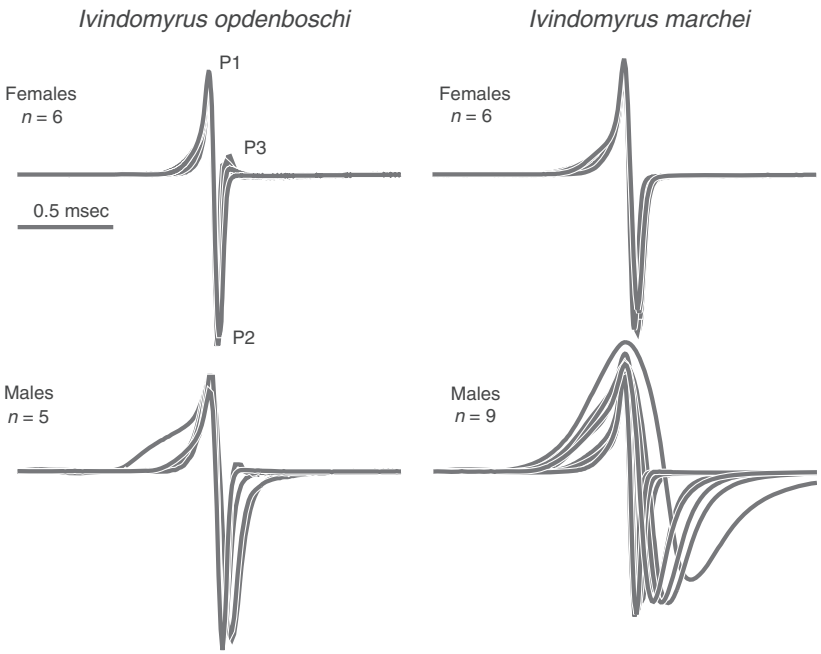

Fig. 3 Representative EOD waveforms produced by *Ivindomyrus* species in Gabon. Within each species and sex, superimposed waveforms are scaled to the same peak-to-peak amplitude and centred on P1 (a 0.5 ms scale bar is shown for reference). Upward in each plot corresponds to head positivity (i.e. current flow in the direction of the head inside the animal). Specimen numbers for the plotted EODs: *I. opdenboschi* females [3954, 2106, 2169, 5753, 5599 and 5682]; *I. opdenboschi* males [3955, 2242, 5652, 5801 and 3957]; *I. marchei* females [4752, 4754, 2939, 2937, 3083 and 3734]; *I. marchei* males [4753, 4751, 1852, 1836, 4945, 3741, 2870, 3704 and 1853].

Table 3 Descriptive statistics of four EOD measurements (shown in the leftmost column, along with units of measurement) and outcomes of statistical comparisons for the same EOD features.

	Ivindomyrus opdenboschi			*Ivindomyrus marchei*			Results of two-way ANOVA		
	Mean ± SD (min., max.) for females (n = 22)	Mean ± SD (min., max.) for males (n = 7)	t-Test between sexes	Mean ± SD (min., max.) for females (n = 29)	Mean ± SD (min., max.) for males (n = 22)	t-Test between sexes	Species	Sex	Species × sex interaction
EOD duration (ms)	0.226 ± 0.046 (0.175, 0.326)	0.555 ± 0.226 (0.295, 0.845)	**$t_{[27]} = -7.27$ $P < 0.0001$**	0.325 ± 0.104 (0.212, 0.654)	0.580 ± 0.380 (0.268, 1.933)	**$t_{[49]} = -4.52$ $P < 0.0001$**	$F_{[1,76]} = 3.40$ $P = 0.0693$	**$F_{[1,76]} = 55.37$ $P < 0.0001$**	$F_{[1,76]} = 3.95$ $P = 0.0504$
FFT peak frequency (kHz)	6.85 ± 1.83 (3.98, 9.67)	2.40 ± 1.14 (1.19, 4.09)	**$t_{[27]} = 6.01$ $P < 0.0001$**	3.82 ± 0.88 (1.84, 5.79)	2.48 ± 1.20 (0.66, 5.53)	**$t_{[49]} = 4.62$ $P < 0.0001$**	**$F_{[1,76]} = 18.92$ $P < 0.0001$**	**$F_{[1,76]} = 72.90$ $P < 0.0001$**	**$F_{[1,76]} = 21.00$ $P < 0.0001$**
P1 amplitude (% peak-to-peak)	37.8 ± 3.0 (35.0, 44.3)	35.2 ± 2.0 (30.9, 37.0)	$t_{[27]} = 2.12$ $P = 0.0436$	40.8 ± 4.1 (35.9, 56.0)	43.2 ± 3.7 (39.0, 54.5)	$t_{[49]} = -2.12$ $P = 0.0390$	**$F_{[1,76]} = 36.62$ $P < 0.0001$**	$F_{[1,76]} = 0.03$ $P = 0.8676$	**$F_{[1,76]} = 7.27$ $P = 0.0086$**

	Ivindomyrus opdenboschi			*Ivindomyrus marchei*			Additional results of Mann–Whitney U-tests	
	Mean ± SD (min., max.) for females (n = 22)	Mean ± SD (min., max.) for males (n = 7)	Mann–Whitney U-test between sexes	Mean ± SD (min., max.) for females (n = 29)	Mean ± SD (min., max.) for males (n = 22)	Mann–Whitney U-test between sexes	Difference in females between species	Difference in males between species
P3 amplitude* (% peak-to-peak)	3.1 ± 1.7 (0.0, 6.9)	0.6 ± 0.9 (0.1, 2.7)	**$U = 17.0$ $P = 0.0022$**	0.2 ± 0.2 (0.0, 0.8)	0.1 ± 0.2 (0.0, 0.7)	$U = 231.0$ $P = 0.0942$	**$U = 32.0$ $P < 0.0001$**	**$U = 10.0$ $P = 0.0006$**

All results are based on temperature-corrected EODs (see text). For the calculation of descriptive statistics, however, data were not otherwise transformed in any way. Outcomes of statistical tests that are significant at the $P < 0.01$ level are shown in boldface type.

*$P < 0.0001$ for the Kruskal–Wallis test of variation in P3 amplitude among the four groups of individuals defined by both species and sex.

species, with *I. marchei* having a lower average peak frequency than *I. opdenboschi*. Relative amplitude of P1 also differed between species (Table 3), being generally higher in *I. marchei*.

Variation in P3 amplitude among the four groups of individuals defined by species and sex (Kruskal–Wallis $P < 0.0001$) was more immediately apparent from the visual inspection of waveform traces (at high gain) than was variation in P1 amplitude (see Fig. 3). Relative P3 voltage tended to be greater in *I. opdenboschi* when either males ($P = 0.0006$) or females ($P < 0.0001$) were compared between species, yet the magnitude of the

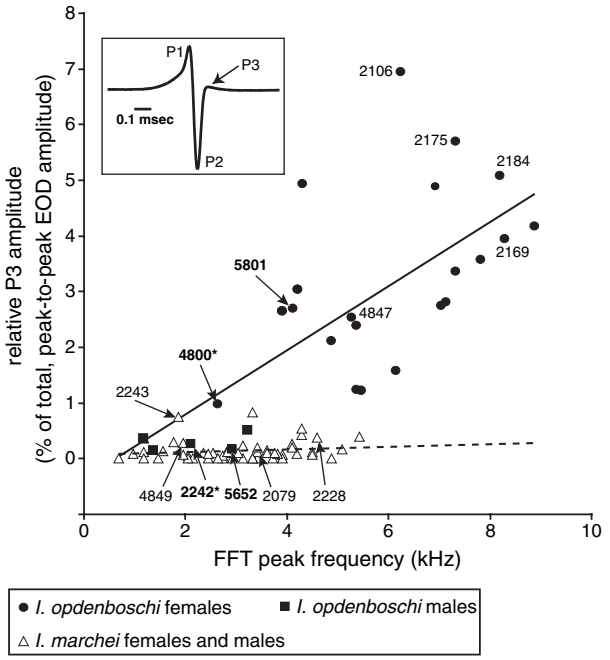

Fig. 4 Plot of relative P3 amplitude vs. FFT peak frequency for EODs recorded from *Ivindomyrus opdenboschi* and *Ivindomyrus marchei*. A correlation exists between these two waveform measurements for *I. opdenboschi* females ($r = 0.69$; $P < 0.001$) but not for *I. marchei* females nor for males of either species ($r < 0.67$; $P > 0.105$). Specimen numbers are shown for individuals genotyped using AFLP markers and for which cyt *b* sequences were generated. Asterisks indicate two specimens (2242 and 4800) which resemble *I. opdenboschi* in morphology but are more similar to *I. marchei* in their AFLP genotypes and cyt*b* haplotypes.

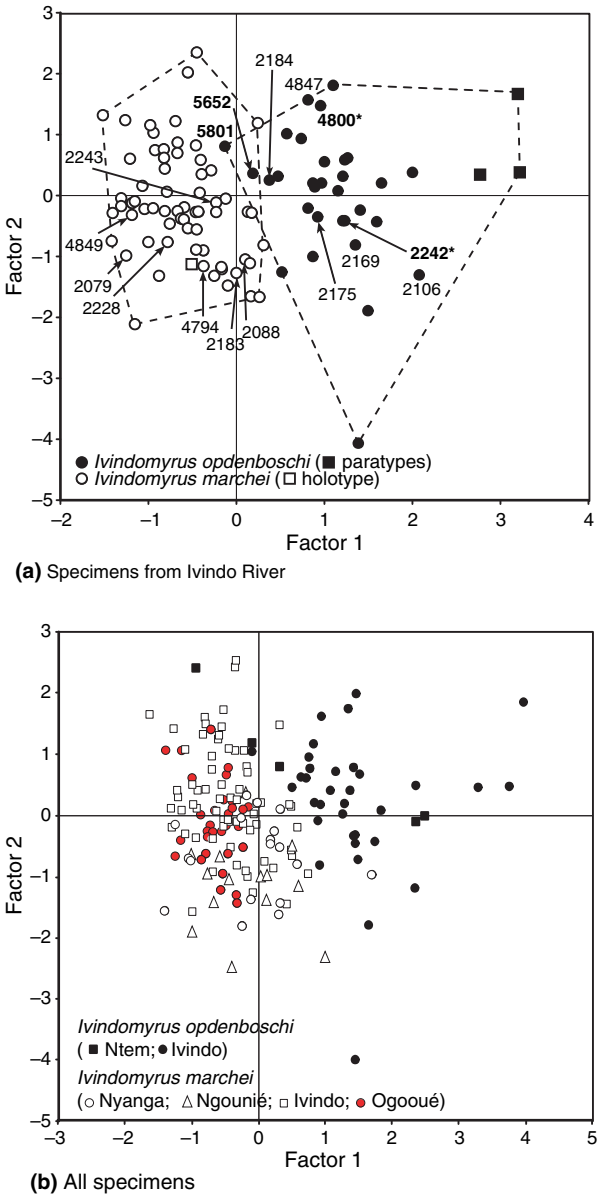

Fig. 5 Plots of scores along the first two factor axes derived from principal components analyses of morphometric data. (a) Scores for sympatric specimens of *Ivindomyrus marchei* and *Ivindomyrus opdenboschi* from the Ivindo River, in addition to the holotype of *I. marchei* and three paratypes of *I. opdenboschi*. (b) Scores for all measured specimens, regardless of origin. Symbols are defined at the bottom of each plot. Specimen numbers in (a) are given for individuals that were subsequently genotyped (using AFLP) and sequenced (across the entire cyt *b* gene). Asterisks indicate two specimens (2242 and 4800) that exhibit morphological similarity to *I. opdenboschi* but possess AFLP genotypes and cyt *b* haplotypes like those of *I. marchei*.

difference was notably larger in females (Table 3). A plot of relative P3 amplitude against FFT peak frequency is shown in Fig. 4, which shows that P3 amplitude is correlated with peak frequency among *I. opdenboschi* females but not *I. marchei* females nor among males of either species. In other words, P3 amplitude is largest in those individuals of *I. opdenboschi* with EODs possessing more energy at higher frequencies (e.g. FFT peak frequency > 5 kHz). The durations of these EODs were among the shortest we measured for this study, as FFT peak frequency and duration are very strongly related to one another (data not shown). Presence of a P3 that is at least 0.9% of the EOD's total peak-to-peak amplitude reliably identifies an individual as *I. opdenboschi* in the set of specimens we recorded, although some members of this species (in particular, most males) exhibit a smaller P3 in their EODs, often scarcely detectable above background noise. Specimen 5801 was originally identified (in the field) as the *I. opdenboschi* morphotype, but this individual was found subsequently to overlap with *I. marchei* in a more detailed shape analysis (Fig. 5a; discussed below). As shown in Fig. 4, this individual of intermediate morphology produced an EOD with a relative P3 amplitude of 2.7%, which is characteristic of *I. opdenboschi*.

Morphological variation

Principal components analysis of sympatric *I. opdenboschi* ($N = 34$) and *I. marchei* ($N = 48$) from the Ivindo River, together with the holotype of *I. marchei* and three paratypes of *I. opdenboschi*, yield first and second factor axes explaining 57% (40% + 17% respectively) of the total variation in our morphometric measurements (Fig. 5a). Factor loadings (not shown) reveal that the first axis is influenced by tail length and several measures associated with head shape, while body depth and head depth are predominant influences on the second axis. Sympatric specimens of *I. opdenboschi* and *I. marchei* almost compose completely distinct groupings from one another in a scatter plot of scores along the first two factor axes (Fig. 5a). However, two specimens (5801 and 5652) initially identified as *I. opdenboschi* (on the basis of the characters shown in Fig. 2a) fall inside the cluster of scores formed by *I. marchei*. Three paratypes of *I. opdenboschi* occur at the far right of the plot as a likely consequence of allometric scaling, as all three specimens are unusually large (SL > 200 mm).

When specimens across the entire geographic distributions of *I. opdenboschi* and *I. marchei* are included in the same PCA (Fig. 5b), the first two factor axes explain 51% (34% + 17% respectively) of total shape variation. The same suites of variables as before influence these axes most strongly. No clear patterns of morphological variation are apparent among geographically separated populations of the more widely distributed species, *I. marchei*. Similarly, *I. opdenboschi* appears rather homogeneous in morphology between the Ntem and Ivindo Rivers.

The two species, as well as their constituent populations, are no more distinct from one another in their meristic variation. *Ivindomyrus opdenboschi* and *I. marchei* form mostly distinct clusters of scores in a PCA of meristic data (not shown), yet the overlap is even greater than for the morphometric data. Characters loading most heavily on the first two factor axes derived from meristic data include number of teeth on the upper and lower jaws and number of branched rays in the dorsal and anal fins.

Pattern of variation estimated from cyt *b* sequences

The aligned matrix of mtDNA sequences for both species contained a total of 29 different cyt *b* haplotypes. Twenty-five of these were each sequenced in only a single individual. Three haplotypes were shared by at least two individuals of the same species, and only one was present in both *I. opdenboschi* and *I. marchei*. The two most common haplotypes were found in the following individuals: 'Ima-Og.1' was shared by 14 specimens of *I. marchei* from various localities along the Ngounié, Nkomi and main Ogooué Rivers; and 'Ima-Iv' was present in 13 specimens of *I. marchei* plus one specimen of *I. opdenboschi* (2242), all from the Ivindo River. The only other shared haplotypes were 'Ima-Og.2' (two *I. marchei* specimens) and 'Iop-Iv' (three individuals of *I. opdenboschi*). Genetic divergence was low among all cyt *b* haplotypes in *Ivindomyrus*, ranging from one nucleotide substitution (*ca.* 0.1% sequence divergence) to a maximum of 21 substitutions (*ca.* 1.8%). The highest divergence, in pairwise comparisons, was found not between species but, rather, between the specimens of *I. opdenboschi* from the Ntem River and all other specimens of *Ivindomyrus*, regardless of location or species (range = 13–21 substitutions).

Only 51 nucleotide positions proved to be variable out of a total of 1140 bp. Twenty-three of these sites were informative for MP analysis within the in-group, which resulted in eight most parsimonious trees when rooting was made using all three out-group taxa. Each resulting tree was 239 steps long, with a consistency index (CI) of 0.816 and a retention index (RI) of 0.766. Figure 6a shows the strict consensus of these trees. Neither *I. opdenboschi* nor *I. marchei* formed monophyletic groups in the strict consensus tree. Rather, relationships among haplotypes depended primarily on geographical origin of the specimens.

We recognize three major clades of in-group taxa. The first (clade 1 in Fig. 6a) is composed exclusively of all *I. opdenboschi* specimens collected from the Ntem River. Clade 1 is the sister group of all remaining *Ivindomyrus* specimens. Nyanga River specimens of *I. marchei* (only two individuals were sequenced) are exclusively contained within clade 2. Its sister group, clade 3, comprises all specimens of both species taken from the Nkomi River and throughout the greater Ogooué basin (including the Ngounié and Ivindo Rivers). Only five nucleotide positions were parsimony-informative in clade 3. Each of these positions represents a unique synapomorphy. Within clade 3, all specimens (except one) of *I. marchei* originating from the Ogooué (proper), Ngounié and Nkomi Rivers form a monophyletic group (clade 3a), which is sister to an Ivindo-specific group (clade 3b) consisting of 17 specimens of *I. marchei* and three specimens of *I. opdenboschi*. A small degree of structure among haplotype relationships is apparent along taxonomic lines within this region of sympatry. That is, clade 3b is dominated by *I. marchei*. All of the remaining Ivindo River specimens are paraphyletic to clades 3a and 3b. Whereas this paraphyletic collection of specimens contains one *I. marchei* individual with a distinctive cyt *b* haplotype (at the base of clade 3), it is dominated by six *I. opdenboschi* individuals with haplotypes of very similar sequence composition (Fig. 6a). The ML tree (not shown) is almost fully congruent with the MP strict consensus tree, except that clades 1 and 2 were recovered as weakly supported sister groups by ML analysis. Bootstrap support values for both trees are summarized in Fig. 6a.

Based on a haplotype network that we reconstructed for clade 3 using a statistical parsimony approach

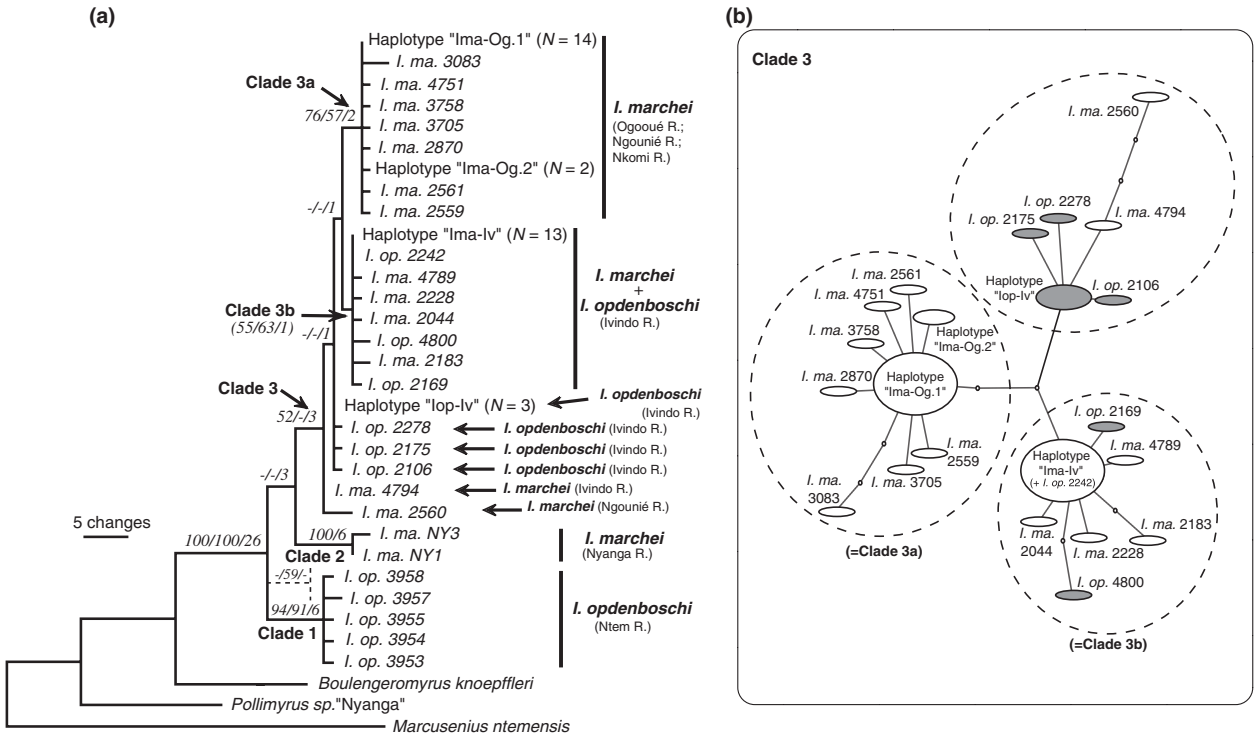

Fig. 6 (a) Strict consensus of eight most parsimonious trees recovered from MP analysis of complete cyt *b* sequences with equal character weighting. Branch lengths are proportional to number of nucleotide changes (see scale bar). The ML tree topology (not shown) is fully congruent with this tree except that clades 1 and 2 were recovered as sister groups (dashed line). Indicated at each in-group node are bootstrap values > 50% (first and second values for MP and ML analyses, respectively) followed by number of changes. Haplotype 'Ima-Og.1' is shared by 14 specimens: 1832, 2903, 2904, 2905, 2959, 3703, 3704, 3734, IK, Nk1, Nk2, 4742, 4753 and 4746. Haplotype 'Ima-Iv' is shared by 14 specimens: 1337, 1340, 2043, 2079, 2081, 2083, 2088, 2243, 2274, 4849, 4837, 4795, 4790 and 2242. Haplotype 'Iop-Iv' is shared by three specimens: 4847, 2184 and 1419. Haplotype 'Ima-Og.2' is shared by two specimens: 3395 and 2644. (b) Statistical parsimony network for cyt *b* haplotypes of clade 3. Ovals representing different haplotypes are drawn proportional to number of individuals sharing each haplotype. Small open circles are missing intermediate haplotypes (each line segment represents a single substitution step).

(Fig. 6b), we recognize three haplogroups that are separated from one another by two to three substitutions. One haplogroup corresponds to the clade 3a and contains all specimens of *I. marchei* from the Ogooué River. The second haplogroup, corresponding to the clade 3b, comprises 17 individuals of *I. marchei* and three individuals of *I. opdenboschi*, all from the Ivindo River. The third haplogroup contains six specimens of *I. opdenboschi* from the Ivindo and two specimens of *I. marchei* (one from the Ivindo River, the other from the Ngounié River).

Phylogeny and genetic partitioning among populations based on AFLP variation

We genotyped AFLP profiles for 28 of the *Ivindomyrus* specimens that we had previously sequenced across the entire cyt *b* gene (see Table 1). Band detection thresholds of 40, 80 and 120 r.f.u. resulted, respectively, in 1124, 813 and 684 variable characters, of which 771, 563 and 473 were parsimony-informative. While topologies of the inferred trees differed somewhat across analyses, general patterns also emerged across the nine approaches we used to estimate phylogenetic relationships. In seven of the nine analyses, we obtained the same four distinctive groups (or genetic clusters), which were defined by geographic origins and morphologically based field identifications of constituent specimens with very few exceptions.

Figure 7 shows the outcome of one such analysis: the single most parsimonious, unrooted tree obtained under Dollo parsimony and a detection threshold of 40 r.f.u. (total tree length = 5930 changes; CI = 0.234; RI = 0.696). One large genetic cluster in this tree (A) groups seven specimens of *I. marchei* from the Ivindo River plus two specimens originally identified morphologically as *I. opdenboschi* (4800 and 2242). These latter two specimens belong to Ivindo River clade 3b in the cyt *b* tree, which is mostly composed of *I. marchei*. A second cluster

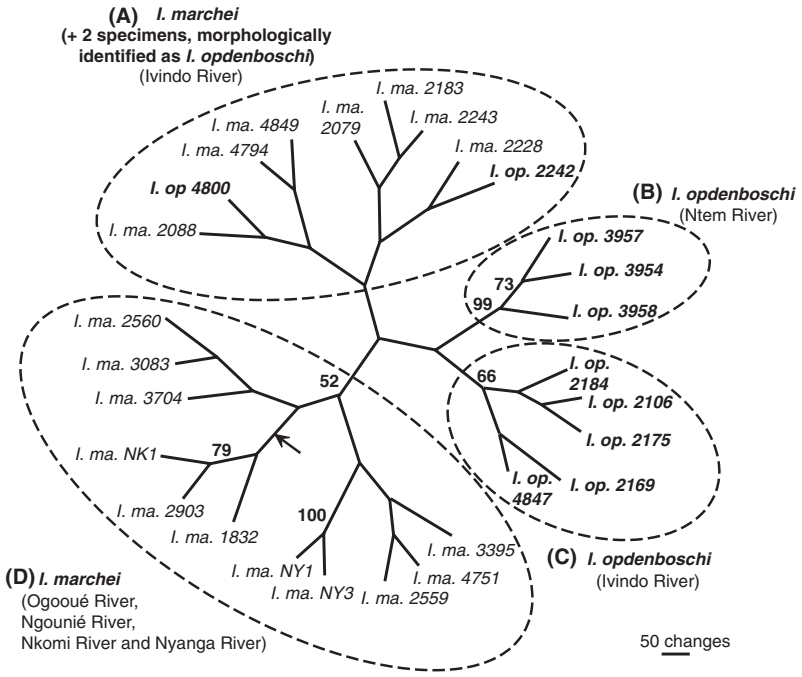

Fig. 7 Single most parsimonious, unrooted tree recovered by MP analysis of AFLP genotypes (band detection threshold = 40 r.f.u.). Each character was weighted equally for this analysis, and Dollo parsimony was assumed. Branch lengths are proportional to number of character state changes (scale bar provided). Bootstrap proportions greater than 50% are shown at all corresponding nodes. Morphological identifications of specimens as *Ivindomyrus opdenboschi* and *Ivindomyrus marchei* are abbreviated with '*I. op.*' and '*I. ma.*' respectively, and unique specimen numbers are provided. An arrow indicates the out-group rooting point when this analysis is conducted with the three out-group taxa (see text). Other analyses also yielded the same four genetically distinct groups (A–D) with similar intra-group topologies.

(B) includes only *I. opdenboschi* from the Ntem River. A third cluster (C) unites five *I. opdenboschi* individuals of Ivindo River origin. The remaining large cluster (D) groups all *I. marchei* individuals sampled from coastal rivers Nkomi and Nyanga with all *I. marchei* individuals collected throughout the greater Ogooué basin outside of the Ivindo. Although the presence of these four groups is robust across seven analyses, no consistency in the branching patterns among or within groups was detected. In the last two (of nine) analyses, we again obtain groups B, C and D. Regardless of rooting point, however, group A does not appear monophyletic in these two analyses. Instead, groups A and C are united as a single genetic cluster from the Ivindo River, within which group A appears monophyletic (results not shown).

Analysis of population structure using a combined sample of all available *Ivindomyrus* specimens from Loa-Loa (a single collection locality on the Ivindo River) yielded additional evidence of genetic partitioning between *Ivindomyrus* morphotypes. As before, assignment of specimens to morphotype was consistently based on field identifications for this analysis. None of the specimens used in this case exhibited intermediate or questionable morphology. Of 539 polymorphic loci in 12 sympatric specimens from the Ivindo River ($N = 6$ of each nominal species), four had the appearance of being diagnostic for morphotype (i.e. all 'recessive homozygotes' in one species vs. all 'dominant homozygotes' or 'heterozygotes' in the other species). Using this entire set of AFLP data, we estimated a small but significant $F_{ST} = 0.0416$ ($P = 0.0284$). When we removed loci for which only one individual lacked a band and all 11 others exhibited the band, this value became $F_{ST} = 0.0387$ ($P = 0.0233$). After further pruning away loci for which two individuals lacked a band, the value became $F_{ST} = 0.0369$ ($P = 0.0212$).

Discussion

Reproductive isolation between sympatric *Ivindomyrus* species

Genetic partitioning between co-occurring morphotypes can provide an important line of evidence for reproductive isolation between similar species when mating is difficult to observe directly in the field (Lu & Bernatchez, 1999; Kai *et al.*, 2002; Arnegard *et al.*, 2005). We found evidence of slight, but significant, genetic differentiation between sympatric populations of *I. marchei* and *I. opdenboschi* in a small stretch of river ($F_{ST} \approx 0.04$). This estimate is relatively small compared to some AFLP-based F_{ST} estimates generated for other species between sympatric morphotypes, conspecific populations in allopatry or hybridizing populations in secondary contact (Wilding *et al.*, 2001; Kai *et al.*, 2002; Paupy *et al.*, 2004; Svensson *et al.*, 2004). However, the level of genetic partitioning between populations of co-occurring *Ivindomyrus* species appears to be comparable to a recently reported estimate for F_{ST} between Caribbean reef fish morphospecies (*Hypoplectrus* spp.), which show strong assortative mating by colour pattern (Barreto & McCartney, 2008). In addition to genetic differences, we also detected significant variation in electric signals between sympatric *Ivindomyrus* morphotypes. These two findings, which

are independent of the morphological characters used for our initial taxonomic identifications, support the validity of the two nominal species of *Ivindomyrus*. Nevertheless, divergence between *I. marchei* and *I. opdenboschi* appears to be rather slight.

Evidence of some impediment to gene flow between *Ivindomyrus* species in the region of sympatry raises the hypothesis that features of their electric signals, such as relative P1 or P3 amplitudes, contribute to behavioural reproductive isolation. Similarly, interspecific divergence in the EODs of three *Petrocephalus* species (Mormyridae) from the Ivindo River is also characterized by differences in the relative magnitude of P3 (Lavoué *et al.*, 2004). Our study adds to a growing list of population genetic investigations demonstrating that mormyrid species boundaries are often congruent with patterns of sympatric EOD variation (Arnegard *et al.*, 2005; Feulner *et al.*, 2006). Relative to the mormyrid groups discussed in these other studies, however, waveform differences between *I. opdenboschi* and *I. marchei* are small. Other cues or recognition signals – for example, temporal patterns of EOD production – may also contribute to species isolation in *Ivindomyrus* and other mormyrid lineages. An understanding of the degree to which EOD waveform features account for reproductive isolation critically depends on behavioural and physiological studies on *I. marchei*, *I. opdenboschi* and other mormyrid species whose EODs differ in relatively fine-scale waveform features such as P3 amplitude (cf. Paintner & Kramer (2003) and Arnegard *et al.* (2006)).

Incongruence between phylogenetic patterns based on cyt *b* and AFLP markers

Despite evidence for the co-occurrence of two *Ivindomyrus* species in the region of sympatry, no single approach employed in this study allowed for the complete taxonomic discrimination of all individuals. Reciprocal monophyly was certainly not detected using cyt *b* or AFLP markers. While many specimens could be unambiguously assigned to species based on morphology and/or EODs, a small number of individuals appeared to be intermediate or spuriously positioned by each approach. The resulting pattern of incongruence was complex, as these spurious specimens were not the same across our analyses of genetic, morphological and electric signal variation.

In comparing our two genetic analyses, for example, different patterns of phylogenetic relationships are inferred from cyt *b* sequences and AFLP markers. Several studies have already shown that mitochondrial phylogenies can differ substantially from species phylogenies when levels of genetic divergence are low (Glemet *et al.*, 1998; Takahashi & Takata, 2000; Ting *et al.*, 2000; Shaw, 2002; Rognon & Guyomard, 2003). By integrating numerous characters across the nuclear genome, we expect an AFLP-based phylogeny to estimate true species relationships more accurately than inferences based on mitochondrial sequences or a small number of nuclear genes (Albertson *et al.*, 1999; Shaw, 2002; Sullivan *et al.*, 2004).

Incongruence between the AFLP and cyt *b* trees has three possible causes, which are not mutually exclusive: (i) overall weak phylogenetic resolution due to recent and/or incomplete genetic divergence between morphotypes; (ii) retained ancestral polymorphism due to incomplete lineage sorting; and (iii) introgressive hybridization (Avise, 2000). A possible signature of incomplete lineage sorting is illustrated by the Nyanga River specimens of *I. marchei*. In the AFLP-based trees, which appear more reflective of a natural pattern of branching among populations, specimens collected from this coastal river are nested within a larger cluster of *I. marchei* (e.g. Fig. 7). This cluster is composed of populations from the more distant Ogooué basin (outside of the Ivindo River) and an intervening coastal drainage (the Nkomi River). In the cyt *b* MP consensus tree, however, the Nyanga individuals form a sister group to all specimens of both species from the greater Ogooué basin (Fig. 6a). One interpretation, assuming an *I. marchei* morphotype did not arise multiple times due to convergence of body shape and EOD waveform, is that a polymorphic cyt *b* lineage was not sorted along species lines prior to colonization of the Nyanga River by *I. marchei*. In this case, current or extremely recent introgressive hybridization is a much less likely cause of incongruence, as substantial barriers to mormyrid dispersal exist between these isolated basins. One or more individuals with novel alleles would first have to migrate into such a population and successfully hybridize, and the migrant allele(s) would then have to sweep to high frequency to be retained and detected. In contrast, hybridization in the distant past cannot easily be distinguished from lineage sorting problems when making inferences based on incongruent genealogies.

Among sympatric populations/species, however, incongruent phylogenetic patterns can implicate comparatively recent hybridization events in some cases. One likely example is provided by specimen 2169, which is assigned to *I. opdenboschi* on morphological grounds and also exhibits an EOD waveform and AFLP profile characteristic of *I. opdenboschi*. The cyt *b* haplotype of this specimen is part of the shallow clade 3b (Fig. 6) composed of very similar haplotypes sequenced in 17 *I. marchei* individuals and two other individuals nominally assigned to *I. opdenboschi*. Unlike specimen 2169, however, these other two *I. opdenboschi* specimens exhibit AFLP genotypes more similar to those of *I. marchei*, which is consistent with their possession of cyt *b* haplotypes from this *I. marchei* clade. Specimen 2169 is the only member of its cyt *b* clade to possess an AFLP genotype characteristic of *I. opdenboschi*. A likely signature of recent introgressive hybridization such as this becomes harder to distinguish from incomplete lineage sorting as it is obscured over time by

events such as colonization, population expansion, lineage splitting, drift and local extinction.

Among vertebrates, interspecific hybridization is particularly common in fishes (Turner, 1999; Scribner *et al.*, 2001). Increases in rates of hybridization may be associated with recent range expansion, skew in population densities among parental species or habitat modification (Rognon & Guyomard, 2003; Johnson *et al.*, 2004; Freyhof *et al.*, 2005; Nolte *et al.*, 2005). One possible explanation for the co-occurrence of *I. opdenboschi* and *I. marchei* in the Ivindo is a past stream capture event that could have introduced *I. opdenboschi* from the Ntem into an Ivindo that had already been colonized by *I. marchei* from the Ogooué (Thys van den Audenaerde, 1966). Statistical evidence of genetic partitioning between *I. marchei* and *I. opdenboschi* at Loa-Loa does not rule out some level of past or ongoing gene flow between species. However, the relative contributions of gene flow and recency of ancestry to the low magnitude of F_{ST} estimated between *Ivindomyrus* species at this site are impossible to tease apart. Both factors may have also contributed to the incongruence between morphological assignments and AFLP genotypes of specimens 2242 and 4800, for example, or to the low bootstrap support for the Ivindo River cluster of *I. marchei* in the AFLP tree (Fig. 7). Furthermore, these factors are likely related, as hybridization may actually be a result of insufficient divergence between allopatric species prior to secondary contact in the Ivindo River.

Despite whatever introgressive hybridization may have occurred or may be ongoing, our significant F_{ST} estimate suggests that some degree of selection has prevented complete fusion of *I. marchei* and *I. opdenboschi*. We hypothesize that such selection may be related, at least in part, to resource-use competition because *I. marchei* and *I. opdenboschi* differ in morphological phenotypes that probably affect resource acquisition (e.g. head shape, relative mouth width and tooth number). We caution that our data, at present, are only suggestive of the possibility of recent hybridization between *Ivindomyrus* species in the current region of sympatry. Further, as our data capture differences between sympatric *I. marchei* and *I. opdenboschi* at a single moment in time, their analysis cannot reveal whether these lineages are on a course of divergence, anastomosis or long-term stable coexistence with some low level of ongoing hybridization.

Hybrid zones can provide unique insights into microevolutionary processes (Harrison, 1990), and in some cases hybridization may play an important evolutionary role in generating genetic diversity and new potential for evolutionary change (Seehausen, 2004). For these reasons, it will be important to determine the magnitude of current gene flow between *I. opdenboschi* and *I. marchei* in the Ivindo River. Collection of larger sample sizes sufficient for the application of genetic techniques to identify individuals of mixed ancestry will facilitate better estimates of current rates of hybridization between these species (e.g. Pritchard *et al.*, 2000).

Concluding remarks

Mormyrids have value as models for studying speciation and signal evolution owing to the discrete and easily quantified EOD component of their electrical signalling system and the relatively recent and extensive radiations some lineages have undergone (Hopkins, 1986, 1999; Sullivan *et al.*, 2002, 2004; Arnegard *et al.*, 2005; Feulner *et al.*, 2007). As illustrated by *I. opdenboschi* and *I. marchei* in the Ivindo River, however, mormyrid species boundaries are in some cases blurry even after extensive study of electric signals, morphology and genetics. Given that we cannot know the future trajectory of *Ivindomyrus* populations in the region of contact, what conclusions can we draw about the taxonomic status of the two entities there?

The Biological Species Concept (BSC) is often criticized because its criterion of reproductive isolation is essentially untestable, at least under fully natural conditions, in cases of allopatrically distributed sister taxa (i.e. the majority of cases). With *Ivindomyrus*, we have a seemingly ideal situation to test 'biological species' status in a region of sympatry between otherwise allopatrically distributed taxa, but our results show we cannot rule out limited gene flow between the morphotypes. Unfortunately, the verdict of the BSC is clear when two entities have incomplete reproductive isolation: they are not 'biological species' (Mayr, 1942). In cases like this, when study of any one phenotypic or genetic trait in isolation is inconclusive, the practical advantages of Mallet's (1995) definition of a species as a morphological and genotypic cluster become readily apparent. Here, a species boundary is identified by discontinuities in the frequency distributions of multiple characters, genetic and/or phenotypic. Turner (1999) points out that this species definition works particularly well for fishes and other sexual organisms that tend to hybridize readily in nature and for which the criteria of other species concepts fail to apply or are impossible to evaluate. Mallet (1995) and Turner (1999) regard this as a genetic reformulation of Darwin's (1859) conception of species, in which the reproductive isolation criterion of the BSC is a sufficient, but not always necessary or applicable, criterion for determining species status. We conclude that *I. opdenboschi* and *I. marchei* – despite less than perfect separability on the basis of any one dataset and inconclusive evidence for complete reproductive isolation between them in the Ivindo River – are, indeed, appropriately considered two different species, even if not 'biological species' in the Mayrian sense. Evidence presented here of complex and dynamic evolutionary interaction between these two species warrants their continued study.

Acknowledgments

Permits to collect fishes in Gabon and export them for this study were granted by l'Institut de Recherche en

Ecologie Tropicale (Dr P. Posso), l'Institut de Recherches Agronomiques et Forestières (Dr J.D. Mbega) and le Centre National de la Recherche Scientifique et Technologique. We are grateful for the valuable assistance we received from persons working in each of these institutions. We also thank A. Kamdem-Toham (WWF) and C. Aveling (ECOFAC) for their tremendous help in the logistic organization of our fieldwork. T. Uschold, M. Onanga, V. Mamonekene, M. Stiassny, J.P. Friel and D. Paugy helped us with specimen collection. J.P. Friel and P. Pruvost curated our specimens at the Cornell University Museum of Vertebrates in Ithaca, New York and the Muséum Nationale d'Histoire Naturelle in Paris respectively. All molecular work was carried out in the Evolutionary Genetics Core Facility in the Department of Ecology and Evolutionary Biology at Cornell University, and we are grateful for technical assistance provided by its director, S. Bogdanowicz. This work was supported by a National Science Foundation grant to C.D. Hopkins (DEB- 0108372).

References

Albertson, R.C., Markert, J.A., Danley, P.D. & Kocher, T.D. 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of Lakes Malawi, East Africa. *Proc. Natl Acad. Sci. U.S.A.* **96**: 5107–5110.

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., 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.

Arnegard, M.E., Jackson, B.S. & Hopkins, C.D. 2006. Time-domain signal divergence and discrimination without receptor modification in sympatric morphs of electric fishes. *J. Exp. Biol.* **209**: 2182–2198.

Avise, J.C. 1994. *Molecular Markers, Natural History and Evolution*. Chapman and Hall, New York.

Avise, J.C. 2000. *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, MA.

Barreto, F.S. & McCartney, M.A. 2008. Extraordinary AFLP fingerprint similarity despite strong assortative mating between reef fish color morphospecies. *Evolution* **62**: 226–233.

Bigorne, R. & Paugy, D. 1991. Note sur la systématique des *Petrocephalus* (Teleostei, Mormyridae) d'Afrique de l'Ouest. *Ichthyol. Explor. Freshw.* **2**: 1–30.

Boden, G., Teugels, G.G. & Hopkins, C.D. 1997. A systematic revision of the large-scaled *Marcusenius* with description of a new species from Cameroon (Teleostei; Osteoglossomorpha; Mormyridae). *J. Nat. Hist.* **31**: 1645–1682.

Boulenger, G.A. 1909–1916. *Catalogue of the Freshwater Fishes of Africa in the British Museum (Natural History)*. Wheldon and Wesley, London.

Bullock, T.H., Hopkins, C.D., Popper, A.N. & Fay, R.R. (eds) 2005. *Electroreception*. Springer Science + Business Media, Inc., New York.

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

Darwin, C. 1859. *On the Origin of Species by Means of Natural Selection*. John Murray, London.

von der Emde, G. 2004. Distance and shape: perception of the 3-dimensional world by weakly electric fish. *J. Physiol. Paris* **98**: 67–80.

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 fishes (Teleostei: Mormyridae: *Campylomormyrus*). *Mol. Phylogenet. Evol.* **39**: 198–208.

Feulner, P.G.D., Kirschbaum, F., Mamonekene, V., Ketmaier, V. & Tiedemann, R. 2007. Adaptive radiation in African weakly electric fish (Teleostei: Mormyridae: *Campylomormyrus*): a combined molecular and morphological approach. *J. Evol. Biol.* **20**: 403–414.

Freyhof, J., Lieckfeldt, D., Pitra, C. & Ludwig, A. 2005. Molecules and morphology: evidence for introgression of mitochondrial DNA in Dalmatian cyprinids. *Mol. Phylogenet. Evol.* **37**: 347–354.

Garnhart, N. 2001. *BinThere, GNU Public License*. University of New Hampshire, Durham, NH.

Glemet, H., Blier, P. & Bernatchez, L. 1998. Geographical extent of Arctic char (*Salvelinus alpinus*) mtDNA introgression in brook char populations (*S. fontinalis*) from eastern Quebec, Canada. *Mol. Ecol.* **7**: 1655–1662.

Graff, C. & Kramer, B. 1992. Trained weakly-electric fishes *Pollimyrus isidori* and *Gnathonemus petersii* (Mormyridae, Teleostei) discriminate between wave-forms of electric pulse discharges. *Ethology* **90**: 279–292.

Harrison, R.G. 1990. Hybrid zones: windows on the evolutionary process. *Oxf. Surv. Evol. Biol.* **7**: 69–128.

Hey, J. 2001. The mind of the species problem. *Trends Ecol. Evol.* **16**: 326–329.

Hollingsworth, P.M. & Ennos, R.A. 2004. Neighbour joining trees, dominant markers and population genetic structure. *Heredity* **92**: 490–498.

Hopkins, C.D. 1986. Temporal structure of non-propagated electric communication signals. *Brain Behav. Evol.* **28**: 43–59.

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

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

Hopkins, C.D., Lavoué, S. & Sullivan, J.P. 2008. Mormyridae. In: *Faune des poissons d'eaux douces et saumâtres de l'Ouest de l'Afrique Centrale (Cameroun, Guinée Equatoriale, Gabon, Congo Brazzaville)*. (M.L.J. Stiassny, G.G. Teugels & C.D. Hopkins, eds), pp. 219–334. MNHN, AMNH, MRAC, IRD, Paris.

Johnson, J.B., Dowling, T.E. & Belk, M.C. 2004. Neglected taxonomy of rare desert fishes: congruent evidence for two species of leatherside chub. *Syst. Biol.* **53**: 841–855.

Kai, Y., Nakayama, K. & Nakabo, T. 2002. Genetic differences among three colour morphotypes of the black rockfish, *Sebastes inermis*, inferred from mtDNA and AFLP analyses. *Mol. Ecol.* **11**: 2591–2598.

Kamdem Toham, A. 1998. *Fish Biodiversity of the Ntem River Basin (Cameroon): Taxonomy, Ecology and Evolution*. PhD thesis. Laboratoire of Ecology and Aquaculture, Department of Biology, Katholieke Universiteit Leuven, Leuven, Belgium.

Kramer, B. & Westby, G.W.M. 1985. No sex difference in the wave-form of the pulse type electric fish, *Gnathonemus petersii* (Mormyridae). *Experientia* **41**: 1530–1531.

Kramer, B., Van Der Bank, H. & Wink, M. 2004. *Hippopotamyrus ansorgii* species complex in the Upper Zambezi River System

with a description of a new species, *H. szaboi* (Mormyridae). *Zool. Scripta* **33**: 1–18.

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., Hopkins, C.D. & Kamdem Toham, A. 2004. The *Petrocephalus* (Pisces, Osteoglossomorpha, Mormyridae) of Gabon, Central Africa, with the description of a new species. *Zoosystema* **26**: 511–535.

Lu, G. & Bernatchez, L. 1999. Correlated trophic specialization and genetic divergence in sympatric lake whitefish ecotypes (*Coregonus clupeaformis*): support for the ecological speciation hypothesis. *Evolution* **53**: 1491–1505.

Lynch, M. & Milligan, B.G. 1994. Analysis of population genetic-structure with Rapd markers. *Mol. Ecol.* **3**: 91–99.

Mallet, J. 1995. A species definition for the modern synthesis. *Trends Ecol. Evol.* **10**: 294–299.

Mayr, E. 1942. *Systematics and the Origin of Species*. Columbia University Press, New York.

Nei, M. 1977. F-statistics and analysis of gene diversity in subdivided populations. *Ann. Hum. Genet.* **41**: 225–233.

Nei, M. & Li, W.H. 1979. Mathematical-model for studying genetic-variation in terms of restriction endonucleases. *Proc. Natl Acad. Sci. U.S.A.* **76**: 5269–5273.

Nelson, J.S. 1999. Editorial and introduction: the species concept in fish biology. *Rev. Fish Biol. Fish.* **9**: 277–280.

Nolte, A.W., Freyhof, J., Stemshorn, K.C. & Tautz, D. 2005. An invasive lineage of sculpins, *Cottus sp* (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. *Proc. R. Soc. Lond., B, Biol. Sci.* **272**: 2379–2387.

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

Paupy, C., Orsoni, A., Mousson, L. & Huber, K. 2004. Comparisons of amplified fragment length polymorphism (AFLP), microsatellite, and isoenzyme markers: Population genetics of *Aedes aegypti* (Diptera: Culicidae) from Phnom Penh (Cambodia). *J. Med. Entomol.* **41**: 664–671.

Pezzanite, B. & Moller, P. 1998. A sexually dimorphic basal anal-fin ray expansion in the weakly discharging electric fish *Gnathonemus petersii*. *J. Fish Biol.* **53**: 638–644.

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

de Queiroz, K. 2005. Ernst Mayr and the modern concept of species. *Proc. Natl Acad. Sci. U.S.A.* **102**: 6600–6607.

Rognon, X. & Guyomard, R. 2003. Large extent of mitochondrial DNA transfer from *Oreochromis aureus* to *O. niloticus* in West Africa. *Mol. Ecol.* **12**: 435–445.

Sanderson, M.J. & Shaffer, H.B. 2002. Troubleshooting molecular phylogenetic analyses. *Ann. Rev. Ecol. Syst.* **33**: 49–72.

Sauvage, H.E. 1879. Notice sur la faune ichthyologique de l'Ogooué. *Bull. Soc. Philomat., Paris* **7**: 90–103.

Scribner, K.T., Page, K.S. & Bartron, M.L. 2001. Hybridization in freshwater fishes: a review of case studies and cytonuclear methods of biological inference. *Rev. Fish Biol. Fish.* **10**: 293–323.

Seehausen, O. 2004. Hybridization and adaptive radiation. *Trends Ecol. Evol.* **19**: 198–207.

Shaw, K.L. 2002. Conflict between nuclear and mitochondrial DNA phylogenies of a recent species radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets. *Proc. Natl Acad. Sci. U.S.A.* **99**: 16122–16127.

Sokal, R.R. & Rohlf, F.J. 1998. *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd edn. W.H. Freeman and Co., New York.

Sullivan, J.P. & Hopkins, C.D. 2005. A new *Stomatorhinus* (Osteoglossomorpha: Mormyridae) from the Ivindo River, Gabon, West Central Africa. *Zootaxa* **847**: 1–23.

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.

Svensson, E.I., Kristoffersen, L., Oskarsson, K. & Bensch, S. 2004. Molecular population divergence and sexual selection on morphology in the banded demoiselle (*Calopteryx splendens*). *Heredity* **93**: 423–433.

Swofford, D.L. 1999. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates, Sunderland, MA.

Takahashi, H. & Takata, K. 2000. Multiple lineages of the mitochondrial DNA introgression from *Pungitius pungitius* (L.) to *Pungitius tymensis* (Nikolsky). *Can. J. Fish. Aquat. Sci.* **57**: 1814–1833.

Taverne, L. & Géry, J. 1975. Un nouveau genre de Mormyridae du Gabon: *Ivindomyrus opdenboschi* gen. nov., sp. nov. *Rev. Zool. Afr.* **89**: 555–563.

Thys van den Audenaerde, D.F.E. 1966. Les *Tilapia* (Pisces, Cichlidae) du Sud-Cameroun et du Gabon: étude systematique. *Mus. R. Afr. Cent. Tervuren Belg. Ann. Ser. Octavo Sci. Zool.* **153**: 123.

Ting, C.T., Tsaur, S.C. & Wu, C.I. 2000. The phylogeny of closely related species as revealed by the genealogy of a speciation gene, Odysseus. *Proc. Natl Acad. Sci. U.S.A.* **97**: 5313–5316.

Turner, G.F. 1999. What is a fish species? *Rev. Fish Biol. Fish.* **9**: 281–297.

Vekemans, X. 2002. AFLP-SURV version 1.0. Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Bruxelles, Belgium.

Wheeler, W.C. 1990. Nucleic acid sequence, phylogeny and random outgroups. *Cladistics* **6**: 363–367.

Wilding, C.S., Butlin, R.K. & Grahame, J. 2001. Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. *J. Evol. Biol.* **14**: 611–619.

Zhivotovsky, L.A. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. *Mol. Ecol.* **8**: 907–913.

Zwickl, D.J. 2006. *Genetic Algorithm Approaches for the Phylogenetic Analysis of Large Biological Sequence Datasets under the Maximum Likelihood Criterion*. The University of Texas. Austin, TX.

Received 26 July 2007; accepted 21 March 2008

Supplementary material

The following supplementary material is available for this article:

Ivindomyrus specimens.

Out-group specimens.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1420-9101.2008.01544.x

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.