



Multiple Cases of Striking Genetic Similarity between Alternate Electric Fish Signal Morphs in Sympatry

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MULTIPLE CASES OF STRIKING GENETIC SIMILARITY BETWEEN ALTERNATE ELECTRIC FISH SIGNAL MORPHS IN SYMPATRY

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Abstract.—Striking trait polymorphisms are worthy of study in natural populations because they can often shed light on processes of phenotypic divergence and specialization, adaptive evolution, and (in some cases) the early stages of speciation. We examined patterns of genetic variation within and between populations of mormyrid fishes that are morphologically cryptic in sympatry but produce alternate types of electric organ discharge (EOD). Other species in a large group containing a clade of these morphologically cryptic EOD types produce stereotyped, species-typical EOD waveforms thought to function in mate recognition. First, for six populations from Gabon's *Brienomyrus* species flock, we confirm that forms of electric fish that exhibit distinctive morphologies and unique EOD waveforms (i.e., good reference species) are reproductively isolated from coexisting congeners. These sympatric species deviate from genetic panmixia across five microsatellite loci. Given this result, we examined three focal pairs of syntopic and morphologically cryptic EOD waveform types that are notable exceptions to the pattern of robust genetic partitioning among unique waveform classes within assemblages. These exceptional pairs constitute a monophyletic group within the *Brienomyrus* flock known as the *magnostipes* complex. One member of each pair (type I) produces a head-negative EOD, while the other member (either type II or type III, depending on location) produces a longer duration EOD differing in waveform from type I. We show that signal development in these pairs begins with juveniles of all *magnostipes*-complex morphs emitting head-positive EODs resembling those of type II adults. Divergence of EOD waveforms occurs with growth such that there are two discrete and fixed signal types in morphologically indistinguishable adults at each of several localities. Strong microsatellite partitioning between allopatric samples of any of these morphologically cryptic signal types suggests that geographically isolated populations are genetically decoupled from one another. By contrast, sympatric morphs appear genetically identical across microsatellite loci in Mouvanga Creek and the Okano River and only very weakly diverged, if at all, in the Ivindo River. Our results for the *magnostipes* complex fail to detect species boundaries between the focal morphs and are, instead, fully consistent with the existence of relatively stable signal dimorphisms at each of several different localities. No mechanism for the maintenance of this electrical polymorphism is suggested by the known natural history of the *magnostipes* complex. Despite a lack of evidence for genetic differentiation, the possibility of incipient sympatric speciation between morphs (especially type I and type II within the Ivindo River) merits further testing due to behavioral and neurobiological lines of evidence implying a general role for stereotyped EOD waveforms in species recognition. We discuss alternative hypotheses concerning the origins, stability, and evolutionary significance of these intriguing electrical morphs in light of geographical patterns of population structure and signal variation.

Key words.—Electric signal polymorphism, geographical variation, microsatellites, ontogeny, population structure.

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The origin and maintenance of polymorphism remains an active research theme of importance for understanding the evolution of phenotypic divergence and specialization (Ravigné et al. 2004; Turelli and Barton 2004). Maintained genetic variance and protected alleles are best empirically studied in relatively few model organisms (e.g., Andolfatto et al. 2001; Dilda and Mackay 2002; Garrigan and Hedrick 2003; Streelman et al. 2003). However, even when the genetic and environmental underpinnings of discrete phenotypes are unknown, investigating newly discovered trait polymorphisms in natural populations of nonmodel species may be warranted. Such trait variation often arises in response to ultimate selective influences that can only be fully understood at organismal and ecological levels of organization. Phenotypic polymorphism can provide insights into an organism's adaptation to its physical, biotic, and/or social environment (Smith 1993; Cook 2003; Svensson and Sinervo 2004). Such variation can act as a starting point for further lineage divergence (Drès and Mallet 2002; Barluenga and Meyer 2004). Generalizations about the evolutionary origins and significance of phenotypic polymorphism will be further refined as work on more tractable genetic models is complimented by

organismal studies in natural contexts across a wide variety of taxa.

Polymorphic variation in communication channels is particularly interesting because it can affect the way individuals are sensed by conspecific competitors, social group members, or mates as well as by heterospecific individuals such as predators or pollinators. Environmental heterogeneity can promote variation in the expression and perception of animal signals, including those affecting mate choice (Endler 1991; Seehausen et al. 1997; Fuller 2002). Signals of importance to courtship and mating can also differ between conspecifics engaging in alternative mating tactics (Gross 1996). In most animals alternative mating tactics, and any associated signal differences, are restricted to males (Andersson 1994; Rhen and Crews 2002; West-Eberhard 2003). Notable exceptions are found among social insects with female mating tactics (Rüppell and Heinze 1999) and in the side-blotched lizard, which exhibits a throat color polymorphism associated with different reproductive tactics in both males and females (Sinervo and Zamudio 2001).

Apparent polymorphism in courtship signals has raised the hypothesis that alternate signal types are, in fact, reproductively isolated. Good biological species that cannot otherwise

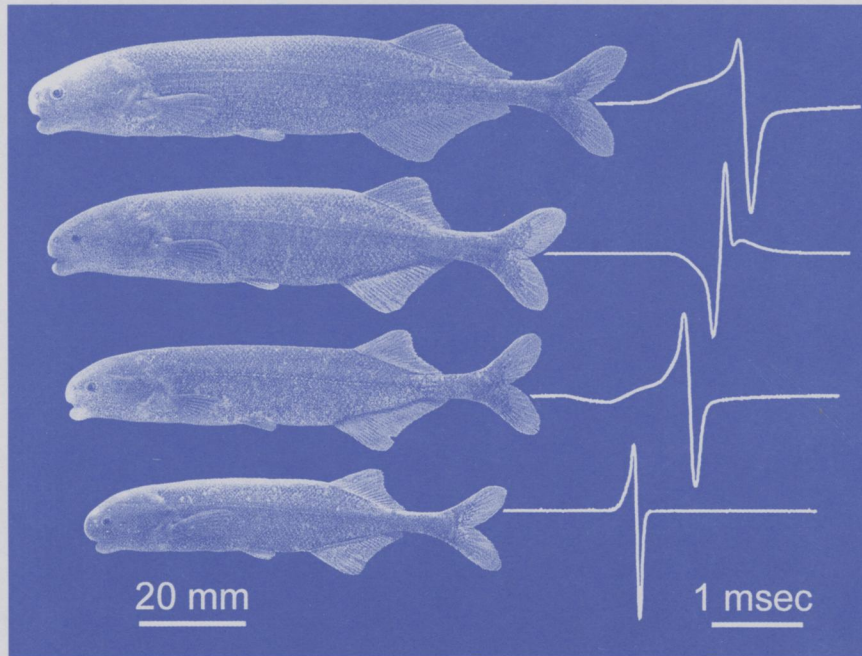
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Sympatric mormyrids from a forested stream in Gabon with the electric pulses they produce. The middle two are nearly identical at five microsatellites, whereas the other two differ significantly. •

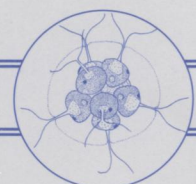
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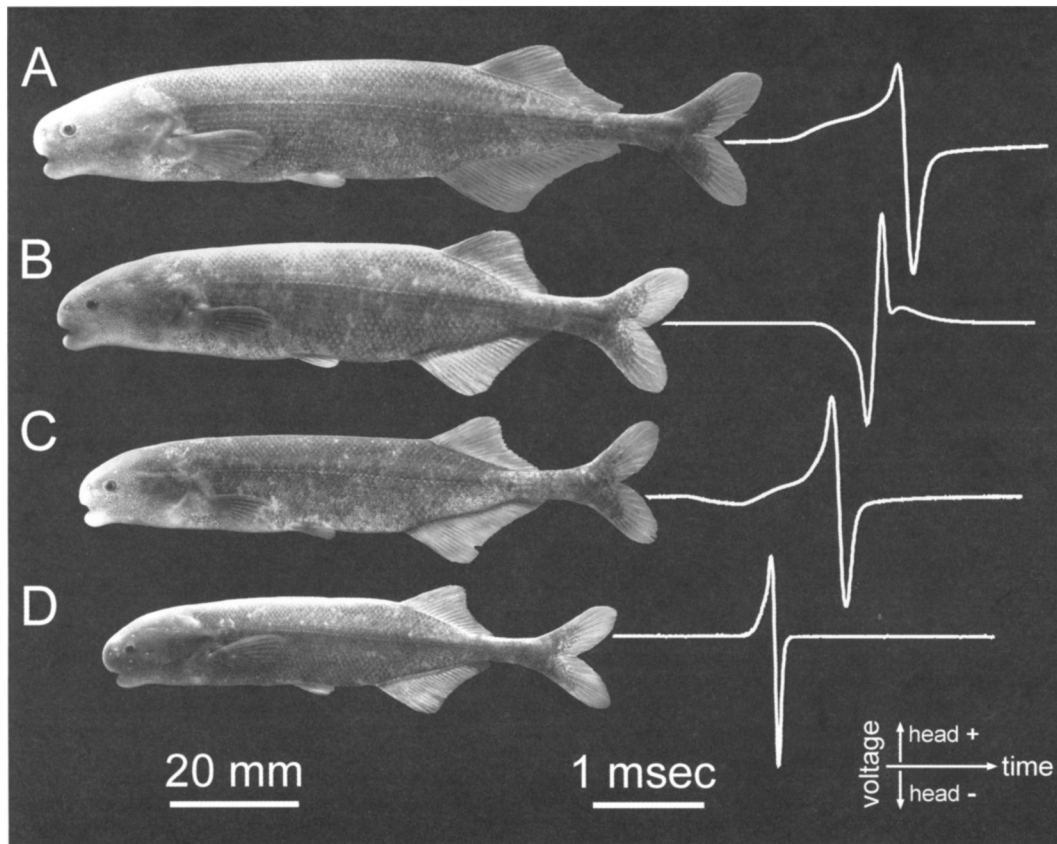


FIG. 1. Example of a sympatric assemblage of morphologically similar mormyrids from the *Brienomyrus* species flock of Gabon. Photographs (scaled to specimen standard length) of the only four *Brienomyrus* signal forms occurring in Mouvanga Creek ($2^{\circ}19'23.5''S$, $11^{\circ}41'18.1''E$) are shown next to voltage traces of electric organ discharges (EODs) recorded from the same individuals: (A) *bp1*, specimen 3016, adult male, CU80355; (B) type I morph, specimen 3025, adult female or nonbreeding male, CU80231; (C) type II morph, specimen 3014, adult female or nonbreeding male, CU80358; and (D) *sn3*, specimen 3027, adult female or nonbreeding male, CU80356. Scale bars of 20 mm and 1 msec are indicated.

be diagnosed using traditional taxonomic characters have been referred to as “sibling species” (Mayr 1942) or “cryptic species” (Mayr 1948). In many cases, reproductive isolation that was initially predicted to exist between cryptic species on the basis of courtship signal differences has later been confirmed by behavioral and/or molecular testing (Stratton and Uetz 1981; Knowlton 1986; Gerhardt 1994; Knight et al. 1998; van Oppen et al. 1998; Wells and Henry 1998; Mendelson and Shaw 2002; Thomas et al. 2003).

We report on a system of closely related African mormyrid fishes exhibiting a striking polymorphism in electric signals thought to play an important role in mate recognition during courtship. Nocturnally active mormyrids produce low voltage electric pulses, electric organ discharges (EODs), for object detection and social communication (for reviews, see Bullock and Heiligenberg 1986; Moller 1995). There are two components to these electric signals: (1) the sequence of intervals between EOD pulses, which varies rapidly in different social or electrolocating contexts (Bratton and Kramer 1989; Serrier and Moller 1989; von der Emde 1992; Carlson and Hopkins 2004); and (2) the waveform of each EOD pulse, which remains comparatively fixed.

Juvenile mormyrids often exhibit slow modifications in EOD waveform with growth (Bass 1986a; Arnegard and Hop-

kins 2003). Signal development of this kind is related to ontogenetic changes in the anatomy of the postlarval electric organ (Szabo 1960; Bennett 1971; Bass 1986b). In addition, EODs of males usually elongate as these individuals enter into social dominance and/or breeding condition (Bass and Hopkins 1985; Carlson et al. 2000). Nevertheless, EODs of subadults as well as mature individuals of both sexes often remain distinct from those of other sympatric mormyrid species (Hopkins 1980; Arnegard and Hopkins 2003).

Study System

Our study focuses on members of what has been termed the Gabon-clade *Brienomyrus* species flock, which has radiated recently and extensively in the Ogooué River system of Central Africa (Sullivan et al. 2002, 2004). In many cases, several different EOD waveforms can be detected in a single forested stream. In Mouvanga Creek, for example, four signal forms coexist (Fig. 1). As many as 11 have been collected syntopically from the most species-rich locality discovered to date for this group: the rapids at Loa-Loa (Fig. 2). The great majority of these sympatric forms, for which species descriptions are forthcoming, can be diagnosed on the basis of external counts and measurements, as well as by their

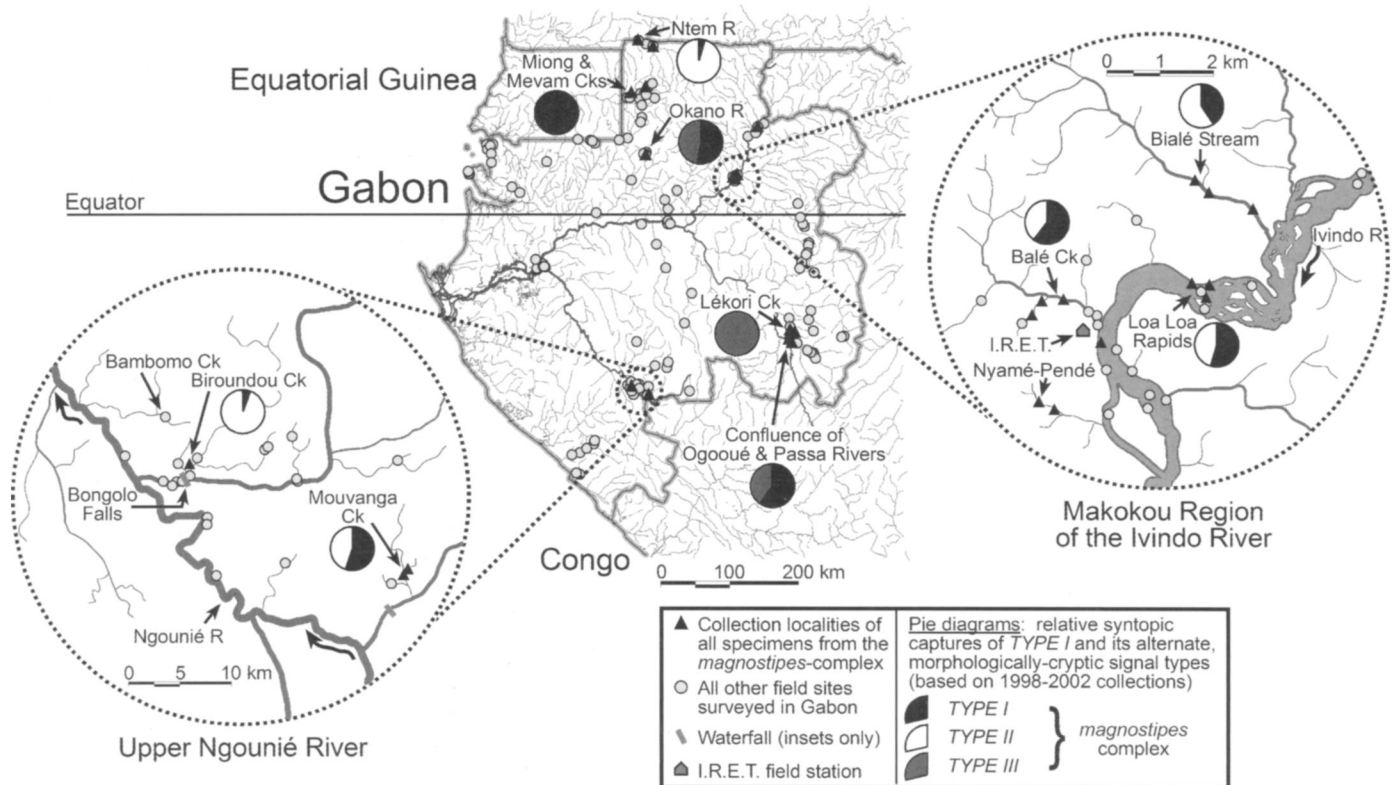


FIG. 2. Drainage map of Gabon naming sites from which specimens were sampled for this study. Black triangles indicate all field sites where at least one *magnostipes*-complex morph has been collected to date. Small gray circles indicate sampled localities where no *magnostipes*-complex morphs have been collected. The type I morph usually coexists with a morphologically cryptic, alternate electric signal morph (either type II or type III). Pie diagrams indicate relative abundances of focal signal morphs at indicated localities, based on all captures of field specimens between 1998 and 2002.

species-typical EOD waveforms (Hopkins 1980; Arnegard and Hopkins 2003). By conducting field playbacks of female EODs to courting males of a Gabon-clade *Brienomyrus* species, Hopkins and Bass (1981) demonstrated the importance of temporal EOD characteristics for species recognition. Laboratory experiments in other groups of mormyrids have also generated results consistent with EOD-mediated recognition (Moller and Serrier 1986; Graff and Kramer 1992), and a neural pathway underlying temporal EOD coding has been described (reviewed in Xu-Friedman and Hopkins 1999).

Here, we investigate pairs of sympatric morphs that are exceptional in the Gabon-clade of *Brienomyrus* because they cannot be diagnosed from one another sympatrically on the basis of external morphological characters. One member of these morphologically cryptic pairs produces an EOD of relatively short duration, in which the first major excursion of the waveform away from baseline is head-negative (i.e., electrical current flow inside the animal is away from the head and the return path for current outside the animal is toward the head). We call this the type I EOD morph wherever it occurs (Fig. 1). At several localities, type I coexists with an alternate signal morph we call type II. This second morph emits a longer duration pulse in which the largest head-positive peak of the waveform occurs before the largest head-negative peak (Fig. 1). A diagnostic internal anatomical difference exists between type I and type II in terms of the size of the stalks penetrating through the electrocytes that

make up their electric organs (C. D. Hopkins, pers. obs.). Throughout this report we refer to focal sympatric morphs as being morphologically cryptic to conveniently describe their indistinctness in external appearance, rather than to downplay the potential importance of an internal anatomical character (i.e., electrocyte morphology). We discovered a third, morphologically cryptic signal class that we designate type III due to its divergent EOD waveform relative to type II (Fig. 3). Like that of type II, the EOD of type III is longer in duration than the EOD of type I. Each of these phenotypic waveform classes exhibits no sex limitation in its expression, nor any obvious skew between the sexes. Site- or region-specific taxon codes previously assigned to these three signal morphs appear in the legend of Table 1. For readability, we lump all such codes under three waveform types (regarded as signal morphs in the present study) on the basis of broad patterns of EOD similarity.

These three signal morphs (types I, II, and III) make up the *magnostipes* complex, one of three major clades of the *Brienomyrus* species flock from Gabon (Sullivan et al. 2004). When sympatric, these morphs share cytochrome *b* haplotypes (Sullivan et al. 2002). Furthermore, a preliminary amplified fragment length polymorphism-based investigation detected no phylogenetic resolution within the *magnostipes* clade but, rather, united geographical locations irrespective of signal type (Sullivan et al. 2004). We present data on the distribution of three morphs composing the *magnostipes* com-

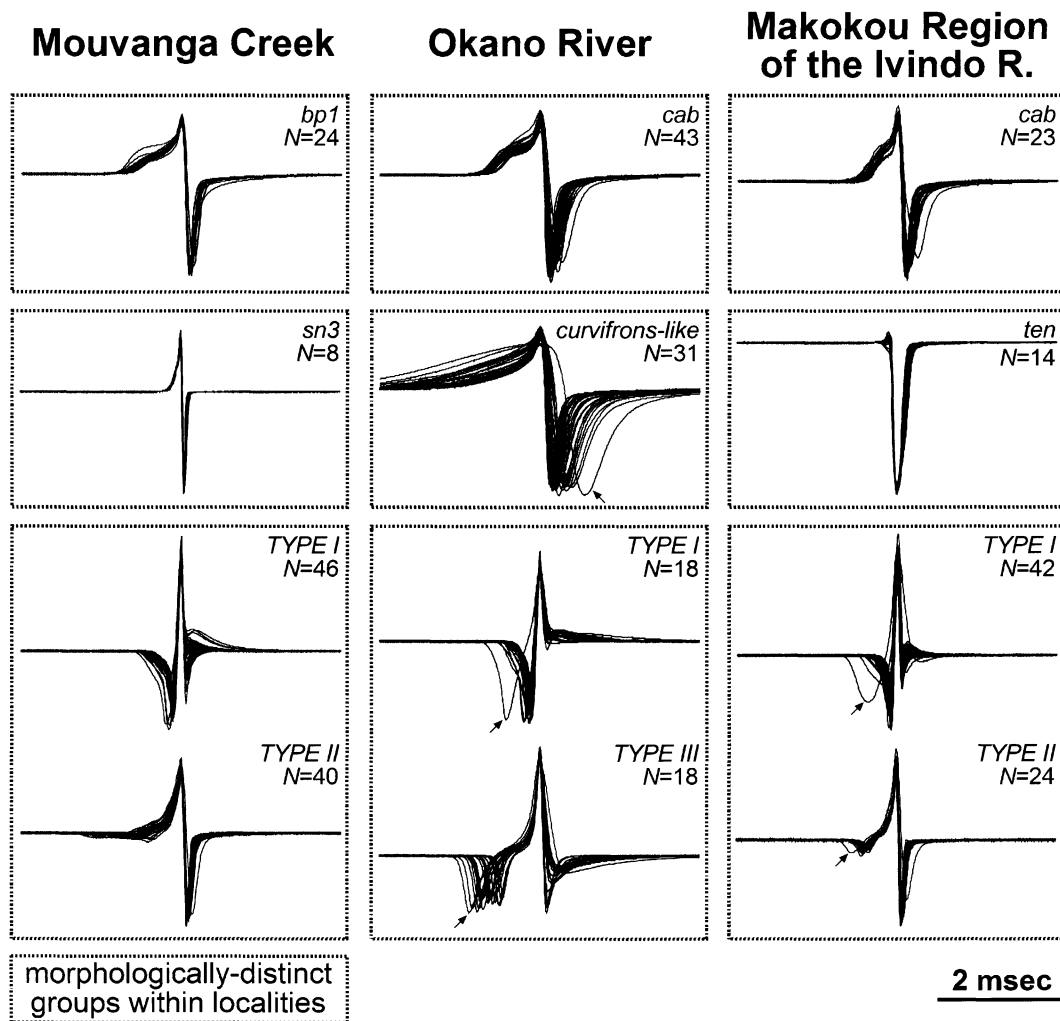


FIG. 3. Overlay plots of electric organ discharges (EODs) recorded from four *Brieno-my-rus* signal forms in each of three collection localities/regions. EODs (recorded in the field from specimens of standard length > 50 mm) are normalized to the same peak-to-peak voltage and plotted on the same time base with head-positivity up (2 msec time scale indicated). EODs in every plot are centered on the major positive peak of each waveform except those of *Brieno-my-rus* sp. *ten*, which are centered on the major negative peak for clarity. Numbers of signals, each from a different specimen, are given by *N*. Dotted rectangles unite specimens forming unique morphological groups within sites, based on external anatomical characters. Small arrows point to examples of elongated EODs of dominant or breeding males.

plex. We quantitatively analyze electric signal variation among all constituent populations so far encountered, and we show the ontogeny of EOD development within some populations. Using five microsatellite markers we: (1) confirm robust genetic differences between several morphologically distinct *Brieno-my-rus* species in sympatry (i.e., good reference species); (2) test for differences between coexisting, cryptic morphs; and (3) describe the genetic structure of geographically isolated *magnostipes*-complex populations.

MATERIALS AND METHODS

Collecting Tissue Samples and Recording Electric Signals

We collected specimens of several Gabon-clade *Brieno-my-rus* forms from numerous streams and rivers in the Ogooué River system from 1998 to 2002 (Fig. 2) using the following three techniques: (1) sundown (especially new moon) fishing

in rivers with worm-baited traps adapted from a design used by local fishermen; (2) daytime localization of fish with an electrode and audio amplifier and chase-capture using hand-held hoop nets; or (3) light application of rotenone to streams, followed by resuscitation of captured fishes in fresh, well-oxygenated water.

We recorded the electric signals of all specimens within a few hours of capture in 5- to 20-L plastic aquaria filled with water from the collection site (conductivity = 12–30 μ S/cm; temperature = 22–26°C). Recordings were made with a bipolar silver/silver-chloride electrode. Positive and negative poles were positioned at opposite ends of the aquarium and connected to a low-noise, BMA-831/XR differential bioamplifier (CWE Inc., Ardmore, PA). We made AC-coupled (bandwidth = 0.1–50 kHz) or DC recordings of each fish when it faced the positive electrode pole. Waveforms were digitized using an IOtech Daqbook (16-bit A/D converter,

TABLE 1. List of study sites from which individuals were sampled for genotyping (*N*, number of individuals collected). Samples of type I and type II were pooled across the Makokou region of the Ivindo River for some statistical comparisons and kept separate for others. Due to constraints on collecting, *Brienomyrus* sp. *cab* and *B.* sp. *ten* were each pooled within the Makokou region for all comparisons. Sullivan et al. (2002, 2004) referred to the super-scripted *magnostipes*-complex populations as *MAG*¹, *SP*², *BEN*³, *SP*⁴, and *SP*⁵. Asterisks denote populations for which exact tests of linkage disequilibrium, heterozygosities, and deviations from Hardy-Weinberg proportions are reported in the text.

Species or EOD form	Site or region	<i>N</i>
Type I ^{1,2}	Loa-Loa Rapids ^{1,*}	30
	Bialé Stream ^{1,*}	20
	Balé Creek ^{1,*}	35
	Nyamé-Pendé Creek ¹	2
	Ivindo River at I.R.E.T. ¹	2
	(Makokou Region Total)	(89)
	Mouvanga Creek ^{2,*}	46
Type II ^{3,4}	Okano River ^{1,*}	18
	Loa-Loa Rapids ^{3,*}	32
	Bialé Stream ^{3,*}	26
	Balé Creek ^{3,*}	20
	Nyamé-Pendé Creek ³	1
	Ivindo River at I.R.E.T. ³	3
	(Makokou Region Total)	(82)
Type III ⁵	Mouvanga Creek ^{4,*}	40
	Biroundou Creek ^{4,*}	29
	Okano River ^{5,*}	18
	<i>Brienomyrus</i> sp. <i>cab</i>	
	Balé Creek	12
<i>Brienomyrus</i> sp. <i>bpl</i>	Nyamé-Pendé Creek	11
	(Makokou Region Total)*	(23)
	Okano River*	43
	Mouvanga Creek*	24
<i>Brienomyrus</i> sp. <i>ten</i>	Bambomo Creek*	30
	Loa-Loa Rapids	6
	Balé Creek	8
<i>Brienomyrus</i> sp. <i>sn3</i>	(Makokou Region Total)*	(14)
	Mouvanga Creek*	8
<i>Brienomyrus</i> cf. <i>curvifrons</i>	Okano River*	31
	Grand Total	495

100-kHz sampling rate) or an IOtech Wavebook (16-bit, 200-kHz; IOtech, Cleveland, OH) and stored on a portable computer using custom-written software.

We euthanized specimens after recording them by administering an overdose of MS222. Standard length (SL) of each was measured to the nearest 0.5 mm. We removed one or more paired fins from specimens selected for molecular analysis and preserved these tissues in 95% ethanol. Some small juveniles from the Okano River or Mouvanga Creek were recorded in the field and imported live to the laboratory to track EOD changes through time. Vouchers were fixed in 10% formalin for two weeks, transferred to 70% ethanol, and deposited in the Cornell University Museum of Vertebrates. All of our methods conform to protocols approved by Cornell University's Center for Research Animal Resources.

Sympatric and Allopatric Comparisons

To investigate sympatric genetic differences between morphs of the *magnostipes* complex, we collected geographically disjunct samples of type II or type III and their morphologically cryptic, sympatric counterparts—the different type I populations—from three sites (or regions) in Gabon:

(1) the Makokou region of the Ivindo River; (2) Mouvanga Creek; and (3) the Okano River (see Fig. 2). In each case, we also collected two morphologically distinct sympatric species for comparison to our focal signal types. The overall study design is summarized in Figure 3. In the Makokou region, such comparisons were based on samples combined across several river-kilometers (Fig. 2), while sampling was essentially from single points in Mouvanga Creek and the Okano River. Due to the wider spacing of collection points in the Ivindo River, we also investigated sympatric differences between type I and type II at three sublocalities within this region: Loa-Loa rapids, Bialé Stream, and Balé Creek. Species used in all sympatric comparisons have overlapping microgeographic distributions and were often collected syntopically (i.e., various combinations of the four species were pulled from individual traps or dip net catches). We also made allopatric comparisons among all the sampled type II/III populations and among all the type I populations. To these comparisons, we added type II from Biroundou Creek, a site where too few type I individuals occur for population-level analysis (Fig. 2). In addition to type I and type II, we collected two additional *Brienomyrus* species at Biroundou Creek with unique EODs and distinctive morphologies (*BON* and *BNI* in Sullivan et al. 2002, 2004; not included in the present study). Collection sites from which samples were taken for genotyping (and sample sizes) are listed in Table 1.

EODs of type I can only be unquestionably distinguished from those of sympatric type II or type III in field-caught specimens greater than 45-mm SL, or in laboratory-reared individuals greater than about 55-mm SL (see Fig. 4B). Therefore, all EOD and genetic comparisons among field populations were based on specimens larger than 50-mm SL. The vast majority appeared to be subadults or adults, and their SL exceeded 60 mm.

Microsatellite Cloning and Development

We constructed a partial genomic library using fresh liver tissue taken from a single type II individual, which we imported live from Mouvanga Creek and euthanized in the laboratory. Total genomic DNA was extracted from liver tissue using the Genomic-tip protocol (Qiagen, Valencia, CA). Extracted DNA was digested with the restriction enzymes *ApoI* and *BstYI*, treated with shrimp alkaline phosphatase, and electrophoresed on an 0.8% agarose gel. A portion of the gel containing fragments ranging in length from 700 to 1400 bp was excised and extracted with a QIAquick Gel Extraction Kit (Qiagen). For ligation of fragments into the plasmid pUC19 (cut with *BamHI* and *EcoRI*), we ran ligation reactions overnight at 16°C using insert:vector ratios of 1:3, 1:1, and 3:1. Restriction enzymes, T4 DNA ligase, ligation buffer, and the cloning vector were obtained from New England Biolabs (Beverly, MA).

We transformed ligation products into Max Efficiency DH5 α competent *E. coli* cells (Life Technologies, Rockville, MD) and grew these cells on Luria-Bertani agar containing ampicillin (50 μ g/ml) and coated with X-gal (50 μ g/ml) and IPTG (1 mM). We transferred recombinant colonies to Magna Graph nylon membranes (Fisher Scientific, Pittsburgh, PA) and probed them with a radiolabeled pool of 24-mer oligo-

TABLE 2. Characteristics of five microsatellite loci cloned from a single type II individual from Mouvanga Creek. For each locus, the table provides: GenBank accession number, primer sequences, polymerase chain reaction annealing temperature (T_a), simple sequence repeat motif, and binned fragment size estimate for the corresponding clone (from genotyping). The table also provides the total number of alleles detected and the corresponding fragment size range for all individuals genotyped in this study. Five additional sequences containing microsatellite loci (NBB006–NBB010) have been posted to GenBank under the accession numbers AY465759–AY465763.

Locus (GenBank)	Primer sequences	T_a (°C)	Repeat motif in clone	Cloned fragment size (bp)	Total alleles	Fragment size range (bp)
NBB001 (AY465754)	5'-TGCAAGATACCGGTCACTGTCTATGAAC-3' 5'-GTGTGCCGTGGAGAGCAAGATGG-3'	64	(TAA) ₂ A(TAA) ₁₄	133	15	112–154
NBB002 (AY465755)	5'-CTTTGCGTTTTGTAGGCCCTTACTAACC-3' 5'-CCCAGACCCCAAGCTTCATTC-3'	64	(TG) ₇ (AG) ₂₁	275	33	245–341
NBB003 (AY465756)	5'-TGGCAAAGGACCGAGGTTTACC-3' 5'-GATCCTCGTCATGTTCCAGTCATAAT-3'	59	(GA) ₁₂	132	26	116–154
NBB004 (AY465757)	5'-AGTGGGGGTATTGTCTCTCC-3' 5'-TGTTCAGCCCAATCATC-3'	59	(GT) ₃₅	243	29	189–263
NBB005 (AY465758)	5'-GTGGAAAAGTGGACAGGGGAAGACC-3' 5'-TCATCCTGTGTTCCGGTTTAACTTTTGA-3'	59	(CA) ₂₀	243	36	225–309

nucleotides composed of AC and TC dimers and all possible trimeric repeats, excluding homopolymers (48°C for 2 days). The hybridization and wash buffer contained 0.01 M NaPO₄, 0.5% SDS, 1 mM EDTA, 1 mg/ml BSA, and 3 M TMAC, which effectively eliminates melting temperature variation due to base-composition differences. Samples from the original colonies, identified as positive clones by autoradiography, were transferred to microtiter wells containing 50 µl of sterile 10 mM Tris buffer (pH 7.6), covered with mineral oil, incubated at 97°C for 15 min, stored at –80°C, and later used directly as templates for polymerase chain reaction (PCR). We amplified inserts with universal M13 flanking primers and sequenced them using BigDye Terminator cycle sequencing and an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Using PrimerSelect v5.05 (DNASar, Madison, WI), we developed primers for five microsatellite loci (NBB001–NBB005). Five additional microsatellites (NBB006–NBB010) were also identified. Sequences for all 10 loci have been posted to GenBank (see Table 2 for accession numbers).

Microsatellite Genotyping

We extracted DNA from fin clips using DNeasy Tissue Kits (Qiagen) and amplified the five microsatellites by PCR. Single-locus amplification reaction volumes were 10 µl and consisted of 1× PCR buffer (20 mM Tris-HCl, 50 mM KCl), 2 mM MgCl₂, 0.2 mM of each dNTP, 2 pmol of each primer (labeled with Applied Biosystems fluorescent dyes FAM, HEX, or NED), and 0.75 unit Taq polymerase (Life Technologies) previously incubated for 20 min at room temperature with 1.05 pmol TaqStart antibody in storage buffer (Clontech, Palo Alto, CA). Thermal cycling (under mineral oil) was started with an initial denaturing step of 90°C for 2 min; followed by 35 cycles of 95°C for 50 sec, T_a (°C) for 60 sec (T_a values given in Table 2), and 72°C for 60 sec; and completed with one step of 72°C for 45 min. We multiplexed loci for each specimen and resolved them by electrophoresis on a 6% denaturing polyacrylamide gel using an ABI 377 automated sequencer (Applied Biosystems). Data collection and scoring were performed with GeneScan v3.1 and Genotyper v2.5 (Applied Biosystems).

Data Analysis

We examined EOD variation among populations of signal forms (whether morphologically cryptic or not), or through time in select individuals, by either overlaying amplitude-normalized plots of electrical recordings (centered on the major head-positive peak and plotted on the same time base) or applying landmark-based ordination to a set of 29 measured EOD characters (described in Arnegard and Hopkins 2003). For each microsatellite locus, we examined possible deviations from Hardy-Weinberg equilibrium within populations using the two-tailed exact test (Weir 1990) implemented by GENEPOP v3.4 (Raymond and Rousset 1995). We considered sympatric type I and type II/III as either united or separate populations for these tests. We also performed exact tests of linkage disequilibrium between all pairs of loci (within and across populations), again in GENEPOP, to test the independent assortment of loci. Statistical significance in both sets of tests was evaluated using Markov chain methods (10,000 dememorization steps; 1000 batches; 5000 iterations per batch).

We assessed genetic differentiation between pairs of nominal populations in two ways for both sympatric and allopatric comparisons: (1) F_{ST} (Weir and Cockerham 1984) was estimated using Arlequin v2.001 (Schneider et al. 2000), the significance of which was evaluated by permuting genotypes between populations 50,000 times; and (2) allelic distributions were examined for differences using the Fisher exact test of genic differentiation in GENEPOP (Markov chain parameters: 10,000 dememorization steps; 10,000 batches; 5000 iterations per batch). We evaluated significance of each pairwise test at the uncorrected $P = 0.05$ level (to highlight possible differences that merit further study), as well as the extremely conservative, Bonferroni-corrected threshold of $P = 0.0045$ (5% divided by the maximum number of comparisons in which any population participated).

We also investigated population structure in the *magnotipes* complex without making any a priori categorizations by EOD waveform or collection locality. To do so, we used the program Structure v2.0 (Pritchard et al. 2000), which employs a model-based Bayesian clustering algorithm to determine the most probable number of homogenous genetic

clusters or populations (K). This program also calculates the probability of each individual's membership, or ancestry, in each cluster. We pooled type I and type II/III within each locality and estimated K for each combined sample to assess the genetic distinctness of morphs. We also investigated the nature of geographical population structure by further combining pooled samples across the Makokou region or across the entire Ogooué basin and, again, estimating K for these combined samples. For each set of most likely clusters we detected, we examined the average probability of membership of individuals belonging to different nominate populations. We ran all simulations under an admixture model, allowing for correlated allele frequencies among populations due, for example, to shared ancestry (Falush et al. 2003). Results are based on 250,000 Markov chain iterations after a burn-in period of 25,000 iterations. Otherwise, default parameter values were used.

RESULTS

Geographic Distribution and Signal Variation

Signal morphs of the *magnostipes* complex were taken from habitats that are generally characterized by swift currents flowing past cobbles, root masses, or debris dams. We detected populations of these morphs at several sites throughout Gabon (Fig. 2), which range from densely forested streams about 2 m wide (e.g., Nyamé-Pendé Creek with six sympatric EOD forms) to collection localities in large open rivers approximately 0.5 km wide (i.e., the Ivindo River at Loa-Loa rapids with 11 sympatric congeners emitting unique EOD waveforms). Only one of three *magnostipes*-complex morphs appears to exist at some sites (Miong, Mevam, and Lékori Creeks), or their overall abundance is heavily skewed toward a single morph (Biroundou Creek and a site in the Ntem River near the Village Doan). By contrast, relatively even numbers of type I and type II/III were collected at all other sites shown in Figure 2. Among adult fish, we never detected a case of type II and type III occurring together. Wherever we found a sympatric pair that included one of these two morphs, the other member of the pair was always type I.

Overlays of EODs recorded from fish exceeding 50-mm SL illustrate that morphologically cryptic individuals fall into two distinct signal classes wherever two *magnostipes*-complex morphs are sympatric (Fig. 3). Several authors have described androgen-mediated EOD elongation in males (socially or seasonally induced) across a large number of mormyrid species (Hopkins 1980; Westby and Kirschbaum 1982; Bass and Hopkins 1985; Bass 1986a; Landsman et al. 1990; Kramer 1997; Carlson et al. 2000). Although elongated EODs of *magnostipes*-complex males deviate from those of adult females and nonbreeding males, the few we have recorded for this study (e.g., Fig. 3, arrows) remain morph-typical. That is, EODs of breeding males are similar in waveform (despite elongation) to those produced by females of the same morph, and they are distinct from EODs produced by breeding males of the alternate, sympatric morph.

Waveforms of type I and type II/III cannot be reliably distinguished in individuals < 50-mm SL. However, the ontogeny of the signal produced by type I can be illustrated by

recordings made at neighboring Miong and Mevam Creeks in the Ntem River basin (Fig. 4A) because only this adult signal type has been detected there (Fig. 2). Juveniles of the smallest size class captured from these two creeks emit EODs resembling those of type II adults from the Ivindo River (cf. Fig. 3). As the juveniles grow, their EODs form an ontogenetic series that progresses toward the waveform typical of type I adults. That is, the relative amplitude of the initial, head-negative EOD peak increases with growth. Type I males in breeding condition are the largest individuals at Miong and Mevam Creeks; they exhibit elongated EODs with a greatly exaggerated initial head-negative peaks (Fig. 4A). We have documented similar developmental series for two wild-caught Okano River juveniles (Fig. 4B), each of which was raised in isolation. Both individuals emitted EODs resembling those of Ivindo River type II adults at the time of capture. As they grew, their EODs followed two different trajectories. One developed into a type I waveform, while the other transformed into a waveform typical of type III adults from the Okano River (cf. Fig. 3). Among adults, the type II waveform has never been detected in our collections from the Okano River.

Once committed to a developmental trajectory, individuals do not appear to switch EOD waveforms. We imported juveniles and subadults (45–65 mm SL) from Mouvanga Creek, recorded them in the laboratory, and placed them in small social groups. Genetic fingerprinting (using the same loci described in this report) showed that each individual's EOD type remained the same after approximately two years of free interaction and growth (Fig. 5). Final recordings revealed elongated EODs in males that had grown large and achieved dominance in their social group, yet these signals were still clearly discernible as either type I or type II (Fig. 5, arrows).

Figure 6 illustrates the results of a principal components analysis (PCA) of waveform landmarks taken from all field-recorded *magnostipes*-complex individuals larger than 50 mm (excluding all eight elongated breeding-male EODs shown in Figs. 3 and 4A and one elongated type II EOD recorded from a breeding male captured in the Ntem River [not shown]). The first factor axis ($\lambda_1 = 15.22$) explains 52.5% of the overall variation in EOD wavelike shape, while the second ($\lambda_2 = 6.895$) explains an additional 23.8%. Each of these axes is heavily loaded by a complex suite of waveform variables (factor loadings not shown). Type II populations cluster together but form three groups corresponding to the Ntem, Upper Ngounié, and Ivindo regions. Type III populations make up a separate cluster of samples from the Okano and Upper Ogooué Rivers. When any two of these type II or III groups are compared alone, they do not overlap with one another in PCA space (not shown), the only exception being two type II individuals from the Upper Ngounié region that slightly overlap with type II individuals from the Ntem. The same does not hold true for type I specimens, all of which form one large cluster even after type II/III cases are removed from the analysis.

Loci Attributes, Linkage Equilibria, and Hardy-Weinberg Expectations

The five microsatellite markers developed for this study can be readily amplified across the Gabon-clade *Brienomyrus*

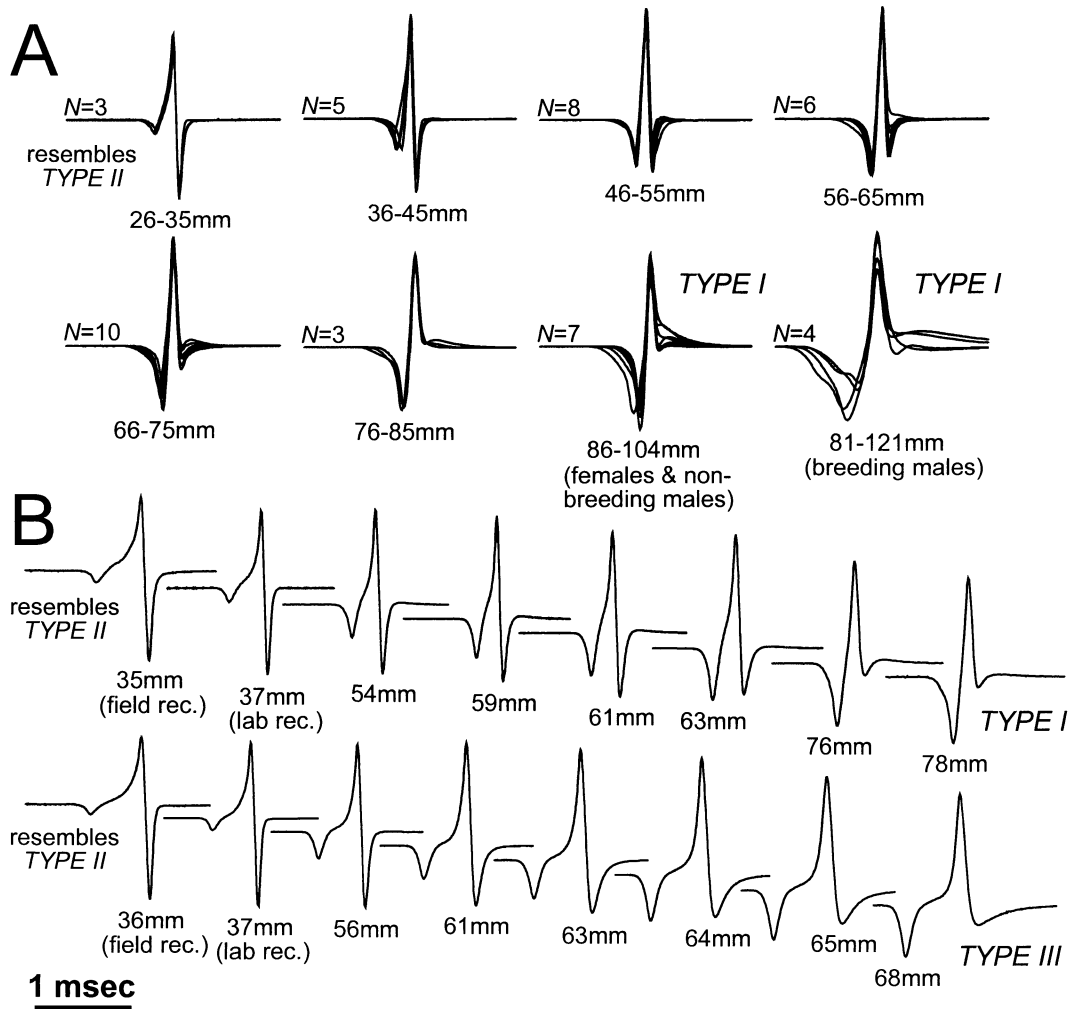


FIG. 4. Examples of ontogenetic changes in electric organ discharge (EOD) waveform in the *magnostipes* complex. (A) EODs (overlaid in groups of N individuals and organized by standard length, SL) recorded in August and September 1999, at the onset of the rainy breeding season, from two nearby sites in the Ntem River basin: Miong Creek ($1^{\circ}37'31.9''N$, $11^{\circ}27'27.3''E$) and Mevam Creek ($1^{\circ}37'25.9''N$, $11^{\circ}27'10.5''E$). Type I is the only *magnostipes*-complex signal morph that has been collected among individuals > 50 -mm SL in these two streams. (B) Ontogenetic trajectories in EOD waveform recorded from two small juveniles (above: type I, specimen 4699, CU89487; below: type III, specimen 4712, CU89488) captured from the Okano River and raised to subadulthood in the laboratory. The first recording of each trajectory was made in the field, while subsequent recordings were made in the laboratory (SL provided for each). As illustrated in both (A) and (B), triphasic EODs produced by small type I or type III juveniles resemble those of type II adults from the Ivindo River (see Fig. 3). Ontogenetic signal divergence in laboratory specimens (B) appears slightly delayed relative to field caught specimens, which are usually fully distinct by the time they reach a SL of 45 mm.

species flock. We successfully assembled five-locus genotypes for all specimens in the present study without detecting any failed amplifications (i.e., possible null homozygotes). Moreover, in a pilot survey, we were able to amplify all five markers in two individuals of *Brienomyrus* sp. *sza*, which has a basal position in the species flock (Sullivan et al. 2002). Genotypes were also recovered for NBB001, NBB003, and NBB005 in four of four specimens of the outgroup species, *Ivindomyrus opdenboschi*.

Total number of alleles detected over all populations (i.e., unique EOD waveforms and different collection sites; see asterisks, Table 1) ranged from 15 to 36 (Table 2), and locus-specific heterozygosities averaged across all populations ranged from 0.409 to 0.720. When averaged across loci, heterozygosities within populations were higher for the three

collection sites in the Ivindo River ($\bar{H}_o = 0.631-0.767$) than outside this region ($\bar{H}_o = 0.422-0.579$), though sample sizes were comparable ($N = 20-35$ vs. $18-46$, respectively). Exact tests produced no evidence of linked loci in any population or across all populations ($P > 0.23$).

We individually examined five loci in each of 23 "population" samples. In addition to the 18 populations defined above, sympatric type I and type II/III were pooled at each of five focal sites within regions. In very few of these samples did observed heterozygosity (H_o) deviate significantly from expected heterozygosity (H_e). The exceptions were: *Brienomyrus* sp. *cab* from the Okano River (locus NBB004; $H_e = 0.280$; $H_o = 0.064$; $P = 0.0001$); *Brienomyrus* sp. *cab* from the Makokou region (NBB002; $H_e = 0.915$; $H_o = 0.739$; $P = 0.0207$); and type II from Mouvanga Creek (NBB004; H_e

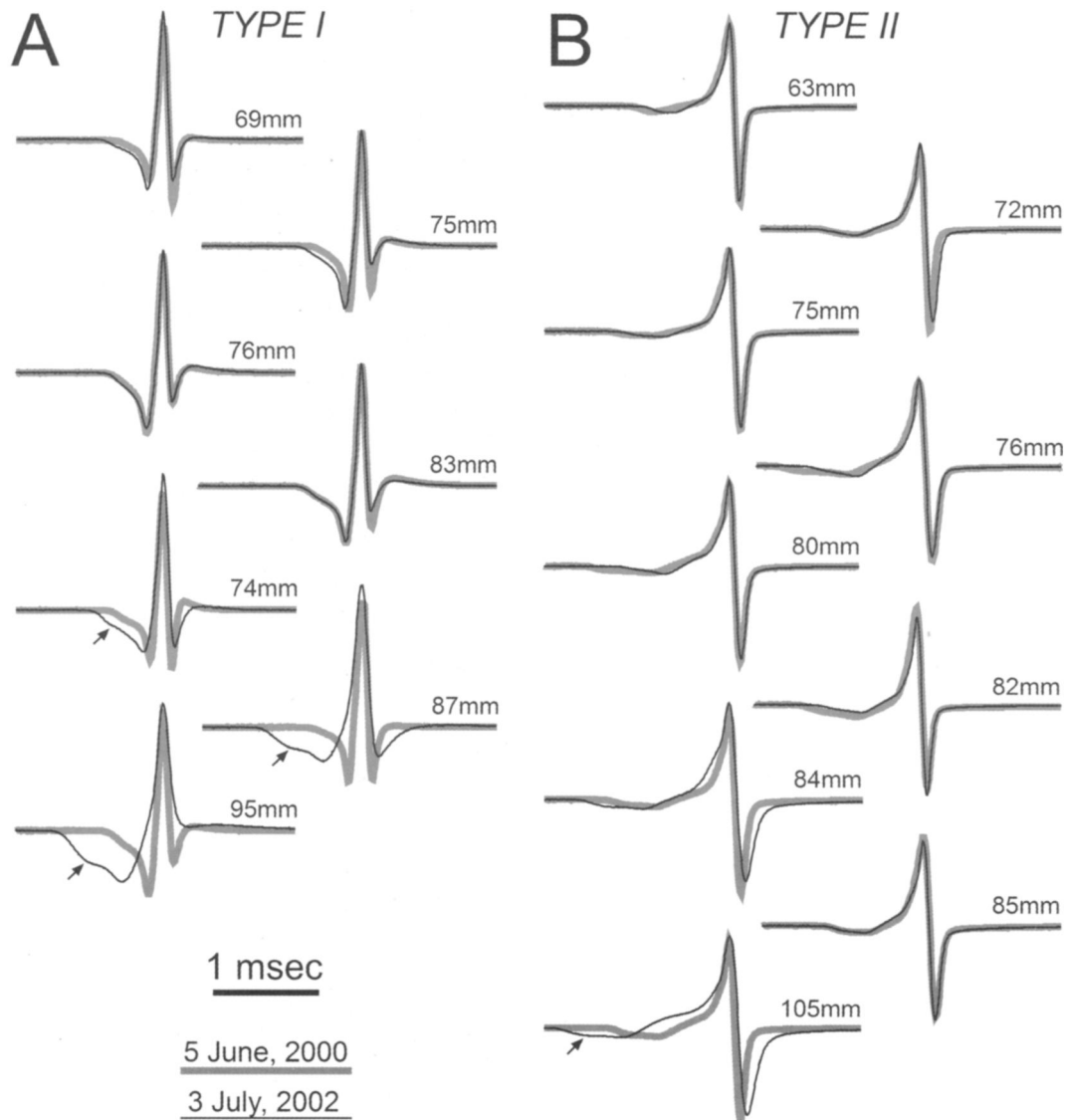


FIG. 5. Overlays of electric organ discharges (EODs) produced by *magnostipes*-complex juveniles captured from Mouvanga Creek (28 July 1999) and reared in laboratory group tanks. Each overlay corresponds to a single individual with a unique five-locus microsatellite genotype. Wide gray traces were recorded on 5 June 2000 in juveniles and subadults ranging from 45- to 65-mm SL (when all specimens were large enough to provide a fin clip under anesthesia). Thin black overlays were recorded on 3 July 2002 (final SL, in mm, provided for each individual). Shown are (A) seven type I and (B) nine type II individuals, none of which switched EOD classes during this two-year period of growth and development in captivity. Some males became dominant in their social group and elongated their EODs (arrows). A scale bar of 1-msec duration is shown for reference.

= 0.684; H_o = 0.550; P = 0.0488), Loa-Loa rapids (NBB003; H_e = 0.418; H_o = 0.281; P = 0.0325), and Balé Creek (NBB001; H_e = 0.768; H_o = 0.450; P = 0.0014). In the combined (type I + type II) samples, significant heterozygote deficiencies were detected for Balé Creek (NBB004; H_e = 0.845; H_o = 0.782; P = 0.0361) and Loa-Loa rapids (NBB001; H_e = 0.738; H_o = 0.597; P = 0.0035). In these combined cases, either both uncombined samples showed no evidence of deviation from expectation (Balé Creek; P > 0.09), or weaker evidence of deviation was detected for only one of the constituent EOD morphs (Loa-Loa type II; P = 0.0060). Combined sympatric samples of *magnostipes*-complex morphs did not deviate significantly from expected Har-

dy-Weinberg proportions outside the Makokou region (i.e., at Mouvanga Creek or the Okano River).

Sympatric Population Genetic Comparisons

Figure 7A shows allele frequency distributions at five microsatellite loci (columns) in four sympatric EOD forms from Mouvanga Creek (rows). Two of these forms, *Brienomyrus* sp. *bp1* and *Brienomyrus* sp. *sn3*, appear to differ from one another and from the type I-type II pair in overall head and body shape (M. E. Arnegard and C. D. Hopkins, pers. obs.). All multilocus F_{ST} estimates and exact tests of genic differentiation are significant for comparisons between *bp1* and

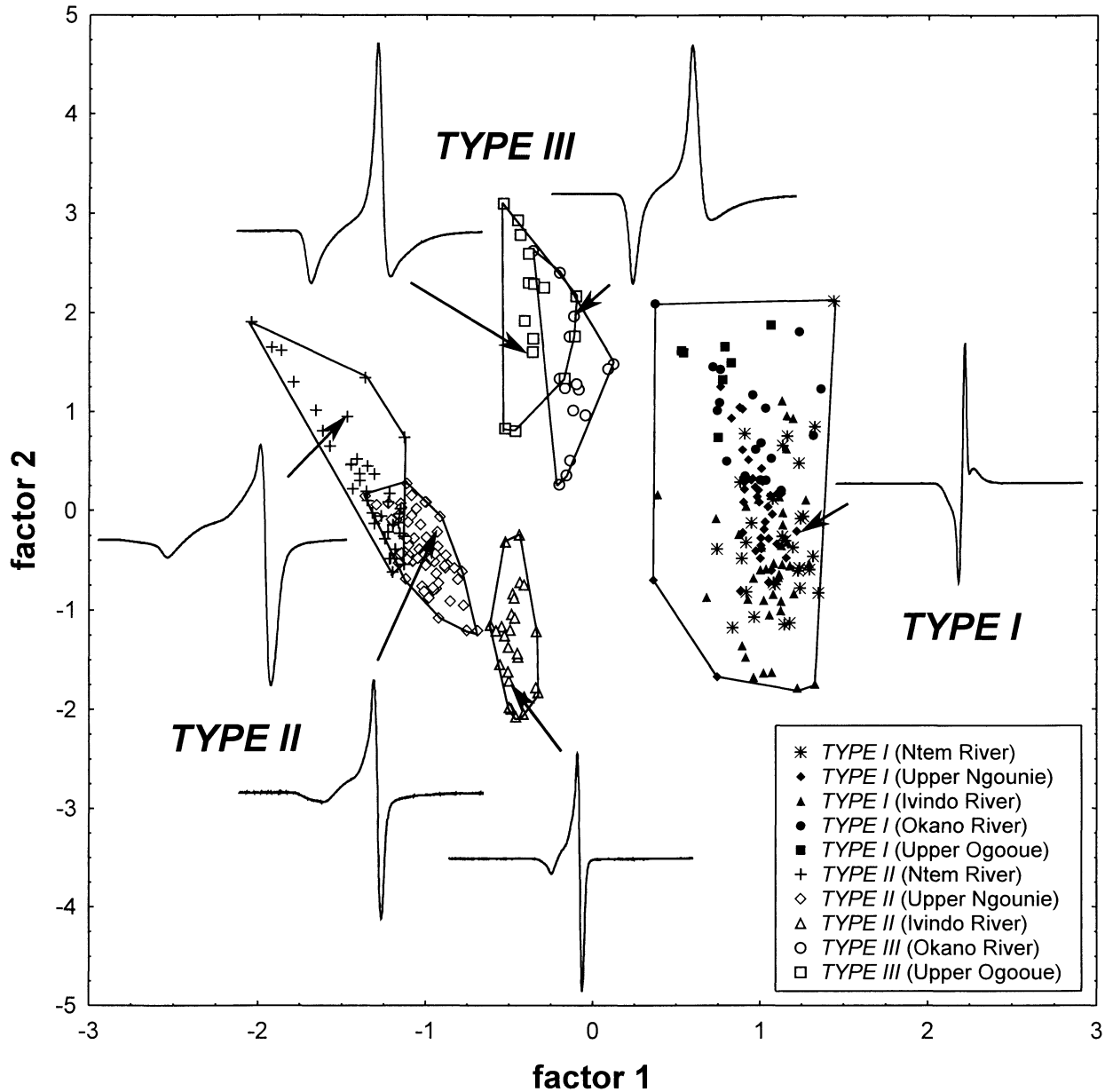
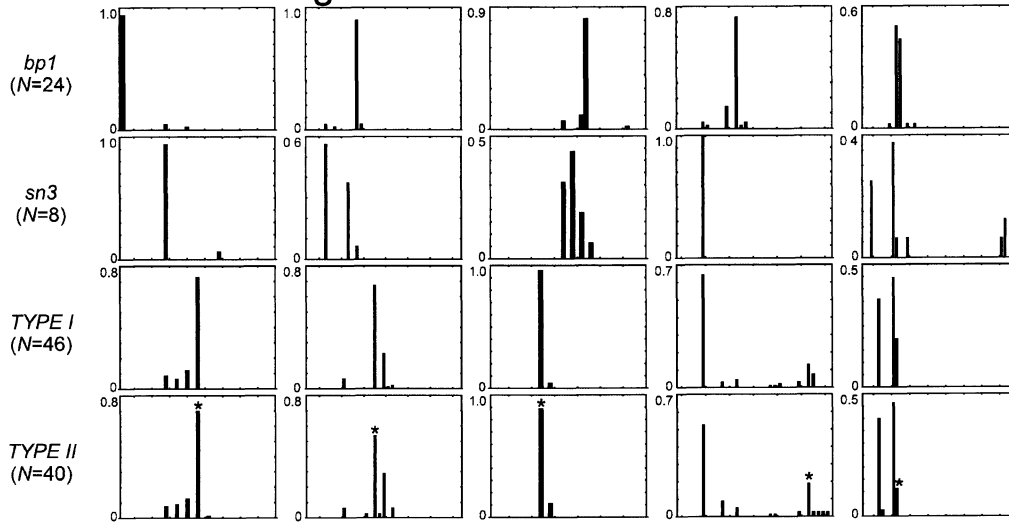


FIG. 6. Plot of the first and second factor axis scores from a principal components analysis (PCA) of 29 electric organ discharge (EOD) characters measured for 291 field-recorded specimens belonging to the *magnostipes* complex. All cases correspond to individuals exceeding 50-mm SL. Minimum polygons circumscribe type II and type III cases from different regions of Gabon, while a single polygon encloses the entire set of type I cases due to lack of discrete geographic variation. Weak apparent differentiation of Upper Ogooué type I cases from other cases of this signal morph probably relates to the large size (139–178 mm SL) of these seven adults. Legend symbols indicate the following regions from which specimens were collected: Ntem River (main channel and, in the case of type I, Miong and Mevam Creeks), Upper Ngounié (Mouvanga and Biroundou Creeks), Ivindo River (Loa-Loa Rapids; adjacent to I.R.E.T.; Balé Creek, Bialé Stream, and Nyamé-Pendé Creek), Okano River (vicinity of the Village Na), and Upper Ogooué (at Franceville; confluence of Ogooué and Passa Rivers; and, in the case of type III, Lékori Creek). EODs of specified individuals are scaled to a constant peak-to-peak amplitude (head-positivity up) and a total trace duration of 4 msec. Recording temperatures (mean = 23.2°C ± 1.4 SD) were only collected for 110 cases, including some individuals from each morph-site combination. Adjusting the time base of these EODs to that expected at the mean temperature using an empirically determined Q_{10} of 1.6 (see also Kramer and Westby 1985) did not appreciably change the pattern of PCA results. Sullivan et al. (2002, 2004) referred to type III (Upper Ogooué), type II (Ntem River), type I (Upper Ogooué), and type III (Okano River) as *SP6*, *SP7*, *SP8*, and *SP9*, respectively. See Table 1 (legend) for other previously used taxon codes.

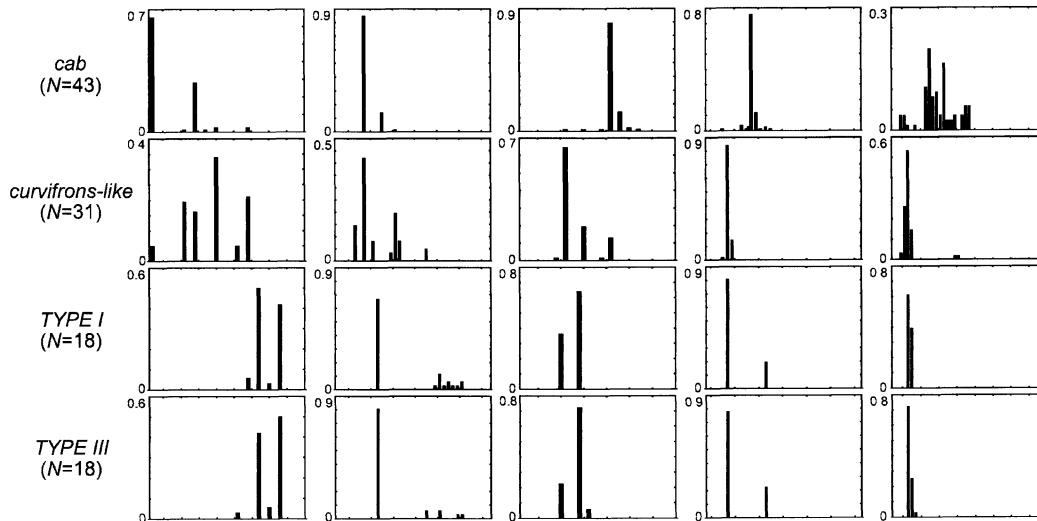
sn3 and between these species and either focal morph, while the same tests reveal no statistical differences between the type I and type II morphs (Table 3). Single-locus tests at four individual microsatellites (NBB001–NBB003 and NBB005) yield the same pattern of outcomes as do the multilocus tests.

That is, we find significant differences at each locus (F_{ST} -values > 0.082; P -values for F_{ST} < 0.00066; exact test P -values < 0.00001) between any two Mouvanga Creek forms that differ in head/body shape and EOD waveform. At the same time, there are no differences at any locus (NBB001–

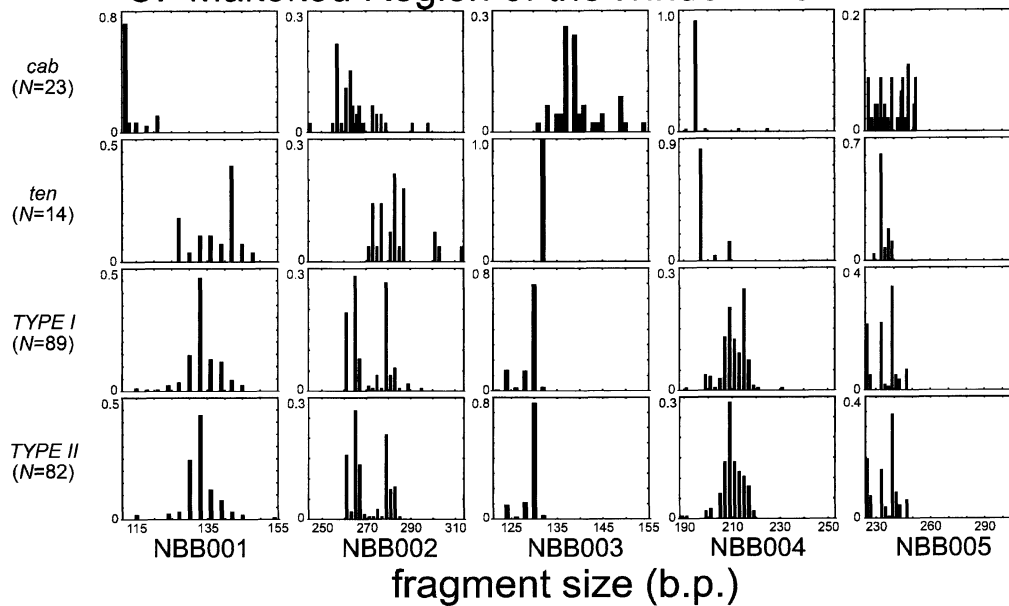
A. Mouvanga Creek



B. Okano River



C. Makokou Region of the Ivindo River



NBB005) between type I and type II ($F_{ST} = 0$ in all cases; P -values for $F_{ST} > 0.07$; exact test P -values > 0.14). Rather than merely lacking statistical differences, type I and type II appear nearly genetically identical across loci when allele frequency distributions are visually compared (Fig. 7A).

We discovered a similar pattern (Fig. 7B) when we examined a pair of cryptic signal morphs from the Okano River (type I and type III) together with two additional sympatric forms that appear distinct in their external morphologies (*cab* and *Brienomyrus* cf. *curvifrons*). Multilocus tests (Table 3) expose strong statistical differences between Okano forms that differ in external morphology and EOD. This is not the case between type I and type III (Table 3). When examined individually, each microsatellite locus yields statistical results consistent with the tabulated multilocus results without exception (not shown).

Results garnered from genetic analyses of four Ivindo River signal forms (Fig. 7C) deviate slightly from the patterns detected at the Okano River and Mouvanga Creek. *Brienomyrus* sp. *cab* and *Brienomyrus* sp. *ten* differ in external morphology from one another and from sympatric type I and type II, which again share a common external morphology. We find robust genetic differences in comparisons between any two morphologically distinct Ivindo forms in terms of multilocus tests (Table 3) or single-locus tests (F_{ST} -values > 0.021 ; P -values for $F_{ST} < 0.0022$; exact test P -values < 0.00004). While a five-locus exact test provides no evidence of genetic partitioning between type I and type II ($P = 0.32528$), we estimate a small, but statistically significant multilocus F_{ST} using the same samples ($F_{ST} = 0.002$; $P = 0.03151$). Aside from a marginally significant exact test outcome for NBB004 ($P = 0.05304$), however, no single-locus test reveals a significant difference between regionally combined samples of focal morphs ($F_{ST} = 0$ in all cases; P -values for $F_{ST} > 0.11$; exact test P -values > 0.23).

More robust indications of genetic partitioning between type I and type II are revealed by separate consideration of three principal sampling sites within the Makokou region (Table 4). The strongest evidence comes from Bialé Stream, for which significant differences are observed at NBB001 and over all five loci, even after we conservatively adjust the alpha probability level using the Bonferroni approach (adjusted $\alpha = 0.0045$). In addition, we estimate a marginally significant five-locus F_{ST} between morphs in Balé Creek. The difference in Balé Creek appears to be based most heavily on a significant difference at NBB004 (after Bonferroni correction). Although multilocus tests do not reveal a difference between type I and type II morphs in the Loa-Loa comparison, a significant F_{ST} is estimated at NBB003. Significance of this F_{ST} estimate disappears after Bonferroni correction (Table 4). Ignoring cases in which very few copies of an

allele are detected, we find no private (i.e., diagnostic) alleles in either morph.

Allopatric Population Genetic Comparisons

Despite the remarkable genetic similarity between cryptic morphs in sympatry, multilocus F_{ST} estimates and exact tests of genic differentiation provide evidence of strong geographic partitioning among type I and among type II/III populations (Table 3). These populations (Mouvanga Creek, Okano River, and Loa-Loa rapids) are separated by hundreds of river-kilometers (Fig. 2). Among these distant sites, statistically significant results of exact tests were found at all individual loci for both the type I and type II/III comparisons (exact test P -values < 0.00001 in all 30 tests). With only three exceptions (not shown), single-locus F_{ST} estimates are also significant for the same allopatric comparisons (F_{ST} -values > 0.023 ; P -values for $F_{ST} < 0.0075$; see Fig. 7).

Samples of either type I or type II from sites only a few river-kilometers apart in the Makokou region (Loa-Loa Rapids, Bialé Stream, and Balé Creek) show no evidence of genetic differentiation in multilocus tests (F_{ST} -values = 0; P -values for $F_{ST} > 0.10$; exact test P -values > 0.13). This pattern is largely the same, even without the application of Bonferroni corrections, when each locus is examined alone (not shown). The only four exceptions are: (1) type I, Loa-Loa versus Bialé Stream, locus NBB001, exact test $P = 0.03337$, F_{ST} not significant; (2) type I, Bialé Stream versus Balé Creek, locus NBB001, exact test $P = 0.01243$, F_{ST} not significant; (3) type II, Bialé Stream versus Balé Creek, locus NBB004, $F_{ST} = 0.010$ ($P = 0.02934$), exact test not significant; and (4) type II, Bialé Stream versus Balé Creek, locus NBB005, $F_{ST} = 0.009$ ($P = 0.04580$), exact test not significant.

We also sampled Biroundou Creek, which harbors type II to the near exclusion of type I (Fig. 2). This site affords us the opportunity to make a comparison of intermediate geographic scale with type II samples from Mouvanga Creek, which is located a few tens of river-kilometers away but is separated by a significant waterfall barrier at Bongolo (Fig. 2). We find robust genetic differences between these two populations when all five loci are considered together (Table 3) or when any single locus is considered alone (results not shown).

Bayesian Assignment Tests

Five-locus genotypes do not resolve alternate signal morphs in sympatry when the model-based clustering algorithm of Pritchard et al. (2000) is applied to the data. However, this Bayesian approach does clarify the geographical nature of population structure among individuals in the *mag-*

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 FIG. 7. Histograms of microsatellite allele frequencies at five loci (columns) in four syntopically collected *Brienomyrus* signal forms (rows) sampled from each of three different sites or regions: (A) Mouvanga Creek near Mbinambi Village; (B) the Okano River in the vicinity of the Village Na; and (C) the Makokou region of the Ivindo River. The number of individual fish genotyped and plotted is given by N for each sample. Within columns, all histograms are aligned by fragment size. An asterisk in each histogram for type II at Mouvanga Creek indicates the allele originally cloned for microsatellite development.

TABLE 3. Microsatellite evidence of genetic differentiation between pairs of populations. Comparisons among four sympatric signal forms collected in each of three different river systems are shown, as are comparisons between allopatric populations of type I, type II/III, or *Brienomyrus* sp. *bpl*. In each case, the table provides sample sizes (number of individuals genotyped), the five-locus F_{ST} estimate, the P -value for that estimate, and the P -value resulting from a five-locus exact test of genic differentiation between samples. Significance at the $P < 0.05$ level is indicated by one asterisk, and significance at the Bonferroni-corrected $P < 0.0045$ level is indicated by two asterisks.

Pairwise comparison between EOD forms (or locations)	Sample sizes	Five-locus F_{ST}	F_{ST} P -value	Five-locus exact test P -value
Sympatric comparisons				
<i>bpl</i> vs. <i>sn3</i> (Mouvanga Creek)	24 vs. 8	0.302	<0.00001**	<0.00001**
<i>bpl</i> vs. type I (Mouvanga Creek)	24 vs. 46	0.296	<0.00001**	<0.00001**
<i>bpl</i> vs. type II (Mouvanga Creek)	24 vs. 40	0.276	<0.00001**	<0.00001**
<i>sn3</i> vs. type I (Mouvanga Creek)	8 vs. 46	0.229	<0.00001**	<0.00001**
<i>sn3</i> vs. type II (Mouvanga Creek)	8 vs. 40	0.219	<0.00001**	<0.00001**
type I vs. type II (Mouvanga Creek)	46 vs. 40	0	0.26596	0.15520
<i>cab</i> vs. <i>curvifrons-like</i> (Okano River)	43 vs. 31	0.192	<0.00001**	<0.00001**
<i>cab</i> vs. type I (Okano River)	43 vs. 18	0.273	<0.00001**	<0.00001**
<i>cab</i> vs. type III (Okano River)	43 vs. 18	0.288	<0.00001**	<0.00001**
<i>curvifrons-like</i> vs. type I (Okano River)	31 vs. 18	0.156	<0.00001**	<0.00001**
<i>curvifrons-like</i> vs. type III (Okano River)	31 vs. 18	0.174	<0.00001**	<0.00001**
type I vs. type III (Okano River)	18 vs. 18	0	0.70430	0.58389
<i>cab</i> vs. <i>ten</i> (Makokou Region of the Ivindo River)	23 vs. 14	0.199	<0.00001**	<0.00001**
<i>cab</i> vs. type I (Makokou Region)	23 vs. 89	0.151	<0.00001**	<0.00001**
<i>cab</i> vs. type II (Makokou Region)	23 vs. 82	0.150	<0.00001**	<0.00001**
<i>ten</i> vs. type I (Makokou Region)	14 vs. 89	0.161	<0.00001**	<0.00001**
<i>ten</i> vs. type II (Makokou Region)	14 vs. 82	0.161	<0.00001**	<0.00001**
type I vs. type II (Makokou Region)	89 vs. 82	0.002	0.03151*	0.32528
Allopatric comparisons				
type I (Mouvanga Creek vs. Okano River)	46 vs. 18	0.259	<0.00001**	<0.00001**
type I (Mouvanga Creek vs. Loa-Loa Rapids)	46 vs. 30	0.165	<0.00001**	<0.00001**
type I (Okano River vs. Loa-Loa Rapids)	18 vs. 30	0.174	<0.00001**	<0.00001**
type II (Mouvanga Creek vs. Loa-Loa Rapids)	40 vs. 32	0.144	<0.00001**	<0.00001**
type II (Mouvanga) vs. type III (Okano)	40 vs. 18	0.240	<0.00001**	<0.00001**
type II (Loa-Loa Rapids) vs. type III (Okano River)	32 vs. 18	0.188	<0.00001**	<0.00001**
type II (Mouvanga Creek vs. Biroundou Creek)	40 vs. 29	0.143	<0.00001**	<0.00001**
<i>bpl</i> (Mouvanga Creek vs. Bambomo Creek)	24 vs. 30	0.268	<0.00001**	<0.00001**

nostipes complex. This method generates outcomes in which one cluster or population ($K = 1$) is most likely wherever we combine type I and type II/III within a site (Table 5). One cluster is also the result when samples from Loa-Loa Rapids, Bialé Stream, and Balé Creek are combined across the Makokou region. Weak evidence of higher order cross-

teering (e.g., $P(K|X) = 0.118$ for $K = 2$ in Bialé Stream) is not associated with any clear divisions by EOD type (assignments not shown). On the other hand, combining all *mag-nostipes*-complex samples across all localities yields $K = 5$ as the most likely number of clusters (Table 5). Mean probabilities of individual membership of 15 a priori groups (de-

TABLE 4. Evidence of genetic differentiation between syntopically collected type I and type II EOD morphs at each of three localities (columns) within the Makokou region of the Ivindo River. For each comparisons, the table gives numbers of individuals (N , first row), per locus F_{ST} estimates (with corresponding P -values), and P -values from exact tests of genic differentiation at each locus. Results of five-locus tests appear in the bottom row. Significance at the $P < 0.05$ level is indicated by one asterisk, and significance at the Bonferroni-corrected $P < 0.0045$ level is indicated by two asterisks.

Makokou region site	Type I vs. type II Loa-Loa Rapids	Type I vs. type II at Bialé Stream	Type I vs. type II Balé Creek
Sample sizes (N)	30 vs. 32	20 vs. 26	35 vs. 20
Locus NBB001 F_{ST}	0 ($P = 0.38383$)	0.015 ($P = 0.01927^*$)	0 ($P = 0.18597$)
Locus NBB001 exact test	$P = 0.85218$	$P = 0.00013^{**}$	$P = 0.11032$
Locus NBB002 F_{ST}	0 ($P = 0.49711$)	0 ($P = 0.70625$)	0 ($P = 0.67518$)
Locus NBB002 exact test	$P = 0.15505$	$P = 0.36981$	$P = 0.84171$
Locus NBB003 F_{ST}	0.006 ($P = 0.04693^*$)	0 ($P = 0.13433$)	0 ($P = 0.99998$)
Locus NBB003 exact test	$P = 0.32014$	$P = 0.10090$	$P = 0.95203$
Locus NBB004 F_{ST}	0 ($P = 0.98533$)	0 ($P = 0.17612$)	0 ($P = 0.10140$)
Locus NBB004 exact test	$P = 0.95162$	$P = 0.18324$	$P = 0.00236^{**}$
Locus NBB005 F_{ST}	0 ($P = 0.95847$)	0 ($P = 0.19824$)	0 ($P = 0.93435$)
Locus NBB005 exact test	$P = 0.94705$	$P = 0.96476$	$P = 0.94488$
Five-locus F_{ST}	0 ($P = 0.60264$)	0.007 ($P = 0.01054^*$)	0.002 ($P = 0.05642$)
Five-locus exact test	$P = 0.76859$	$P = 0.00183^{**}$	$P = 0.07289$

TABLE 5. Posterior probabilities of different numbers of populations (K) inferred for combined samples (type I + type II and/or type III) using the Bayesian clustering method implemented in Structure v2.0. Probabilities, $P(K|X)$, where X denotes the genotypes of sampled individuals, assume a uniform prior on $K = \{1, \dots, 6\}$. These are shown for samples from: single localities (Mouvanga Creek, Okano River, Loa-Loa Rapids, Bialé Stream, and Balé Creek); across the entire study region (including Biroundou Creek); and across the Makokou region only. The most likely K in each case is indicated in boldface type.

K	Posterior probability						
	Mouvanga Creek	Okano River	Loa-Loa Rapids	Bialé Stream	Balé Creek	All <i>magnostipes</i> -complex populations combined (including Biroundou Creek)	Makokou populations combined (Loa-Loa, Bialé, Balé)
1	0.986	0.702	0.994	0.644	0.972	0.000	1.000
2	0.000	0.001	0.004	0.118	0.028	0.000	0.000
3	0.000	0.029	0.000	0.001	0.000	0.000	0.000
4	0.014	0.234	0.000	0.000	0.000	0.000	0.000
5	0.000	0.017	0.000	0.000	0.000	0.957	0.000
6	0.000	0.017	0.002	0.237	0.000	0.043	0.000

fined on the basis of EOD type and site) in each of these clusters indicate no breakdown by signal morph (Table 6). Rather, they show clear geographical partitioning among four sites or regions: Okano River, Mouvanga Creek, Biroundou Creek, and the Makokou region. Individual assignments provide no convincing evidence of geographically admixed individuals. Two of the five derived clusters lie within the Makokou region, but these are not associated with any divisions between type I and type II or among sample sites within this area.

DISCUSSION

Reproductive Isolation in the Briemomyrus Species Flock

Deviations from genetic panmixia can be used to infer reproductive isolation among sympatric forms (e.g., Lu and Bernatchez 1999). Six of the populations we investigated are well differentiated from sympatric congeners at four to five (of five) unlinked microsatellite loci, including: *bp1* and *sn3* from Mouvanga Creek, *cab* and *Briemomyrus* cf. *curvifrons* from the Okano River, and *cab* and *ten* from the Ivindo River. Mutation and drift can cause genetic divergence if gene flow between lineages becomes sufficiently low. In this case drift is expected to affect all neutral nuclear loci with equal probability, which is the pattern seen among these electric fish species across several, anonymously cloned microsatellites (cf. Emelianov et al. 2004). Robust differentiation across microsatellites is noteworthy for some of the good *Briemomyrus* species examined considering the close similarities of their mitochondrial genomes. In particular, cytochrome *b* sequence divergence between *ten* and the two *magnostipes*-complex morphs in the Ivindo River ranges from 0.18 to 0.44%, while most specimens of *sn3* and *bp1* from Mouvanga Creek exhibit only 0–0.09% sequence divergence (Sullivan et al. 2002). These species exhibit among the least amount of cytochrome *b* divergence in the flock, possibly due to the effects of mitochondrial introgression (Sullivan et al. 2004). Sympatric morphological differences appear to characterize all EOD forms of the Gabon-clade except the *magnostipes*-complex morphs. Given this observation and previous findings based on other molecular markers (Sullivan et al. 2002, 2004), it is probable that all of the many morphologically

distinct EOD forms in this recently discovered species flock are reproductively isolated from one another in sympatry.

Population Structure of the Magnostipes Complex

The same five loci reveal remarkable genetic similarity between type I and type II/III wherever they are sympatric. Slight, but statistically significant, differences between samples of type I and type II only occur in the Makokou region of the Ivindo River, yet with some inconsistencies among tests. Significant F_{ST} estimates in this region range from 0.002 (multilocus; all Makokou region samples pooled) to 0.015 (locus NBB001; Bialé Stream only). These values are much lower than those found among other closely related forms of fish that exhibit behavioral evidence of isolation ranging from at least moderate assortative mating to rather complete premating reproductive isolation (van Oppen et al. 1998; Taylor and McPhail 2000; Wilson et al. 2000; Schliewen et al. 2001; Barluenga and Meyer 2004). Makokou region sites also offer some evidence of heterozygote deficiencies in pooled samples of type I + type II, but only for a single locus that differs at each of two field sites. Heterozygote deficiencies in these pooled samples could be the result of EOD-based assortative mating, although other potential causes exist, such as the undetected presence of null alleles (Morand et al. 2002). Despite these population-level differences, Bayesian assignment of individual genotypes to signal type is no better than random within any locality. Thus, the microsatellites developed for this study would not appear to be closely linked to any genes underlying signal differences or experiencing divergent selection between sympatric morphs. Assuming EODs were associated with reproductive isolation in the *magnostipes* complex, which appears to be the case for the rest of the species flock based on our findings, such linkage would be expected to yield more accurate genotypic assignment of individuals to signal type. If disruptive selection or drift now acts differently between sympatric *magnostipes*-complex morphs of the Makokou region, either its effect on the genotyped microsatellites has just begun or its outcome has been negligible.

All tests yield a consistent picture of geographical population structure in the *magnostipes* complex—one that con-

TABLE 6. Average probabilities of ancestry of predefined samples (i.e., electric signal morph/location) in each of five “populations” or clusters ($K = 5$), the number found to be most likely across the entire Ogooué basin (see Table 5). Sample sizes for predefined groups (rows) are indicated by N . Maximum mean probability for each a priori group is given in boldface type.

Predefined sample (signal morph/location)	N	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Type I/Balé Creek	35	0.497	0.470	0.015	0.006	0.012
Type II/ Balé Creek	20	0.508	0.448	0.015	0.009	0.020
Type I/Bialé Stream	20	0.382	0.573	0.019	0.006	0.020
Type II/Bialé Stream	26	0.552	0.419	0.018	0.005	0.006
Type I/Loa-Loa Rapids	30	0.512	0.464	0.010	0.005	0.009
Type II/Loa-Loa Rapids	32	0.506	0.458	0.013	0.009	0.014
Type I/Ivindo River at I.R.E.T.	2	0.281	0.629	0.007	0.006	0.077
Type II/Ivindo River at I.R.E.T.	3	0.180	0.725	0.079	0.005	0.011
Type I/Nyamé-Pendé	2	0.634	0.347	0.006	0.005	0.008
Type II/Nyamé-Pendé	1	0.501	0.480	0.005	0.005	0.009
Type I/ Mouvanga Creek	46	0.005	0.006	0.977	0.004	0.008
Type II/ Mouvanga Creek	40	0.007	0.012	0.960	0.007	0.014
Type I/Okano River	18	0.005	0.005	0.003	0.982	0.005
Type III/Okano River	18	0.005	0.006	0.003	0.979	0.007
Type II/Biroundou Creek	29	0.014	0.011	0.023	0.006	0.946

trasts sharply with the genetic similarities in sympatry. Sites in the Makokou region are very weakly spatially structured, if at all. These sites lie only a few kilometers apart and are not separated by any obvious barriers to fish dispersal. On the other hand, levels of microsatellite divergence among Mouvanga Creek, Biroundou Creek, Okano River, and the Makokou region rival those noted among coexisting, good *Brienomyrus* species. These sites/regions are separated by tens to hundreds of river kilometers and by waterfall barriers, such as the major cataracts (Mingouli and Kongou; not shown in Fig. 2) that isolate the Makokou region and upper Ivindo from the rest of the Ogooué basin. These kinds of habitat discontinuities are a common topographical feature of the untamed Ogooué.

Additional evidence of the importance of waterfalls comes from *Brienomyrus* sp. *bp1*, which is not a member of the *magnostipes* complex (Sullivan et al. 2004). Strong genetic partitioning exists between populations of *bp1* at Bambomo and Mouvanga Creeks (Table 3), most likely due to the isolating effects of tens of river kilometers and at least one waterfall (indicated on a topographical map of this region and reproduced in Fig. 2). Not unexpectedly, therefore, waterfall barriers appear to have played a generally important role in promoting allopatric divergence within the *Brienomyrus* species flock of Gabon—much like the role that sand and deep water play, albeit on a much finer scale, in the isolation of rock-dwelling cichlid populations in the Lake Malawi species flock (van Oppen et al. 1997; Arnegard et al. 1999; Rico and Turner 2002).

Ontogenetic and Geographic Signal Variation

All electrical recordings in the present report correspond to adult (i.e., postlarval) electric organs. When very small (< 40 mm SL), type I and type III juveniles from the Okano River produce indistinguishable EODs that are similar in waveform to those of type II adults from the Ivindo River. This is also true for type I–type II pairs from Mouvanga Creek and the Ivindo River itself (M. E. Arnegard, pers. obs.). Subsequent postlarval EOD changes are more dramatic in type

I than in type II. The amplitude of the initial head-negative peak increases with growth in type I (Fig. 4), implying slow and continued electrocyte stalk growth (Bass 1986b). At the same time, the second head-negative peak shrinks (possibly due to the increasing influence of stalk currents on the EOD), until the type I waveform fully inverts by the time individuals reach 60- to 70-mm SL. A dramatic postlarval transformation also occurs in type III, although its EOD never fully inverts (i.e., the EOD waveform produced by type III adults retains a second negative peak). Elongated EODs are found in breeding males of all three signal morphs around the onset of the major rainy season (September–November). This androgen-dependent sex difference in EODs has been noted in numerous mormyrid species.

Despite strong evidence of genetic divergence among geographically isolated populations, type I does not exhibit discrete variation in EOD waveform from region to region. Conversely, populations of the alternate morphs (i.e., types II and III) exhibit measurable geographic variation in waveform, which might be expected given the degree of isolation among regions. Nevertheless, in spite of all ontogenetic and geographic sources of signal variation, we have never identified cases of intermediate EODs between coexisting type I and type II/III adults. Below, we outline two nonexclusive hypotheses concerning the origin and maintenance of heterotypic signal variation in the *magnostipes* complex given what we know about the biology of these animals.

Hypothesis 1: Persistent Dimorphisms in Electric Organ Discharge

Considering the striking genetic similarity of focal morphs in sympatry, the best supported conclusion to be drawn for our microsatellite results is that type I and type II/III are conspecific signal types comprising undifferentiated gene pools in each regionally defined and phenotypically polymorphic population. Genetic polymorphisms can be maintained by influences such as negative frequency dependent selection, selection regimes that vary in space or time, differential selection between the sexes, antagonistic pleiotropy,

or an advantage to heterozygotes (Levene 1953; Haldane and Jayakar 1963; Kidwell et al. 1977; Hedrick 1986; Curtsinger et al. 1994; Ravigné et al. 2004; Turelli and Barton 2004). Condition-dependent selection can give rise to alternate mating tactics, which may be associated with discrete signal differences (Gross 1996). Phenotypic plasticity and environmental influences on developmental switchpoints have also been described for a variety of animal signals and ornaments (Emlen 1994; Brakefield et al. 1996; Jia et al. 2000).

In 2002, we examined gonads of numerous type II and type I individuals during the period of heaviest rains in the Makokou region (September–November). These observations confirmed that both morphs comprise gravid females and mature males in roughly equal proportions (in this region) and that reproductives of both sexes of the type II morph coexist in the same microhabitats (and at the same time) with those of the type I morph. Congruent with our genetic results, an apparent lack of appreciable microhabitat partitioning is consistent with the presence of persistent EOD dimorphisms in each of several panmictic gene pools. A strict association between signal types and alternative mating tactics would appear unlikely since these kinds of tactics are almost always sex-limited (Andersson 1994; Gross 1996; Ruppell and Heinze 1999; Rhen and Crews 2002). Nevertheless, the possibility that alternative reproductive tactics are expressed in both sexes (Sinervo and Zamudio 2001) deserves attention in future behavioral and ecological studies of this system of polymorphic electric fishes. Although environmental effects on the earliest stages of postlarval waveform development have not been studied, it appears that EODs remain fixed once discernible heterotypic divergence is achieved between morphs.

We have not yet explored the possibility that spatially or temporally varying selection acts on the alternate signal morphs. Varying selection pressures can be extremely fine scaled, yet they can still be evolutionarily significant and maintain signal polymorphisms (e.g., Svensson and Sinervo 2004). Frequency-dependent predator-prey interactions can also play an important role in the evolution of trait polymorphisms (Hori 1993; Bond and Kamil 2002). Predatory catfishes are equipped with ampullary electroreceptors tuned to low electrical frequencies (Lissmann and Machin 1963). Hanika and Kramer (2000) have demonstrated that a species of *Clarias* catfish can detect relatively low-frequency mormyrid EODs. In all likelihood, however, the focal morphs evade electrical eavesdropping by catfishes owing to the spectral composition of *magnostipes*-complex EODs (e.g., see fig. 12 of Bass and Hopkins 1985). While the Ogooué River basin is free of piscivorous mormyrids large enough to prey on fully grown type I and type II/III adults, other mormyrid species (whose feeding habits remain unknown) could conceivably exert EOD-mediated selection on *magnostipes*-complex juveniles.

A pair of *magnostipes*-complex EOD types occurs under the same (i.e., syntopic) environmental conditions at several locations, yet only a single morph emerges in, or at least inhabits, others (Fig. 2). This pattern of distribution may reflect genetically determined signal differences, with only one EOD type occurring at some sites due to colonization by a single morph or subsequent loss of alleles from those

populations. Other explanations for this pattern are spatio-temporally variable selection regimes or signal plasticity, the effects of which could not be detected during our field efforts or the subsequent housing of live specimens. Our unexpected discovery of genetically panmictic EOD morphs in sympatry raises numerous questions that can only be addressed by subsequent investigation. Some answers will be found through breeding studies aimed at determining the environmental and genetic underpinnings of EOD differentiation. Field investigations are also needed to explore the possibility that spatio-temporally variable selection has generated and continues to maintain the observed dimorphisms in EOD waveform.

Hypothesis 2: Early Stages of Incipient Sympatric Speciation between Morphs

Two lines of evidence and a well-studied central nervous system mechanism suggest a general role for EODs in species isolation in the Gabon *Brienomyrus* flock. First, among the two dozen or more morphologically distinct species that have already been discovered, each one produces a different, species-typical EOD waveform (Hopkins 1980; Sullivan et al. 2002; Arnegard and Hopkins 2003). Second, in situ electrical playback experiments have demonstrated EOD-mediated species recognition in the context of courtship in *Brienomyrus* sp. *vad* (Hopkins and Bass 1981). Lastly, the EODs of type I and type II/III appear to differ sufficiently for waveform discrimination via a neural pathway described by Xu-Friedman and Hopkins (1999; and references therein). Therefore, instances of dramatic signal difference between sympatric morphs appear somewhat paradoxical in this group of fishes when genetic evidence for reproductive isolation between them is lacking.

Divergence at only a few loci is required for speciation (Ritchie and Phillips 1998; Orr 2001; Broughton and Harrison 2003). While gene flow between incipient species may be effectively prevented at genes under selection, it may be relatively unimpeded throughout much of the genome (Harrison 1991; Wang et al. 1997; Ting et al. 2000; Emelianov et al. 2004). If alternate EODs are, in fact, associated with nascent reproductive isolation within *magnostipes*-complex populations, five microsatellites might fail to detect the very earliest effects selection or drift due to a combination of porous species boundaries, recombination, and/or large population sizes.

If genes are found to play an overarching role in determining signal fate, the lack of intermediate EOD waveforms between type I and type II/III in sympatry would imply either a relatively simple Mendelian basis to the signal differences (with or without hybridization) or a more complicated polygenic architecture with very little gene flow between sympatric morphs. Behavioral tests of assortative mating, examination of artificially produced hybrid signals, and application of additional molecular markers are warranted to better examine the possibility of incipient speciation between signal types. Evidence of weak genetic differentiation between type II and type I in the Makokou Region points to this population, in particular, for further testing. If evidence of reproductive isolation is found, the genetic similarity of syntopic morphs would suggest a more recent beginning to speciation than

has occurred between notable examples of nascent fish species in postglacial lakes of the Holarctic region or in certain crater lakes of Cameroon (Taylor and McPhail 2000; Schlieven et al. 2001).

Striking genetic similarity between coexisting morphs provides the signature of a fully sympatric process, whether it involves incipient speciation or the maintenance of phenotypic dimorphism by some other mechanism. An additive, polygenic signal architecture, if found, would cast doubt on the viability of a scenario of postdivergence hybridization (upon secondary contact) as an alternate explanation for the genetic patterns we describe. Under such an architecture, introgression sufficient to homogenize allele frequencies across neutral loci would have almost certainly led to a breakdown of signal differences, rather than the discrete signal classes we observe.

Ecological selection plays the key role in several prevailing models of sympatric speciation or reinforcement upon secondary contact (e.g., Schluter and Nagel 1995; Kondrashov and Kondrashov 1999; Kirkpatrick 2001; Schluter 2001; Drès and Mallet 2002; Doebeli and Dieckmann 2003). However, the morphologically cryptic signal forms examined in this study exhibit no obvious signs of ecomorphological divergence like those described by Skúlason and Smith (1995). Due to the spectral similarity of EOD waveform types in the *magnostipes* complex (M. E. Arnegard and C. D. Hopkins, pers. obs.), we do not expect that selection for the electrolocation of different targets has operated on the focal morphs. By contrast, disruptive selection on acoustic signals functioning in both echolocation and mate recognition appears to have occurred between some species of horseshoe bats (Kingston and Rossiter 2004). Arnegard and Kondrashov (2004) reason that relatively cryptic initial responses to ecological selection (e.g., behavioral resource partitioning) can accompany sympatric divergence in species-packed communities, such as the electric fish assemblage that inhabits Loa-Loa rapids. In addition, other causes of divergence under gene flow (e.g., Gavrilets and Waxman 2002; Seehausen and Schluter 2004) might not lead to immediate changes in external morphology and should not be ruled out for this intriguing system.

Phylogenetic and Geographical Considerations

All three morphs of the *magnostipes* complex express type Pa electrocytes, which have penetrating stalks with anterior innervation (Alves-Gomes and Hopkins 1997; Lavoué et al. 2000; Sullivan et al. 2000). In the best resolved tree available to date—the AFLP-based phylogeny of Sullivan et al. (2004)—the immediate outgroups to the *magnostipes* complex exhibit type NPp electrocytes (i.e., nonpenetrating stalks and posterior innervation). Therefore, Pa appears to be the derived electrocyte type for the *magnostipes* complex (Wiley 1981). Pa electrocyte anatomy is not unique to the *magnostipes* complex but also occurs in other lineages within the *Brienomyrus* species flock of Gabon (Sullivan et al. 2004). All EOD waveforms produced by Pa electrocytes have an initial head-negative peak, whereas those produced by NPp electrocytes lack an initial negativity (Bennett 1971; Bass 1986c). The three *magnostipes*-complex waveforms (types I,

II, and III) vary in the relative size of their initial head-negative peak. Due to the absence of an initial negativity in the EODs of the immediate outgroups and to a lack of phylogenetic resolution within the *magnostipes* complex, however, it is not possible to determine which EOD waveform (type I, type II, or type III) is primitive within the complex.

Geographically isolated populations of the *magnostipes* complex are also genetically isolated. Among *magnostipes*-complex EODs, the type I waveform is the most geographically widespread (Fig. 2) and does not vary appreciably in waveform from region to region. The alternate EOD waveforms (type II and type III) are geographically more restricted. Either of these EODs can be found together with type I, but each one can also occur alone. Across the Ogooué River basin, type II and type III EODs exhibit obvious and quantifiable waveform variation, yet patterns of distribution and signal variation cannot establish the evolutionary polarity of EODs. Geographical signal variation in the *magnostipes* complex is consistent with type I being ancestral and types II and III being derived. However, it is equally consistent with multiple origins of type I due to recurrence (West-Eberhard 2003) or developmental bias (McCune and Carlson 2004) that may or may not be coupled with parallel effects of selection in isolated populations (Schluter and Nagel 1995). Why geographic variation appears strong for type II and type III EODs but negligible for type I remains a pivotal question for the *magnostipes* complex. Regardless of the answer, repeated instances of dimorphism in an electrical communication channel offer a replicate system in which influences maintaining discrete signal variation in sympatry (or factors possibly driving divergence) can be studied within multiple populations.

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