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AFLPs RESOLVE PHYLOGENY AND REVEAL MITOCHONDRIAL INTROGRESSION WITHIN A SPECIES FLOCK OF AFRICAN ELECTRIC FISH (MORMYROIDEA: TELEOSTEI)

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Abstract.—Estimating species phylogeny from a single gene tree can be especially problematic for studies of species flocks in which diversification has been rapid. Here we compare a phylogenetic hypothesis derived from cytochrome *b* (cyt *b*) sequences with another based on amplified fragment length polymorphisms (AFLP) for 60 specimens of a monophyletic riverine species flock of mormyrid electric fishes collected in Gabon, west-central Africa. We analyze the aligned cyt *b* sequences by Wagner parsimony and AFLP data generated from 10 primer combinations using neighbor-joining from a Nei-Li distance matrix, Wagner parsimony, and Dollo parsimony. The different analysis methods yield AFLP tree topologies with few conflicting nodes. Recovered basal relationships in the group are similar between cyt *b* and AFLP analyses, but differ substantially at many of the more derived nodes. More of the clades recovered with the AFLP characters are consistent with the morphological characters used to designate operational taxonomic units in this group. These results support our hypothesis that the mitochondrial gene tree differs from the overall species phylogeny due at least in part to mitochondrial introgession among lineages. Mapping the two forms of electric organ found in this group onto the AFLP tree suggests that posteriorly innervated electrocytes with nonpenetrating stalks have independently evolved from anteriorly innervated, penetrating-stalk electrocytes at least three times.

Key words.—Amplified fragment length polymorphism, cytochrome *b*, electric fish, gene trees, hybridization, signal evolution.

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The riverine Gabon-clade *Brienomyrus* of central Africa, a recently discovered fish species flock (Sullivan et al. 2002), is a compelling system in which to study competing models of speciation and the diversification of ecologies, morphologies, behaviors, and signals. Unlike the cichlid species flocks of Africa (Schliewen et al. 1994; Kornfield and Smith 2000) in which visual cues mediate reproductive isolation among sympatric species (Seehausen et al. 1997), these mormyrid fishes use a complex electric sense to recognize mates, sense prey, and locate objects in the nocturnal environment in which they are active (Hopkins 1986). Understanding the relationship between speciation and phenotypic diversification in any group requires knowledge of species phylogeny. However, the prolific nature of speciation within species flocks poses particular difficulties for phylogenetic reconstruction with molecular markers.

Because the phylogenies of species lineages can differ from the genealogy of sampled alleles at any given genetic locus, single-locus hypotheses of species phylogeny can be misleading (Pamilo and Nei 1988; Doyle 1992, 1997; Moore 1995; Brower 1996; Maddison 1997; Slowinski and Page 1999; Sota 2002). Here we take the view, expressed by Maddison (1996) and others, that species phylogeny can be thought of as a ''cloudogram'' formed by the central tendency among all the gene trees represented in the genomes of the species under study, not all of which have identical topologies. Phylogenies of species flocks often may be particularly ''cloudy'' because diversification may outpace the fixation of alleles within populations and incomplete barriers to hybridization may allow some degree of genetic introgression among forms. In these cases, species phylogeny will be best estimated by assessing genealogical concordance (Avise and Ball 1990; Avise 2000) among many independently segregating loci.

In our first attempt to investigate species-level phylogeny of the Gabon-clade *Brienomyrus* with mitochondrial cytochrome *b* (cyt *b*) sequences (Sullivan et al. 2002), we found that few of the entities we identified as putative species, or higher-level groups of species, corresponded to monophyletic (or otherwise coherent) groups of haplotypes. Instead, collection site was often a better predictor of haplotype affinities than were the phenotypes of the fish from which they were sampled, suggesting local introgression of the mitochondrial genome across forms. Other parts of the cyt *b* tree were more suggestive of incomplete lineage sorting among the mitochondrial haplotypes. However, because no independent estimate of phylogeny was available for these fishes, we were unable to rule out the possibility that unnatural (i.e., polyphyletic) operational taxonomic units (OTUs) and widespread convergent evolution contributed to these patterns. While failing to produce a convincing species phylogeny, the cyt *b* analysis supported the monophyly of the Gabon-clade *Brienomyrus* and the low level of haplotype divergence among species suggested a recent origin for this species flock.

Apart from such studies of quickly evolving mitochondrial sequences—that, because of the nonrecombining nature of the mitochondrial genome, estimate a single gene tree—practical options for researchers wishing to investigate phylogeny within recently diversified groups are limited. Even relatively quickly evolving nuclear loci such as introns may not evolve at a sufficiently fast rate to be informative. Our own assay of several nuclear introns revealed limited allelic variability among Gabon-clade *Brienomyrus* lineages (Sullivan et al. 2002). Furthermore, for study groups in which incomplete

mitochondrial lineage sorting is suspected, far fewer fixed allelic differences between lineages are to be expected in neutral autosomal loci due to their fourfold greater effective population size (Avise 1994; Palumbi and Cipriano 1998). Phylogenetic inference from quickly evolving nuclear microsatellite loci is hampered by lack of consensus on how these regions evolve, and, as for allozyme data, how these data should be coded and analyzed for phylogenetic analysis (Kornfield and Parker 1997). Phylogenetic analysis of short interspersed element (SINE) retroposons (Hillis 1999; Shedlock and Okada 2000) has shown promise in cichlid fish species flocks (Takahashi et al. 1998, 2001a,b), but loci are relatively few, require substantial time for development, and are subject to lineage sorting problems (Terai et al. 2003).

Use of amplified fragment length polymorphism (AFLP) data for reconstructing phylogeny among closely related organisms emerged in the botanical and mycological literature (Kardolus et al. 1998; Caicedo et al. 1999; Labra et al. 1999; Baayen et al. 2000; Bakkeren et al. 2000; Hodkinson et al. 2000; van Raamsdonk et al. 2000; Zhang et al. 2001; Després et al. 2003) and has been increasingly applied in animal systems for which sequence-based studies have been inconclusive (Albertson et al. 1999; Giannasi et al. 2001; Parsons and Shaw 2001; Buntjer et al. 2002; Allender et al. 2003; Seehausen et al. 2003). The AFLP technique uses PCR to amplify specific subsets of restriction fragments from whole genome digests that are then resolved by gel or capillary electrophoresis (Vos et al. 1995; Mueller and Wolfenbarger 1999). The specificity is achieved by PCR primers that extend one or more bases into the unknown portion of the restriction fragments and thus limit amplification to those that match. Varying these primers and their combination generates independent sets of anonymous, replicable markers derived from loci distributed across the genome. Homology of AFLP bands across samples is assumed if they have equal electrophoretic mobility; studies that have sequenced and mapped comigrating AFLPs have shown them predominantly to be homologues, at least when AFLP profiles of closely related organisms are compared (Waugh et al. 1997; O'Hanlon and Peakall 2000; Parsons and Shaw 2001). Importantly for phylogenetic analysis, most AFLP fragments amplified by a particular primer combination are independent, unlinked markers. Because only relatively short AFLP fragments (50–625 bp in this study) are scored, a substitution within a restriction site will usually result in the absence of all fragments from that locus in the AFLP profile (Albertson et al. 1999). Origin of a new restriction site within an amplifiable fragment will usually have the same effect, because the flanking bases will infrequently match the selective PCR primers. Indels evolving inside fragments can produce nonindependent bands, but a recent study found the percentage of such codominant AFLPs to be less than 10% of the total (Parsons and Shaw 2002). While a single AFLP character is no more likely to be phylogenetically informative than a nuclear character in a sequence-based study, the promise of the technique derives from the ability to sample hundreds or even thousands of independent loci.

In this study we assess the phylogenetic utility of AFLP characters in the Gabon-clade *Brienomyrus*. Given the incomplete taxonomic sampling of this study, our goal is not to provide a definitive phylogeny for the group, but to examine whether AFLP characters better support the monophyly and plausible higher-level grouping of OTUs than do cyt *b* haplotypes sampled from the same individuals. If they do, larger-scale application of AFLP studies to this system will be appropriate. We explore the implications of conflict between AFLP and mitochondrial trees and the evolutionary history of electric signals and electric organs in this group.

Background: Systematics of the Study Group and Electric Signals

Our field collections in the country of Gabon in west-central Africa (Fig. 1) and our previous molecular phylogenetic work convince us that current taxonomy misclassifies these fish and greatly underestimates species-level diversity (Sullivan et al. 2002). The monophyletic group of mormyrid fishes studied here includes three species described within the genus *Brienomyrus* Taverne (1971), a single species described as *Paramormyrops gabonensis* Taverne (1977a), and many additional undescribed forms. Sequence-based studies using two nuclear and three mitochondrial markers indicate that these species form a monophyletic group apart from *Brienomyrus brachyistius*, the type species of the genus, and also apart from *Brienomyrus niger*, which belongs to a third independent lineage (Alves-Gomes and Hopkins 1997; Lavoué et al. 2000, 2003; Sullivan et al. 2000). There are an additional five valid species of *Brienomyrus* (Daget et al. 1984; Harder 2000) that we have been unable to identify with certainty among our collections that may belong to this clade. Because they dominate the mormyrid fish fauna of Gabon, we informally refer to this group as the ''Gabon-clade *Brienomyrus*'' pending a taxonomic revision of this group that is in preparation.

All mormyrids produce weak pulastile electric discharges from an organ near the tail for the purposes of orientation and communication. These fishes modulate the repetition rate of the electric organ discharge (EOD) according to activity level and behavioral context, but the waveform characteristics of each EOD are fixed and encode information on the species identity and sex of the animal. EOD waveforms are stereotyped within a species but often differ markedly among sympatric species (Hopkins 1981, 1986; Arnegard and Hopkins 2003). The EOD waveforms of Gabon-clade *Brienomyrus* differ from each other in total duration (0.1 to 10 msec), the number of peaks (two to four), polarity, and fine structure (Hopkins 1999). Playback experiments suggest that these fish can determine the species identity and sex of nearby fish on the basis of EOD waveform cues alone (Hopkins and Bass 1981). In Gabon, many species of this group occur in syntopy. For example, at the Loa-Loa rapids of the Ivindo River near Makokou, Gabon, we collect 11 different forms together, each with a unique and recognizable EOD waveform. In constrast with the diversity of their electric signals, external morphological differences among species are often subtle, and little trophic diversification is evident in their dentition, snout, and jaw anatomies.

We can broadly classify the EOD waveforms in these fishes according to the presence or absence of an initial head-negative peak or phase to the waveform which correlates with

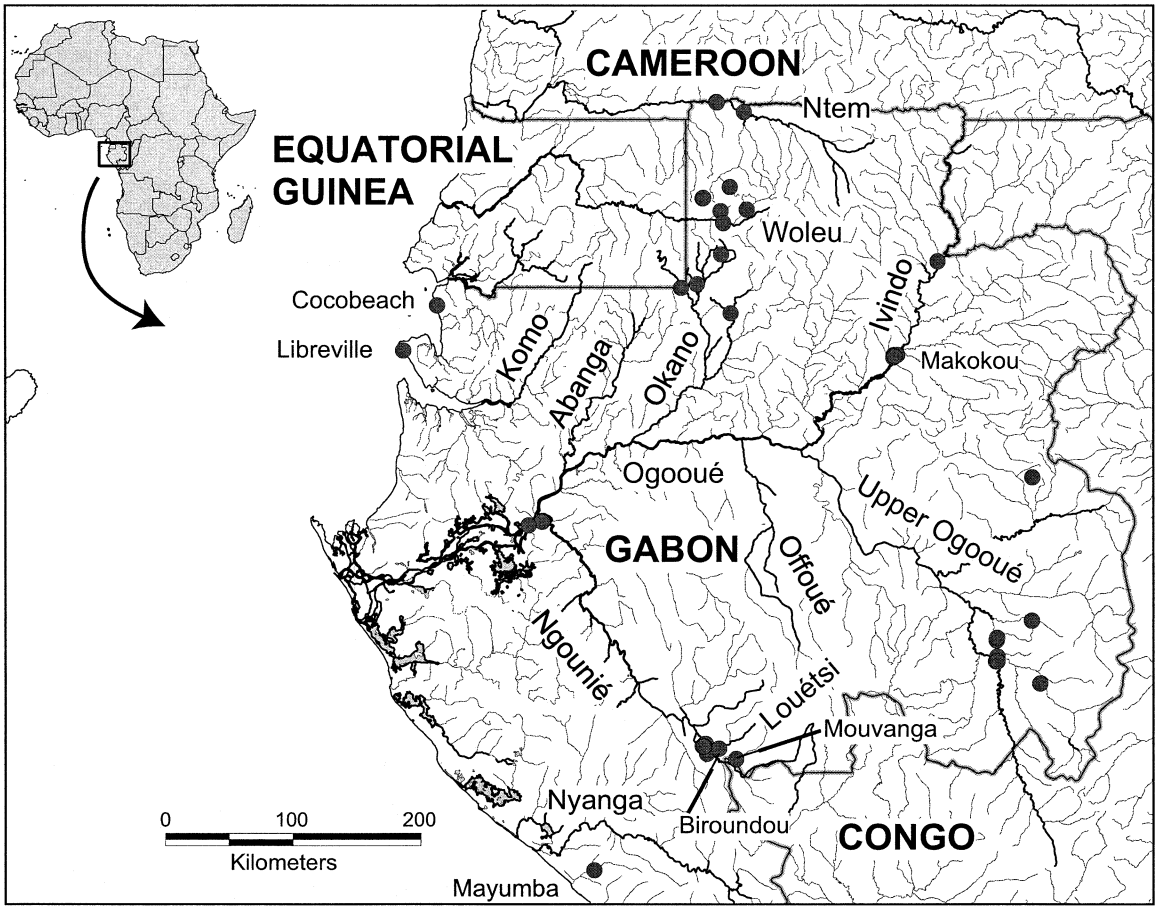

FIG. 1. Map of Gabon. Closed circles indicate collection localities of Gabon-clade *Brienomyrus* specimens used in this study. Localities listed in Table 1 and in Figure 2 are indicated.

a difference in the anatomy and innervation of electrically active stalks emerging from the posterior faces of electrocytes within the electric organ (Bennett and Grundfest 1961; Szabo 1961; Bennett 1970, 1971; Bass 1986; Alves-Gomes and Hopkins 1997; Lavoué et al. 2000; Sullivan et al. 2000, 2002). Species with this initial phase in their EODs have electrocytes with penetrating stalks and anterior innervation (type Pa), while those that lack this phase have electrocytes with nonpenetrating stalks and posterior innervation (type NPp). In fish with Pa electrocytes the initial head-negative peak is caused by the inward flow of current in the stalk being directed caudally through the electrocytes at the point where it penetrates through the electroplate membranes. The result is a head-negative prepotential (P_0) that precedes sequential activation of posterior and anterior faces of the cell that produce first a large head positive peak (P_1) and then a head negative peak (P_2). Parsimony reconstruction of the evolution of this electric organ character on the mormyrid phylogenetic tree (Sullivan et al. 2000; Lavoué et al. 2003) suggests that type Pa is the ancestral electrocyte character state for the Gabon-clade *Brienomyrus*.

MATERIALS AND METHODS

Specimens

We included 60 specimens of Gabon-clade *Brienomyus* in this study and one specimen each of the sequential outgroups *Marcusenius ntemensis* and *Pollimyrus marchei* (Lavoué et al. 2000, 2003; Sullivan et al. 2000). The 60 ingroup specimens include four OTUs that are described species and 28 provisional OTUs. We designated the latter OTUs when collected specimens differed in morphology and/or electric organ discharge (EOD) waveform from specimens of known, described species. While we believe many of these OTUs represent new species, in other cases these designations may have split single species into subspecific geographical variants. We recognize for the purpose of phylogenetic analysis that splitting single species into multiple OTUs is less problematic than mistaken lumping of nonsister lineages (Sullivan et al. 2002).

Specimens are listed in Table 1 and collection sites are mapped in Figure 1. Collection, OTU designation, EOD recording and DNA extraction methods are described by Sullivan et al. (2002). OKA 4150, SP9 4164, and *B. cf. curvifrons* 4149 represent new OTUs sampled from the Okano River in 2001 and these plus SN8 4210, CAB 4253, MAG 4250, and *P. gabonensis* 4185 are the only individuals not included in our earlier cyt *b* study (Sullivan et al. 2002). The OTU designations of all other included specimens follow those used in Sullivan et al. (2002), as do methods for polymerase chain reaction (PCR) and sequencing of the cyt *b* gene. Because we wished to avoid introducing possible error from cross-

TABLE 1. List of the 62 specimens used in this study, organized alphabetically by operational taxonomic unit (OTU). Specimen numbers, GenBank accession numbers for cytochrome *b* sequences, museum accession numbers, collection localities and total number of specimens of each OTU in our collections are indicated. All localities are within Gabon. Origin of OTU designation is indicated in third column. ''Blunt'' and ''sharp'' describe the snout profile in dorsal view; Pa and NPp refer to electrocyte morphology. Numerals distinguish unique forms with similar snout and electrocyte morphologies.

Specimen No.	OTU	OTU description	No.	Locality	GenBank	Museum no.
2050	*B. curvifrons*	described species	55	Ivindo R. near Makokou	AF477469	CU81661
4149	*B. cf. curvifrons*	similar but not identical to above		Okano R. near Mitzic	AY475209	AMNH231500
2285	*B. hopkinsi*	described species	24	Ivindo R. near Makokou	AF201575	CU78352
1611	*B. hopkinsi*	described species		Ntem R. at Bikondom	AF477420	CU80928
4210	*B. hopkinsi*	described species		Ntem R. at Ayengbé	AY475210	AMNH231522
2289	*B. longicaudatus*	described species	5	Ivindo R. near Makokou	AF201576	CU78355
2681	BN1	blunt, NPp, #1	87	Louétsi/Ngounié	AF477457	CU84579
2718	BN1			Louétsi/Ngounié	AF477459	CU84566
3542	BN2	blunt, NPp, #2	53	Upper Ogooué R. basin	AF477431	CU80474
2659	BON	manuscript abbrev.	44	Louétsi R., Ngounié R. basin	AF477471	CU84649
3358	BP1	blunt, Pa, #1	210	Coastal creek near Mayumba	AF477478	CU80520
2704	BP1			Mouvanga creek/Ngounié R.	AF477445	not catalogued
3771	BP1			Woleu R. basin	AF477477	CU80892
3547	BP6	blunt, Pa, #6	17	Woleu R. basin	AF477430	CU80476
4018	BX1	blunt, mixed, #1	30	Coastal creek near Cocobeach	AF477479	CU81264
2116	CAB	manuscript abbrev.	73	Ivindo River	AF477466	CU80816
3848	CAB			creek near Oyem, Ntem R. basin	AF477422	CU80893
4253	CAB			Okano R. near Mitzic	AY475211	AMNH231527
1844	IN1	intermediate, NPp, #1	15	Lower Ogooué R.	AF477439	CU80591
2427	LIB	from Libreville	15	Coastal creek near Libreville	AF477446	CU80867
3366	LIS	manuscript abbrev.	25	Upper Ogooué R.	AF477427	CU81090
2297	MAG	manuscript abbrev.	69	Ivindo R. near Makokou	AF477452	CU78326
2168	MAG			Ivindo R. near Makokou	AF477451	CU78323
3945	MAG			Ntem R. at Ayengbé	AF477415	CU80904
3999	MAG			creek near Oyem, Ntem R. basin	AF477416	CU80902
4250	MAG			Okano R. near Mitzic	AY475212	AMNH231526
2186	*Marcusenius ntemensis*	described species	21	Ivindo R. near Makokou	AF201593	CU79706
2710	NGO	from Ngounié River	11	Louétsi R., Ngounié R. basin	AF477464	CU84665
2643	OFF	from Offoué River	16	Louétsi R., Ngounié R. basin	AF477447	CU84667
3394	OFF			Upper Ogooué R.	AF477402	CU80526
4150	OKA	manuscript abbrev.	12	Okano R. near Mitzic	AY475213	AMNH231501
3461	PAR	manuscript abbrev.	42	Upper Ogooué R.	AF477419	CU80934
2048	*Paramormyrops gabonensis*	described species	31	Ivindo R. near Makokou	AF201603	CU79702
3980	*Paramormyrops gabonensis*			Ntem R. at Ayengbé	AF477425	CU80713
4185	*Paramormyrops gabonensis*			Woleu R.	AY475214	AMNH231509
03-0425	*Pollimyrus cf. marchei*	similar to described species	1	Coastal creek near Mayumba	AY475215	MNHN-2003-0425
2969	SN2	sharp, NPp, #2	52	Louétsi R., Ngounié R. basin	AF477437	CU80299
2595	SN2			Louétsi R., Ngounié R. basin	AF477438	CU84664
2606	SN3	sharp, NPp, #3	11	Mouvanga creek/Ngounié R.	AF477465	CU84603
2619	SN3			Mouvanga creek/Ngounié R.	AF477456	CU84603
3396	SN4	sharp, NPp, #4	76	Upper Ogooué R.	AF477434	CU80458
3465	SN4			Upper Ogooué R.	AF477433	CU80463
3666	SN7	sharp, NPp, #7	2	Upper Ogooué R.	AF477428	CU80496
3203	SP2	sharp, Pa, #2	40	Louétsi R., Ngounié R. basin	AF477398	CU81312
2672	SP2			Louétsi R., Ngounié R. basin	AF477399	CU84571

TABLE 1. Continued.

Specimen No.	OTU	OTU description	No.	Locality	GenBank	Museum no.
2638	SP2			Mouvanga creek/Ngounié R.	AF477405	CU84582
2542	SP2			Mouvanga creek/Ngounié R.	AF477406	CU84578
2995	SP4	sharp, Pa, #4	72	Louétsi R., Ngounié R. basin	AF477408	CU80305
2673	SP4			Louétsi R., Ngounié R. basin	AF477449	CU84602
2671	SP4			Louétsi R., Ngounié R. basin	AF477448	CU84602
3026	SP4			Louétsi R., Ngounié R. basin	AF477411	CU80357
3658	SP6	sharp, Pa, #6	19	Upper Ogooué R.	AF477413	CU80485
3966	SP7	sharp, Pa, #7	32	Ntem R. at Ayengbé	AF477414	CU80877
3657	SP8	sharp, Pa, #8	8	Upper Ogooué R.	AF477417	CU80488
4164	SP9	sharp, Pa, #9	20	Okano R. near Mitzic	AY475216	AMNH231503
2008	SZA	manuscript abbrev.	80	Ivindo R. near Makokou	AF477475	CU80848
3839	SZA			creek near Oyem, Ntem R. basin	AF477440	CU80881
2011	TEN	manuscript abbrev.	61	Ivindo R. near Makokou	AF477453	CU80809
2191	TEN			Ivindo R. near Makokou	AF477454	CU80807
3850	TEN			creek near Oyem, Ntem R. basin	AF477426	CU81311
2425	VAD	manuscript abbrev.	42	Ivindo R. near Makokou	AF201578	CU79740
3814	VAD			Woleu R.	AF477476	CU80888
Total 62	Total OTUs 35	Specimens examined	1371			

gel comparisons of AFLP data, PCR products for each AFLP primer combination were run on single, 64-lane polyacrylimide gels. Due to this constraint, we included specimens from only 30 of the 38 different OTUs we recognized in our earlier cyt *b* study. These 30 (plus two new) OTUs were selected for being representative of the morphological and signal diversity we observe in this group.

In this study we wish to assess correspondence of a small number of phenotypic characters common to groups of OTUs to tree topologies derived from two sources of genetic data. We do not code these phenotypic characters and combine them with our molecular datasets prior to analysis, preferring to avoid any circularity this would introduce to the comparisons.

Fluorescent Amplified Fragment Length Polymorphism Methods and Dataset Construction

We followed the AFLP procedure as described by Vos et al. (1995) with certain modifications for fluorescent AFLP developed by Berres (2003). Digestion reactions in 20-μl volumes consisted of 4 μl RNase-purified total genomic DNA from each specimen at a concentration of approximately 25 ng/μl, 1 μl of the restriction enzyme *Bfa* I (5 units), 1 μl *Eco*R I (20 units), 2 μl 10× restriction buffer, and 12 μl H_2O. Enzymes and restriction buffer are from New England Biolabs, (Beverly, MA). Following a 3-h incubation at 37°C, we added a 20 μl ligation mixture consisting of 12 μl H_2O, 4 μl 10× T4 DNA ligase buffer, 1 μl T4 DNA ligase, and 1.5 μl (75 pmoles) of each adaptor to each restriction mixture. We constructed the double stranded adaptors from the following complementary single-stranded oligonucleotides: *Eco*R I adaptor: 5′-CTC GTA GAC TGC GTA CC-3′ and 5′-AAT TGG TAC GCA GTC TAC-3′; *Bfa* I adaptor: 5′-GAC GAT GAG TCC TGA G-3′ and 5′- TAC TCA GGA CTC AT-3′. We performed ligation at 16°C overnight. Following ligation, we diluted each mixture in 160 μl 10 mM Tris (pH 8.5).

For the preselective PCR step, we added 10 μl of the Tris-diluted ligation mixture to 40 μl of a preselective PCR amplification mixture consisting of: 25μl H_2O, 1 μl deionized formamide, 5μl 10× PCR buffer, 3μl $MgCl_2$ (25 mM), 4μl dNTP (2.5 mM of each), 0.75μl (15 pmoles) of each preselective primer, and 0.5 μl (2.5 units) Taq DNA polymerase. We used two preselective primers: *Eco*R I + G: 5′-GAC TGC GTA CCA ATT CG-3′ and *Bfa* I+T: 5′-GAT GAG TCC TGA GTA GT-3′. Our PCR cycling parameters were a preliminary 72°C extension for 60 sec followed by 20 cycles of 94°C for 50 sec, 56°C for 60 sec, and 72°C for 2 min. Following PCR, we diluted 40 μl of this mixture in 720 μl 10 mM Tris (pH 8.5).

For the selective PCR step, we added 5 μl of the diluted preselective mixture to 20 μl of the selective PCR amplification mixture consisting of 11.25 μl H_2O, 0.5 μl deionized formamide, 2.5 μl 10× PCR buffer, 1.5 μl $MgCl_2$ (25 mM), 3μl dNTP (2.5 mM of each), 0.25 μl (0.5 pmoles) of the flourophore (6-FAM)-labelled *Eco*R I selective primer, 1.25 μl (25 pmoles) of the unlabelled *Bfa* I selective primer, and 0.25 μl (1.25 units) Taq DNA polymerase. Our selective PCR parameters were 30 cycles of 94°C for 50 sec, 65–56°C (1°C reduction for first 10 cycles) for 60 sec, and 72°C for 2 min. These 30 cycles were followed by a 10-min extension step at 72°C.

We screened 64 selective primer combinations with four taxa, choosing 10 combinations (Table 2) for all 62 specimens

TABLE 2. Summary of amplified fragment length polymorphism (AFLP) data and analysis statistics. To the right of each primer combination is shown the number of scored AFLP characters (number of unique bands scored for 62 specimens/mean number of bands scored per individual) at each of three peak detection thresholds in GeneScan 3.1 (rfu, relative fluorescence units). Total characters, number of informative characters for parsimony, and the g_1 statistic calculated for 1 million random trees are shown for each dataset as are statistics for the distance, Wagner parsimony, and Dollo parsimony analyses.

Primer combination	Primer 1	Primer 2	40 rfu	80 rfu	120 rfu
1	*Eco*RI G-C	*Bfa*I T-CG	338/54	274/41	249/35
2	*Eco*RI G-AT	*Bfa*I T-TA	317/60	257/43	221/35
3	*Eco*RI G-CG	*Bfa*I T-CT	257/34	198/22	165/17
4	*Eco*RI G-CG	*Bfa*I T-TT	348/73	290/53	258/41
5	*Eco*RI G-CG	*Bfa*I T-TC	227/34	176/24	152/20
6	*Eco*RI G-A	*Bfa*I T-CC	272/55	204/37	167/29
7	*Eco*RI G-CG	*Bfa*I T-AT	277/45	200/30	171/24
8	*Eco*RI G-C	*Bfa*I T-CC	332/71	286/58	263/50
9	*Eco*RI G-AT	*Bfa*I T-AT	230/30	176/22	145/19
10	*Eco*RI G-AT	*Bfa*I T-CG	208/27	130/18	106/14
total characters			2806	2191	1897
informative characters for parsimony			2208	1579	1326
g_1 for 1 million random trees			−0.257**	−0.256**	−0.251**
Nei-Li distance, neighbor-joining					
sum of branch lengths			2.759	2.746	2.708
nodes resolved ≥50% bootstrap			50	42	43
Wagner parsimony			1 MP tree, 12,766 steps	1 MP tree, 9,024 steps	11 MP trees, 7,117 steps
mean CI			0.17	0.18	0.19
informative characters with CI = 1			83 (3.8%)	82 (5.2%)	91 (6.9%)
nodes resolved ≥50% bootstrap			37	34	37
Dollo parsimony			1 MP tree, 25,415 steps	1 MP tree, 17,136 steps	1 MP tree, 13,887 steps
mean CI			0.09	0.09	0.1
informative characters with CI = 1			68 (3.1%)	78 (5.0%)	77 (5.9%)
nodes resolved ≥50% bootstrap			38	38	38

** $P < 0.01$ (Hillis and Huelsenbeck 1992).

that produced between 30 and 70 well-defined bands per sample, distributed widely across the 50–625-bp scoring window. The selective amplification products were purified by gel filtration in 0.7 ml spin columns using G-50 (fine) Sephadex (Sigma-Aldrich Corp., St. Louis, MO). We loaded 0.25 μl of purified product (diluted 4:1) per sample along with 0.5 μl GeneFlo-625 ROX ladder (CHIMERx, Milwaukee, WI) onto a RapidLoad membrane comb (The Gel Company, San Francisco, CA). We electrophoresed these samples through 5% polyacrylamide gels (Long Ranger-BioWhittaker Molecular Applications, Rockland, ME) on an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA) for 7 h of collection time at 40 W of electrophoresis power.

We identified and sized the peaks between 50 and 625 bp in the ABI gel image using GeneScan 3.1 (Applied Biosystems). Because we observed a nearly continuous distribution of peak intensities in all electropherograms and some nonuniformity of signal intensity across samples, we analyzed each gel three times using minimum peak detection thresholds of 40, 80, and 120 relative fluorescence units (rfu) to assess the effect of threshold selection on the analysis results.

We binned peaks from ABI trace files with the software BinThere (Garnhart 2001) using the expert binning algorithm and 1-bp bin width. From the BinThere output, we created three datasets for phylogenetic analysis consisting of the combined data from all 10 primer combinations, for the 40-, 80-, and 120-rfu peak detection thresholds (hereafter AFLP40, AFLP80, and AFLP120). We calculated the g_1 statistic (Hillis and Huelsenbeck 1992) for all three datesets.

Phylogenetic Analysis of Cytochrome b Sequences and Amplified Fragment Length Polymorphisms

We reconstructed cyt *b* and AFLP phylogenies in PAUP* 4.0b10 (Swofford 2003). For cyt *b*, we performed a heuristic unweighted (Wagner) parsimony search. Starting trees for tree bisection-reconnection (TBR) branch swapping were obtained by 100 iterations of the random stepwise addition sequence. We evaluated relative support for nodes common to all most parsimonious cyt *b* trees by 1000 bootstrap pseudoreplicates, each with 10 iterations of random stepwise addition. In our previous cyt *b* study in which we included sequences from more individuals (Sullivan et al. 2002), we found the cyt *b* tree topology obtained by a maximum likelihood analysis to be identical to that obtained by parsimony. We did not repeat a maximum likelihood analysis here.

For analysis of AFLP data we employed distance and parsimony methods. A complete comparative study of all possible methods of AFLP analysis is beyond the intended scope of this paper. Here we examine results from three methods to focus only on those relationships best supported by the AFLP data for comparison to the cyt *b* topology. Little consensus exists regarding the most appropriate and effective method(s) of phylogenetic analyis of AFLP characters, although distance methods and Wagner parsimony are most frequently employed. DeBry and Slade (1985) suggested that Dollo parsimony was more appropriate for analysis of restriction fragment data than Wagner parsimony, while others have argued that neither the free reversibility of Wagner par-

simony nor Dollo's constraint of evolutionary singularity is a good fit for analysis of data of this kind (Backeljau et al. 1995). Others have proposed ''relaxed Dollo'' or ''enhanced Wagner'' approaches through step-matrix weighted parsimony for restriction fragment data (Albert et al. 1992; Swofford et al. 1996). Determination of appropriate weights, however, requires a model of evolution for AFLP fragments that currently does not exist. Here we have employed both Wagner and Dollo parsimony analyses, which necessarily miss a theoretical (unknown) best character-state transition weight, but which bracket the full range of possible weights. Hierarchic structure in the AFLP data that is recovered in both analyses we viewed as robust.

For the distance analyses, we converted the data into a matrix of pairwise genetic distances using the algorithm of Nei and Li (1979), which they derived for restriction fragment data. From this matrix, we used the neighbor-joining method to construct a starting tree for a TBR branch swapping search for the minimum evolution tree. We conducted Wagner and Dollo parsimony heuristic searches in which the TBR branch swapping starting trees were the shortest found in 100 iterations of random stepwise addition. To assess support for nodes in these trees, we performed 1000 bootstrap pseudoreplicates for distance and Wagner parsimony, but only 100 pseudoreplicates for Dollo parsimony due to the much greater computational time required. For the Wagner and Dollo parsimony bootstraps, the starting tree for each pseudorepicate was obtained by 10 iterations of random stepwise addition. For the Nei-Li distance bootstrap, the starting tree was obtained by neighbor-joining. PAUP's default settings were used for all other parameters. Trees for the AFLP and cyt *b* analyses were rooted with the taxon *Pollimyrus marchei*. Monophyly of the Gabon-clade *Brienomyrus* taxa was not constrained with respect to the taxon *Marcusenius ntemensis*.

Finally, we optimized the two electrocyte character states found in this group, Pa and NPp, onto the AFLP and cyt *b* topologies using unweighed parsimony in MacClade 4.05 (Maddison and Maddison 2002). The AFLP gel files, GeneScan trace files, and the AFLP and cyt *b* Nexus files used in PAUP* are available from the authors by request.

Results

Cytochrome b Phylogeny

The cyt *b* dataset consisted of 58 aligned 1140-bp sequences from the 60 Gabon-clade *Brienomyrus* ingroup specimens and two outgroup specimens used in the AFLP study. Cyt *b* haplotypes were identical between four pairs of these specimens: SN4 3396/3465, SN2 2595/2969, SP2 2542/2638, and SP2 2672/3203. These sequences were entered only once into the data matrix for phylogenetic analysis. In one case, identical haplotypes were obtained from two specimens belonging to different OTUs, SN3 2619 and BP1 2704, and were both maintained in the data matrix. The dataset contains 188 characters informative for parsimony. The heuristic Wagner parsimony search yielded 2864 shortest trees, each of 472 steps and a consistency index (CI) = 0.49 with uninformative characters excluded. A semistrict (combinable component) consensus tree (Bremer 1990) of these 2864 trees is shown in Figure 2 (left), with bootstrap proportions indicated at nodes where these are at or above the 50% level. The topology of this tree is consistent with that obtained by Sullivan (2002).

Amplified Fragment Length Polymorphism Phylogeny

The number of fragments scored for each primer combination at each detection threshold as well as statistics on distance, Wagner parsimony, and Dollo parsimony analyses are presented in Table 2. The combined AFLP 40, 80, and 120 datasets contain 2208, 1579, and 1326 informative characters for parsimony analysis, respectively. The g_1 value for each dataset is far lower (i.e., more significant) than the $P = 0.01$ critical value for this statistic (Hillis and Huelsenbeck 1992), indicating significant nonrandom structure in the data. For each dataset, the Dollo parsimony tree is nearly twice as long as the tree(s) produced by Wagner parsimony, reflecting the fact that Dollo parsimony allows as many reversals to the band-absent character state as necessary to limit each character to a single origin of the band-present character state. Only a small proportion of the binary characters exhibit single character state changes on these trees. The very low CI values (Table 2) are consistent with the expected high level of homoplasy in AFLP data, while the higher CIs at the higher peak detection thresholds is probably due to fewer spurious peaks included in these datasets. Fragments scored as present in all taxa are few: in the AFLP40 dataset, only nine were scored as present in all taxa, including the outgroup taxon *P. marchei*, six are present in all taxa excluding *P. marchei*, and a single fragment is present in all ingroup taxa excluding *P. marchei* and *M. ntemensis*.

We observed limited conflict (discussed below) among the well-supported nodes of the nine analyses (AFLP40, AFLP80, and AFLP120 datasets by three analysis methods: distance, Wagner parsimony, and Dollo parsimony) and show a semistrict consensus (Bremer 1990) of the nodes of all analyses that were supported at or above the 50% bootstrap level in Figure 2 (right). This consensus tree contains 38 nodes. Of these, 29 nodes (76%) receive support from each analysis method at or above the 50% bootstrap level in at least one of the datasets, four are supported at this level by distance analysis and Dollo parsimony only, and five exclusively by the distance analysis.

Twelve of 50 nodes of the best-resolved 50% bootstrap tree (AFLP40 Nei-Li distance) conflict with nodes supported in the other eight bootstrap analyses. Ten of these conflicts are at terminal nodes that receive no more than 65% bootstrap support in any one tree. The two exceptions are worth noting. In the first of these, a sister-group relationship between the *B. curvifrons* specimen 2050 from the Ivindo River and the *B. cf. curvifrons* specimen 4149 from the Okano River is supported by five analyses (AFLP40 Wagner and Dollo; AFLP80 Nei-Li distance, Wagner and Dollo). Bootstrap support values for this relationship are 50%, 71%, 83%, 84%, and 50%, respectively. The pairing of these specimens in the analysis makes sense given their close similarity in both morphology and EOD characteristics. However, this relationship is not represented in the consensus tree in Figure 2 because three other analyses (AFLP40 Nei-Li, AFLP120 Nei-Li and Wagner) support a sister group relationship between *B. cf. curvifrons* 4149 and OKA 4150 with bootstrap support values

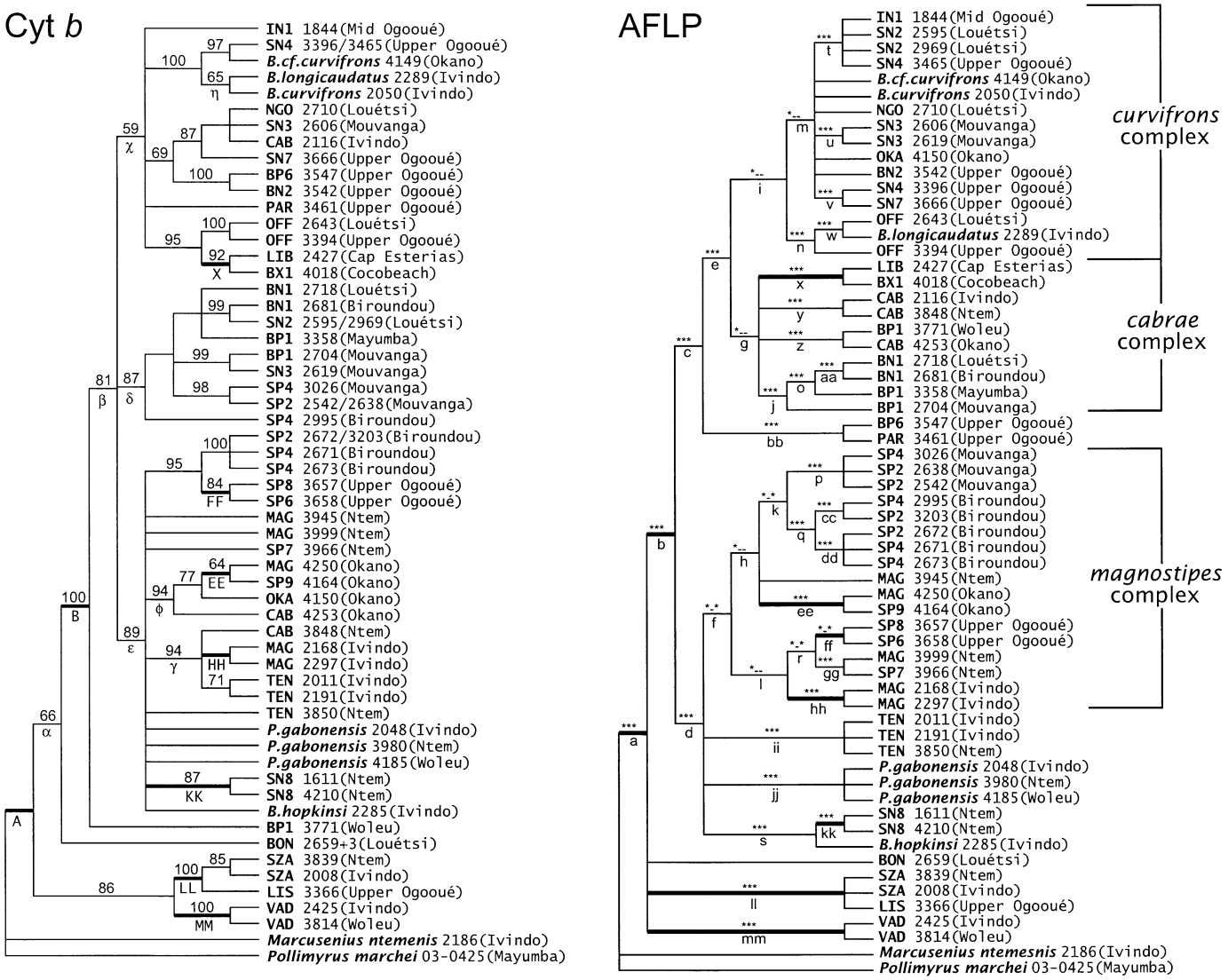

FIG. 2. Phylogenetic hypotheses for 60 Gabon-clade *Brienomyrus* specimens derived from cytochrome *b* (cyt *b*) sequences and amplified fragment length polymorphisms (AFLP). At left is shown the semistrict consensus of 2864 most parsimonious trees produced from an unweighted (Wagner) parsimony analysis of complete cyt *b* sequences. Bootstrap values at nodes supported at or above the 50% level are indicated. At right is shown the semistrict consensus of nodes supported at or above the 50% bootstrap level among nine analyses of AFLP data. These consisted of three analysis methods (minimum evolution Nei-Li distance, Wagner parsimony, and Dollo parsimony) applied to each of three AFLP datasets in which bands were scored at three different peak detection thresholds (40, 80, 120 rfu). Asterisks above nodes indicate bootstrap support at or above 50% in at least one of the distance, Wagner parsimony, and Dollo parsimony analyses, respectively. Bootstrap support values for each node in each AFLP analysis are shown in Table 3. Nodes on AFLP tree labeled a–mm. Nodes shared by both AFLP and cyt *b* trees are given the same letter code (capitalized on cyt *b* tree) and indicated by thickened internal branches. Other cyt *b* tree nodes discussed in text labeled by a greek letter.

of 75%, 67%, and 65%, respectively. The second notable conflict across analyses concerns the position of specimen BON 2659. Nei-Li distance and Wagner parsimony analysis of the AFLP40 dataset place it as sister to the two VAD specimens (clade mm in Fig. 2) with high bootstrap support (84% and 98%, respectively), but as sister to the two SZA specimens plus LIS 3366 (clade ll in Fig. 2) in the analyses of the AFLP120 dataset (70% and 74% bootstrap support, respectively). Inferred interelationships among these basal lineages and with respect to the well-supported large clade b appear poorly supported and variable across the nine analyses.

Comparison of Amplified Fragment Length Polymorphism and Cytochrome b Tree Topologies

The AFLP and cyt *b* tree topologies for the Gabon-clade *Brienomyrus* conflict with each other at many nodes. While each of the most parsimonious topologies recovered from the cyt *b* dataset require 470 steps, constraining this dataset to the AFLP dataset-derived topologies require between 762 and 787 steps. Conversely, while the most parsimonious topology recovered for the AFLP80 dataset by Wagner parsimony requires 9024 steps, constraining this dataset to the cyt *b* tree topologies requires between 10450 to 10639 steps.

TABLE 3. Bootstrap values for each node appearing in the consensus amplified fragment length polymorphism tree shown in Figure 2 for each of nine analyses. Details of bootstrap procedure are given in the text.

	Threshold 40			Threshold 80			Threshold 120		
Node	Nei-Li	Wagner	Dollo	Nei-Li	Wagner	Dollo	Nei-Li	Wagner	Dollo
a	96	100	100	74	99	100	89	100	100
b	84	71	96	79	68	74	79	39	38
c	88	98	96	95	86	74	88	87	38
d	96	100	100	84	100	98	93	100	100
e	95	97	<5	99	97	20	97	96	51
f	70	20	34	33	<5	50	10	<5	43
g	53	33	<5	15	20	<5	30	8	21
h	85	46	<5	43	7	<5	39	<5	<5
i	56	11	9	30	<5	<5	43	<5	27
j	88	65	66	57	35	48	82	47	29
k	57	47	46	35	45	52	14	43	60
l	66	<5	12	31	<5	20	41	<5	19
m	62	12	20	24	10	14	39	6	5
n	100	94	86	100	100	94	100	100	100
o	98	87	58	100	96	29	96	82	51
p	99	100	76	100	100	94	100	99	84
q	89	17	53	86	47	56	92	61	70
r	70	9	20	52	<5	45	45	<5	54
s	100	100	69	100	100	72	100	99	98
t	84	54	53	83	37	66	92	61	55
u	100	100	92	98	93	82	93	78	58
v	99	98	81	76	51	54	64	58	40
w	98	94	88	90	74	91	94	86	83
x	100	100	97	100	100	100	100	100	100
y	67	88	81	89	86	89	96	99	90
z	85	91	74	89	87	78	94	94	77
aa	97	100	93	97	97	88	99	99	98
bb	100	100	100	100	100	99	100	100	100
cc	80	62	60	94	67	84	98	85	93
dd	71	14	36	96	63	64	96	77	81
ee	100	100	100	100	100	100	100	100	100
ff	57	25	71	51	44	64	68	20	71
gg	100	93	90	97	81	84	98	61	89
hh	100	100	98	100	100	98	100	100	99
ii	82	77	61	86	86	61	73	83	75
jj	98	84	98	78	69	90	74	83	86
kk	100	100	61	100	100	73	100	100	54
ll	100	98	99	100	100	100	100	100	100
mm	100	100	100	100	100	100	100	100	100

Nodes common to both the cyt *b* and AFLP trees are A/a, B/b, X/x, EE/ee, FF/ff, HH/hh, KK/kk, LL/ll, and MM/mm (Fig. 2, left and right). Node A/a excludes the taxon *M. ntemensis* and defines the clade we refer to as the Gabon-clade *Brienomyrus*. Previous molecular phylogenetic studies of the Mormyridae indicated that *M. ntemensis* is the sister group to the Gabon-clade *Brienomyrus* (Lavoué et al. 2000, 2003; Sullivan et al. 2000). Node B/b defines a large group of taxa that excludes the OTU referred to as BON, clade LL/ll (composed of two specimens of the OTU SZA plus LIS 3366), and clade MM/mm (two VAD specimens). In the cyt *b* analysis these latter two clades are each other's sister group and BON is sister to clade B. In contrast, these are unresolved with respect to each other and to clade b in the AFLP tree (but compatible with the cyt *b* topology). The remaining commonalities are at terminal nodes: node X/x groups LIB 2427 and BX1 4018; node EE/ee groups MAG 4250 and SP9 4164; node FF/ff groups SP8 3657 and SP6 3658; HH/hh groups two MAG specimens from the Ivindo River; and KK/kk groups two SN8 specimens from the Ntem River. Of the remaining nodes on the AFLP consensus tree, four are compatible with an unresolved topology on the cyt *b* tree (s, dd, gg, jj), and the other 26 nodes conflict with nodes of the cyt *b* tree. We discuss the most important of these differences.

Within the shared clade b, major groupings of taxa differ substantially in the AFLP and cyt *b* trees. On the AFLP tree, clade b is divided into two large, well-supported nodes, c and d. AFLP nodes i and g (included within node c) and node f (included within node d) represent large clades that are coherent with respect to their morphology and EOD/electric organ type. These groups are broken apart in the cyt *b* tree by node β and its three subclades, χ, δ, and ε.

For example, AFLP node f groups seven OTUs that are recognizable as a group by their narrow heads and sharp snouts (viewed from above), somewhat protruding lower jaw, the presence of a strong initial negative peak in their EOD waveforms (Figs. 3, 4), Pa-type electrocytes, and their preference for fast-flowing water. In the cyt *b* tree, by contrast, they are divided between clades δ and ε. These two well-supported nodes of the cyt *b* tree divide conspecifics from single populations. An unusual characteristic common to the fishes unified by AFLP node f is that in most localities one

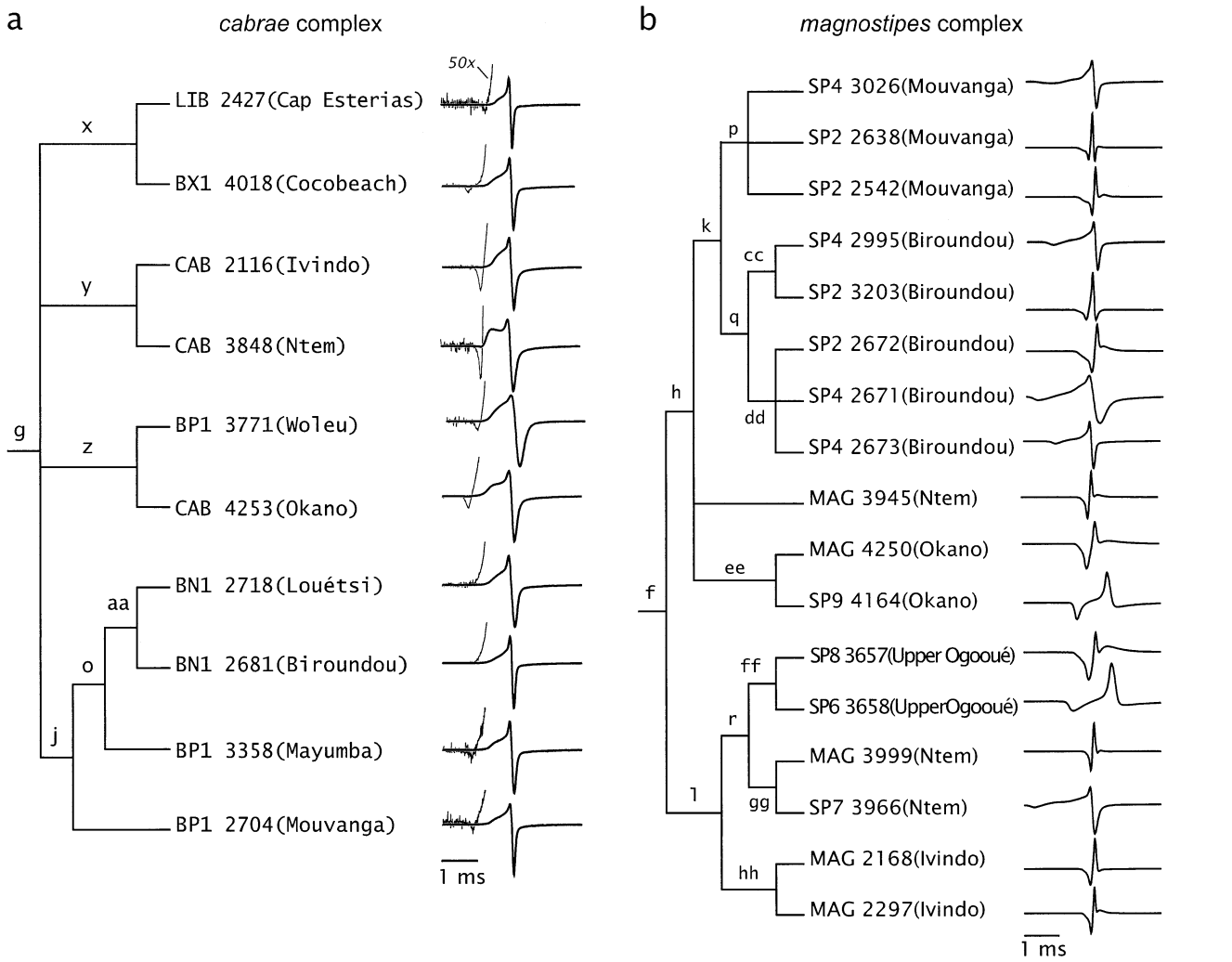

FIG. 3. Two examples from the larger amplified fragment length polymorphism (AFLP) tree shown in Figure 2 of clades of phenotypically similar species/populations recovered as monophyletic groups by AFLP data but not by cytochrome *b* sequences. (a) OTUs of the ''*cabrae* complex'' have blunt snouts, colored scales on their undersides, and electrocytes with penetrating stalks. In some species the head negative prepulse produced by the penetrating stalks is substantial, while it is weak in others. (b) Members of the sharp-snouted ''*magnostipes* complex'' are recognizable by their distinctive head morphology. Electric organ discharge diversity in this group is categorized in Figure 4.

finds two forms of adult EOD among otherwise identical fish. In each case, some males and females possess an EOD of the *magnostipes*-type (Fig. 4, left), while others possess a single alternate form (Fig. 4, right). We included a relatively large number of these specimens in this study to gain insight into the question of whether these EOD types represent cryptic species. The fact that the AFLP tree groups specimens by population sampled and not by EOD type at nodes p, q, ee, ff, and gg suggest that these may be EOD morphs and not reproductively isolated species. We refer to the entities grouped by node f as the *magnostipes* complex pending determination of the number of species in this clade.

AFLP clade g is a second group coherent with respect to morphology and EOD type that are recovered by the AFLP characters, but not by cyt *b* sequences. These are wide-headed, blunt-snouted forms with golden scales along the underbelly in life. All have EOD waveforms with pronounced to weak head-negative prepulses (Fig. 3a) and electric organs containing Pa-type electrocytes (or a mix of NPp and Pa-type electrocytes in BX1 and BN1). These fishes are most common in creeks with low to moderate flow. These taxa are divided among clades χ, δ, and ε of the cyt *b* tree. All of the BN1 and BP1 individuals from southern Gabon are grouped together at node j of the AFLP tree. BP1 and BN1 specimens are externally indistinguishable and often found at the same sites. These specimens were placed in two different OTUs because of their subtly different EOD waveforms (in contrast to the very different EODs of SP2/SP4, MAG/SP7/SP9 described above). BP1s have EOD waveforms with a weak head negative prepulse that is visible at high gain amplification. This feature is either absent or not discernable in the BN1s (Fig. 3a). Closer examination of these EOD records suggests a continuous gradation from weak to indiscernible prepulse in these specimens consistent with their belonging to a single species. A similar phenomenon was observed among specimens of the OTU BX1 from near Cocobeach, Gabon, which

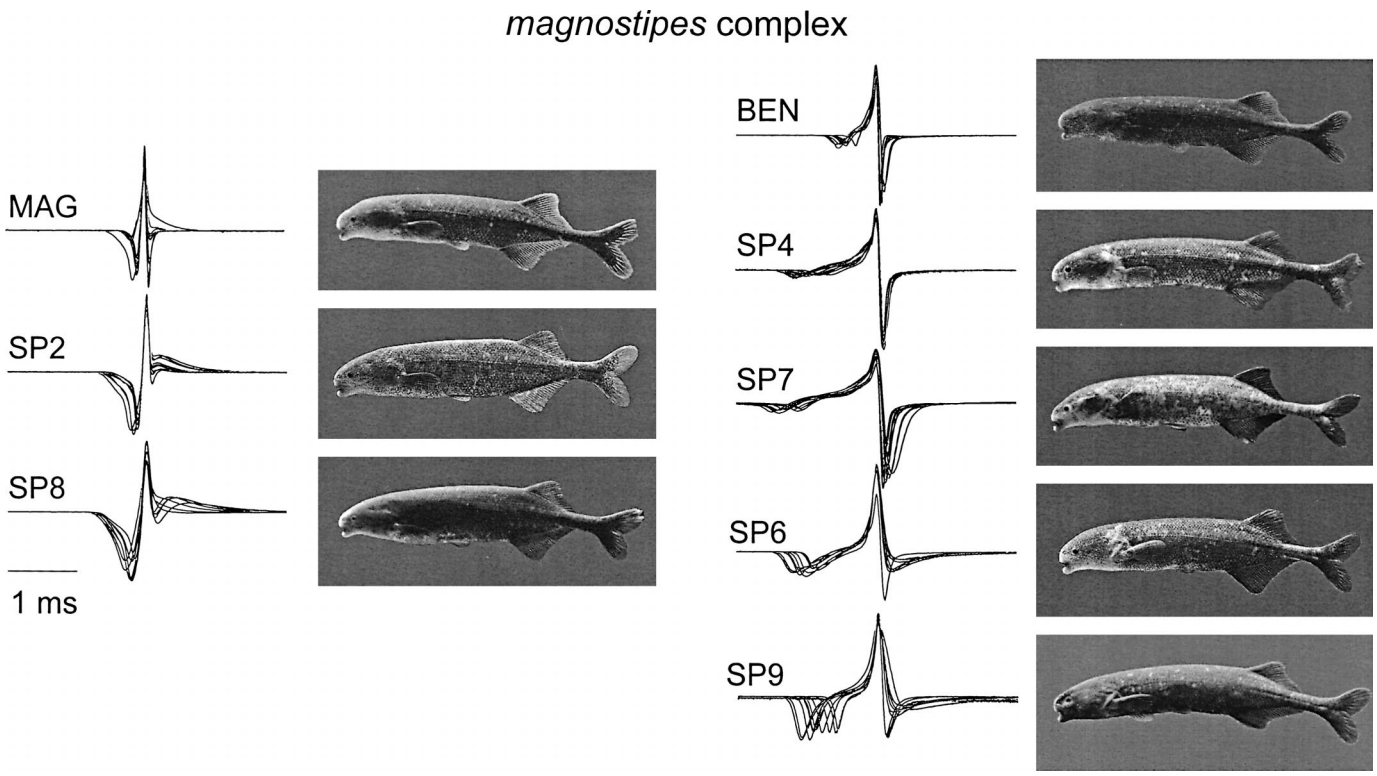

FIG. 4. Electric organ discharge (EOD) overlays and photographs of ''magnostipes complex'' operational taxonomic units (OTUs) whose interrelationships based on amplified fragment length polymorphism (AFLP) characters are shown in Figure 3b. Two distinct EOD types and concomitant differences in electrocyte stalk development are often found among otherwise phenotypically identical forms. Within sites one finds male and female individuals exhibiting a *magnostipes*-type EOD waveform (left) alongside others exhibiting a locality-specific alternate EOD waveform (right). Intermediates between these types are not found among adults. The AFLP tree (Figs. 2, 3b) groups specimens by locality, not by EOD type, suggesting that these may be EOD morphs and not separate species. Note: OTU BEN (upper left) was not included in the present study, but is found together with the externally identical MAG from the Ivindo River, Gabon.

have closely similar EOD waveforms and morphology to the other members of AFLP clade g. We refer to the members of AFLP clade g as the *cabrae* complex for their similarity to *Marcusenius cabrae* (Boulenger 1900), described from Mayombe, Republic of Congo.

A third large recognizable group of taxa uniquely recovered by AFLP characters is clade i. These are intermediate- to sharp-snouted forms all with biphasic EODs and electric organs containing NPp-type electrocytes. These taxa are dispersed among clades χ, δ, and ε of the cyt *b* tree. We refer to this AFLP clade as the *curvifrons* complex, for *Brienomyrus curvifrons* (Taverne et al. 1977b), a described species from this group.

In addition to these large groupings of taxa, AFLP data recovers single OTUs as monophyletic groups to a greater extent than do cyt *b* sequences. For example, SN3 and TEN each appear as monophyletic groups on the AFLP tree, but are split by well-supported nodes on the cyt *b* tree. Additionally, the AFLP tree groups together the two OFF specimens with the single *B. longicaudatus* specimen at node n. These two very similar taxa, which share a distinctive head profile and long caudal peduncle (Sullivan et al. 2002), are split apart within clade χ of the cyt *b* tree. The two specimens *B. curvifrons* 2050 (Ivindo) and *B. cf. curvifrons* (Okano) form a sister pair in five out of the nine AFLP analyses with >50% bootstrap support (>80% in two of these), but this node is not represented in the AFLP consensus tree due to an alternate topology supported in three analyses.

While the fit of the AFLP consensus tree to the observed phenotypes of the Gabon-clade *Brienomyrus* specimens is generally good, some OTUs do not appear as monophyletic groups on this tree. In some cases, as for the BN1/BP1 we believe that these are probably two designations for a single species in the southern Gabon localities, as discussed above. In other cases, our application of OTU designations needs to be reexamined, such as for BP1 3771 (Woleu) and CAB 4253 (Okano), and for OFF and *B. longicaudatus* in which differences between the forms are slight. More surprising is the nonmonophyly of the OTU SN4 in the AFLP tree with the placement of specimen 3396 as the sister taxon to phenotypically distinct SN7 3666 at node v. Inclusion of more individuals of these two OTUs in a future study is may be informative. BP6 3547 and PAR 3461 paired at node bb in the AFLP tree are morphologically similar, but have different EOD characteristics. In no other cases on the AFLP tree are phenotypically dissimilar specimens grouped.

Geographic Signal in the Mitochondrial Tree

Interestingly, collection locality and not phenotype is the feature shared by many of the groups recovered on the cyt

b tree. For instance, at the well-supported node ϕ, the MAG/SP9 pair, also recovered on the AFLP tree, is placed in a clade with the very dissimilar fishes OKA 4150 and CAB 4253. All of these specimens were collected at the same site on the Okano River. In the AFLP tree, these two specimens are placed with phenotypically similar fish in clades i and g, respectively, irrespective of collection site. At node γ, two specimens of TEN from the Ivindo River are placed together in a clade with two MAG specimens from the same site along with CAB 4253 from the Ntem, to the exclusion of TEN 3850 from the Ntem. In the AFLP tree, all three TEN specimens form a clade (ii) and the two Ivindo MAG specimens appear as the sister clade to the morphologically similar SP8/SP6 and MAG/SP7 sister pairs from the Upper Ogooué and Ntem River sites, respectively. At node η, *B. curvifrons* 2050 is grouped with *B. longicaudatus* 2289 from the same Ivindo site to the exclusion of *B. cf. curvifrons* 4149 from the Okano River. Another example of geographic signal in the cyt *b* tree is node δ, the disparate members of which share collection sites in the south of Gabon.

Discussion

Amplified Fragment Length Polymorphism Versus Mitochondrial Phylogeny

Agreement between the AFLP consensus tree and the cyt *b* tree is good at basal nodes, but with the exception of several terminal nodes mentioned above, differs above node b/B. By contrast with the cyt *b* tree, the topology supported by the AFLP characters above node b/B generally groups same-OTU specimens together and further groups similar OTUs together into higher-level clades, irrespective of collection locality. Because of their agreement with phenotypic characters, we infer that the well-supported nodes of the AFLP tree better reflect the common ancestry of the species lineages of Gabon-clade *Brienomyrus*, while many nodes of the cyt *b* tree, although probably accurately reflecting mitochondrial geneaology, depart from the larger phylogeny of these species lineages. The concordance of the AFLP tree with phenotypes of Gabon-clade *Brienomyrus* allow us to reject the hypothesis of widespread convergent evolution suggested by interpreting the cyt *b* tree as a species tree. Instead, the AFLP data generally support the phylogenetic integrity of the phenotypically defined groups and indicates their independent dispersal to multiple localities. These conclusions are similar to those reached in a number of other recent phylogenetic studies in which morphologically inferred relationships among closely related organisms were found to conflict with mitochondrial geneaology, but were supported by nuclear sequence data (Sota and Vogler 2001, 2003; Taggart et al. 2001; Shaw 2002; Sota 2002; Rognon and Guyomard 2003) or AFLPs (Albertson et al. 1999; Parsons and Shaw 2001).

Evidence for Mitochondrial Introgression

The departure of the cyt *b* tree from species phylogeny could result from the persistence of multiple mitochondrial lineages across speciation events (lineage sorting) or from introgression of the mitochondrial genome from one species to another by hybridization followed by backcrossing into a parental population. The patterns created by these two processes can be difficult to distinguish from one another, and the effects of both could be interwoven in the mitochondrial tree. However, evidence for introgression is persuasive in cases in which conflicting cyt *b* topology is associated with haplotypes sampled from phenotypically distinct individuals from the same collection site. For instance, two SN3 individuals from the Mouvanga locality, monophyletic on the AFLP tree at clade u within the *curvifrons* complex, are separated into cyt *b* haplotype clades χ (SN3 2606) and ∂ (SN3 2619). Specimen SN3 2619 shares an identical cyt *b* haplotype with BP1 2704, collected at the same locality. Without additional sampling the polarity of the introgression cannot be definitively determined, but because cyt *b* clade ∂ contains the haplotypes from all other BP1/BN1 individuals collected at nearby localities, mitochondrial introgression from OTU BP1/BN1 to SN3 seems most likely. Another example of cyt *b* topology best explained by introgression is cyt *b* tree node ϕ that unites the externally identical MAG/SP9 pair from the Okano River with the very dissimilar OKA and CAB specimens collected at the same site. On the AFLP tree, these individuals are grouped with phenotypically similar fish from other localities in the *magnostipes*, *curvifrons*, and *cabrae* species complexes, respectively. Mitochondrial introgression across at least two species boundaries at this Okano River site is the only scenario that adequately reconciles the cyt *b* tree with the AFLP topology and the phenotypes of the specimens.

Comparison of the mitochondrial and AFLP trees in this study suggests that introgressive hybridization has been a persistent characteristic of Gabon-clade *Brienomyrus* evolutionary history, albeit without concomitant breakdown of species-specific morphologies and signals, or phylogenetic structure as assessed from nuclear loci. Such nondestructive interspecific introgression and replacement of mtDNA may be relatively common in fishes (e.g., Bernatchez et al. 1995; Chow and Kishino 1995; Glemet et al. 1998; Takahashi and Takata 2000; Gerber et al. 2001; Ruber et al. 2001; Redenbach and Taylor 2002; Rognon and Guyomard 2003) as well as in other organisms (examples cited in Avise 2000). Repeated instances of mitochondrial introgression within the phylogeny of a single clade, including introgression across multiple species boundaries, have also been hypothesized in recent studies of Hawaiian *Lauapala* crickets (Shaw 2002) and Japanese carabid beetles (Sota and Vogler 2001, 2003; Sota et al. 2001; Sota 2002).

Because the methods of phylogenetic reconstruction we have used enforce a dichotomously branching structure to tree topology, these results do not allow us to evaluate what role, if any, more complete lineage reticulation has played in the evolutionary history of Gabon-clade *Brienomyrus*. However, hybrid origin of species has been an important evolutionary phenomenon in some groups of freshwater fishes and other animals (DeMarais et al. 1992; Smith 1992; Dowling and DeMarais 1993; Arnold 1997; Dowling and Secor 1997).

AFLPs as Phylogenetic Characters

Despite their higher reproducibility than random amplified polymorphic DNA (RAPDs; Jones et al. 1997) and a lower

incidence of nonindependence than restriction fragment length polymorphisms (RFLPs), AFLPs share some characteristics of these other markers that are problematic for phylogenetic analysis (Backeljau et al. 1995; Swofford et al. 1996), particularly the high probability of parallel losses of characters relative to gains. However, homoplasy, nonindependence, and character state change asymmetries are potential sources of error for every kind of molecular and morphological character. We believe the best evaluation of AFLPs as phylogenetic markers will continue to be empirical studies such as this one in which congruence between AFLP-derived topologies and independent evidence of relationships such as morphology can be assessed. Our study joins a growing consensus of others that indicate that these multilocus markers can resolve relationships among closely related species and populations, groups for which sequence data have often been inconclusive or problematic (Kardolus et al. 1998; Albertson et al. 1999; Labra et al. 1999; Baayen et al. 2000; Bakkeren et al. 2000; Ganter and Lopes 2000; Hodkinson et al. 2000; Kanzaki et al. 2000; van Raamsdonk et al. 2000; Giannasi et al. 2001; Parsons and Shaw 2001; Buntjer et al. 2002; Allender et al. 2003; Després et al. 2003; Seehausen et al. 2003).

Examination of the results from three AFLP analysis methods and three different peak detection thresholds in this study yields few clear patterns by which to choose a single method or threshold as superior. While our distance analysis at the lowest peak detection threshold (AFLP40) recovered the most nodes above the 50% bootstrap level, we do not interpret this result as a reason to prefer of this analysis over the others. Some relationships supported in the AFLP40 distance analysis, such as the nonmonophyly of *B. curvifrons* 2050 and *B. cf. curvifrons* 4149 (discussed above) are contradicted by well-supported conflicting topologies in other analyses. This probable sister-group relationship receives high support in all three Dollo parsimony analyses and in two out the three Wagner parsimony analyses, but the distance method favors this relationship only in the analysis of the AFLP80 dataset. In this case and others, examining the results of multiple analyses proves useful. Bootstrap values in Table 1 reveal a complex relationship between peak detection threshold and analysis method. For example, among the three Nei-Li distance analyses, the highest level of bootstrap support for node k is obtained with the AFLP40 dataset (57%) and lowest (14%) with the 120 threshold dataset. This pattern is reversed at this node in the Dollo parsimony analyses, with bootstrap values increasing from 46% with the AFLP40 dataset to 60% with the AFLP120 dataset. A similar pattern exists for clade r. Overall for the Dollo analyses, bootstrap values for the consensus nodes increase somewhat with increasing peak detection thresholds as one would expect if higher peak detection thresholds reduce the number of nonhomologous bands scored as the same character. Given the stringent requirement that Dollo characters evolve once and only once, homoplasy in the band-present character state will likely cause more serious problems for this method than for others.

One extremely powerful feature of AFLP characters is their ability to recover population structure below any putative species level as well as phylogenetic structure above it. One application of this information can be as an aid to species delimitation. For example, within our AFLP clade f, which groups all the *magnostipes* complex forms, subgroups exist that correspond to geographic zone and population. If future studies show consistent morphological traits differentiating these subgroups and an absence of intermediate forms, several different monophyletic species belonging to a new genus erected at node f could be recognized. Alternatively, all specimens below node f might be more appropriately considered geographical variants of a single species. Additionally, future AFLP studies incorporating more individuals of the *magnostipes* complex may help resolve whether the two EOD waveform types collected together at single sites represent phenotypic polymorphism within single species or reproductively isolated sibling species. Because so many independently segregating, variable markers can be generated and analyzed, AFLPs may provide the resolving power to detect divergence of lineages at the earliest stage, the point at which predominant nonconcordance among shallow allelic genealogies are converted to patterns of predominant concordance in deeper allelic trees (Avise and Wollenberg 1997).

Evolution of Electric Organs and Signals

Hypotheses of character evolution are only as robust as the phylogenetic hypotheses on which they are based. In the Gabon-clade *Brienomyrus*, species have electric organs composed of electrocytes of one of two types, NPp or Pa. Species with NPp electrocytes produce EOD waveforms with a head-positive phase followed by a head-negative phase. Species with Pa electrocytes produce EOD waveforms with an additional head-negative prepulse that precedes the first head-positive phase. In Figure 5 we mapped the two electrocyte types onto the AFLP topology in MacClade 4.0. Although the AFLP tree is incompletely resolved, the unweighted parsimony analysis of this binary character indicates a minimum of three reversals to the NPp condition from the hypothesized ancestral Pa electrocyte condition. In no case is the evolution of Pa electrocytes from an NPp ancestor indicated. By contrast, the cyt *b* topology requires a minimum of nine steps for this character, including two NPp-to-Pa transitions. The AFLP tree indicates a much greater degree of phylogenetic conservatism for this electrocyte and signal character than does the cyt *b* tree, if interpreted as a species phylogeny. Futhermore, the AFLP analysis suggests a unidirectionality of character state change in keeping with previously proposed evolutionary scenarios for this character within the subfamily Mormyrinae (Sullivan et al. 2000; Lavoué et al. 2003) in which a single origin of penetrating-stalk type electrocytes was hypothesized early in the history of the group from an NPp ancestor, followed by multiple reversals to the simpler and ontogenetically antecedent NPp character state. We are encouraged by the results of this study that an expanded AFLP dataset generated with the complete complement of known Gabon-clade *Brienomyrus* forms may make possible stronger conclusions about the evolution of electrocyte and EOD characters and their relationship to the remarkable diversification in this clade of fishes.

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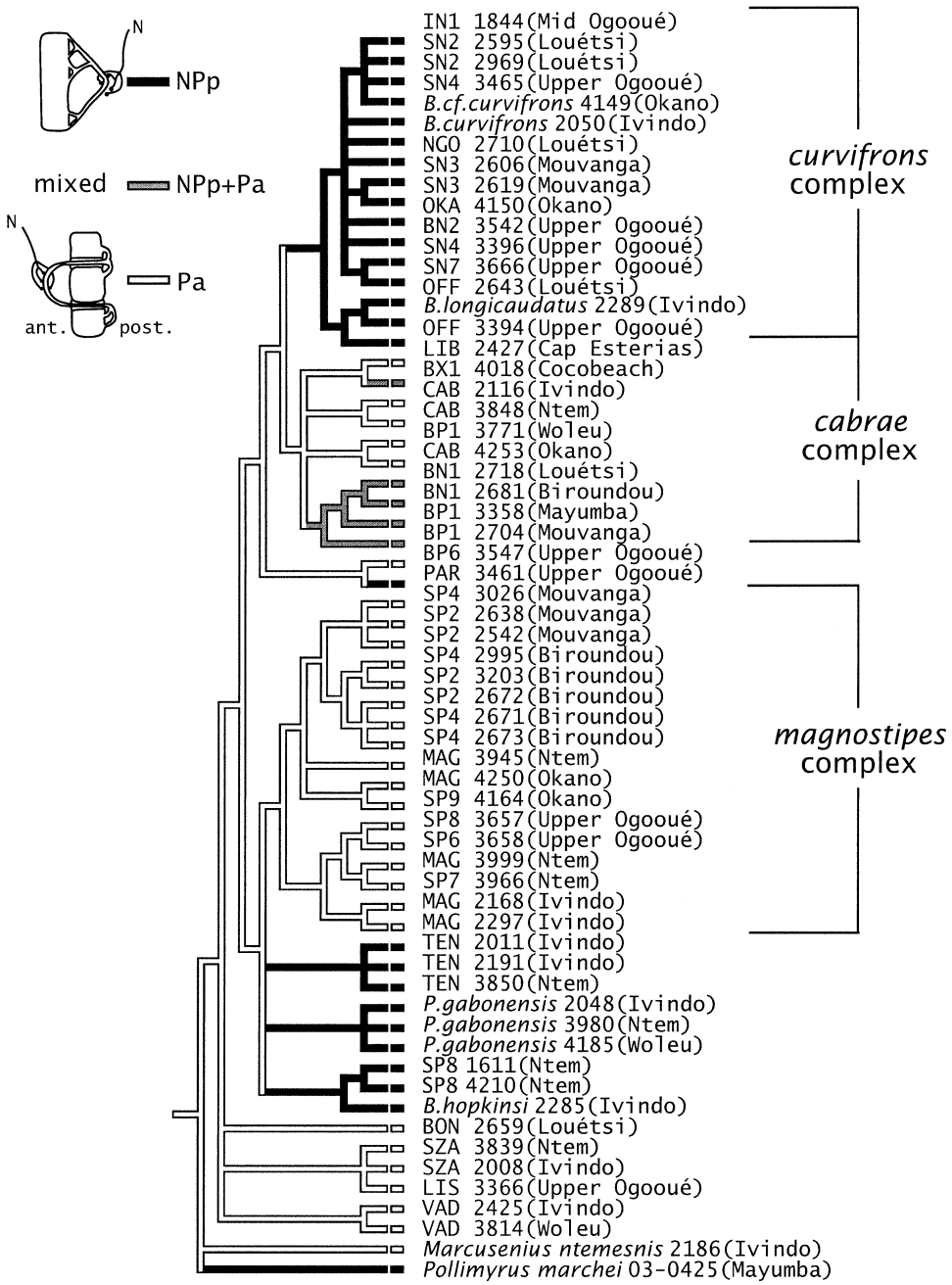

FIG. 5. Evolution of electrocyte structure within the Gabon-clade *Brienomyrus* on the amplified fragment length polymorphism (AFLP) tree. Electrocyte stalks in this group are of two types (illustrated upper left): nonpenetrating with posterior innervation (NPp) or penetrating with anterior innervation (Pa). The electric organs of species with Pa electrocytes produce electric organ discharges (EODs) with an initial head-negative prepulse, which is lacking in those produced by electric organs containing NPp electrocytes. NPp is the primitive condition for the entire family Mormyridae, but Pa is primitive condition for the species studied here (Sullivan et al. 2000; Lavoué et al. 2003). Unweighted parsimony reconstruction of these two character states in Gabon-clade *Brienomyrus* using MacClade 4.0 indicates minimally three independent Pa (open) to NPp (black) reversals on the AFLP consensus tree. No NPp-to-Pa transitions are required. Gray fill branches indicate OTUs in which electric organs have some Pa and some NPp electrocytes and in which EOD prepulse is small to invisible in recordings. These were coded as ''Pa'' in the analysis. Reconstruction of this character on the cytochrome *b* tree (not shown) requires minimally nine steps, including at least two NPp-to-Pa transitions.

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