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SCIENCE DIRECT
Molecular Phylogenetics and Evolution 40 (2006) 332–346
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogenetic relationships and phylogeography of the Killifish species of the subgenus *Chromaphyosemion* (Radda, 1971) in West Africa, inferred from mitochondrial DNA sequences

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Received 10 March 2006; accepted 14 March 2006
Available online 19 April 2006

Abstract

We have analyzed the phylogenetic relationships of 160 specimens from 88 samples representing all defined species of the African Aplocheiloid subgenus *Chromaphyosemion* in order to examine the monophyly of this group, the species interrelationships, and to reveal trends in chromosomal evolution and formulate hypotheses about their evolutionary history. The data set comprised 1153 total nucleotides from the mitochondrial 12S rRNA, cytochrome oxidase I, and D-loop. The molecular-based topologies were analyzed by maximum parsimony, maximum likelihood, distance method and Bayesian inference support the monophyly of the subgenus *Chromaphyosemion*. All populations with ambiguous taxonomic status were assigned to an already described species except *A.* sp. Rio Muni which corresponds to a still undescribed species. *Aphyosemion alpha* and *A. lugens* were in basal position in the different trees that indicate a possible origin of the subgenus *Chromaphyosemion* in the South Cameroon–North Gabon region. Furthermore, the South Cameroon region (between 2° and 3° of North latitude) that accommodates half of the *Chromaphyosemion* species is considered to have been a refuge zone during the late quaternary dry events that Africa experienced. Phylogenetic relationships among the subgenus also revealed that chromosomal evolution is complex and should be studied at the intraspecific level.

Keywords: Cyprinodontidae; Mitochondrial DNA; Taxonomy; Biogeography; Refuge zone

1. Introduction

Fish of the subgenus *Chromaphyosemion* inhabit small rivers and freshwater streams in the coastal lowlands from Togo to Gabon (Scheel, 1990). *Chromaphyosemion* was described by Radda (1971) as a subgenus of *Aphyosemion* Myers, 1924. These fishes can be easily distinguished from all other *Aphyosemion* species by several characters including primarily the presence of two dark lateral bands in both sexes (versus one or none) and the capacity of males to change their coloration rapidly depending on stress or hierarchical status (versus less conspicuous changes in *Aphyosemion*). Seegers (1981), Amiet (1987), Murphy and Collier (1999), and Sonnenberg (2000) considered *Chromaphyosemion* as a likely monophyletic group even if they did not give any demonstration. This trend led some authors in more recent publications to consider *Chromaphyosemion* as a genus (Legros et al., 2005; Sonnenberg, 2000; Völker et al., 2005).

It is easier to distinguish a species belonging to *Chromaphyosemion* from that of all the other *Aphyosemion*, than it is to differentiate those within the subgenus *Chromaphyosemion*. There exists no meristic difference among them

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1055-7903/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2006.03.018

although species or forms are distinguished only by male coloration and sometimes female color pattern as well. Despite the efforts made by many authors to clarify the taxonomic confusion in this group (Amiet, 1987; Scheel, 1990; Seegers, 1986; Sonnenberg, 2000; Wildekamp et al., 1986), questions exist concerning the validity of some described species and the taxonomic status of some populations. Color patterns are difficult to interpret when species exhibit a high degree of variability. A classic example of this is *A. splendopleure* (Brüning, 1929) for which several phenotypes have been identified: Dizangue, Kopongo, and Meme. For other populations that exhibit a unique color pattern it would be difficult to know if these differences correspond to species differentiation or simply correspond to intraspecific variations: *A.* sp. No6 appears to be very close to *A. lugens* (Amiet, 1991), *A.* sp. Rio Muni, *A.* sp. Likado or *A.* sp. Mboro are all different but seem close to *A. splendopleure*, etc. Some described taxa are also questioned: *A. volcanum* (Radda and Wildekamp, 1977) is sometimes considered as a junior synonym of *A. splendopleure* and *A. pappenheimi* (Ahl, 1924) considered as a synonym of *A. loennbergii* (Boulenger, 1903). This high degree of phenotypic differentiation is paralleled by a high amount of karyotype diversity. Chromosome number and chromosome morphology have been reported to vary not only among species but often also among populations of the same nominal species (Scheel, 1990; Völker et al., 2005). For example, diploid numbers are ranged from $2N=20$ to $2N=40$. Populations of *A. riggenbachi* exhibit different karyotypes varying from $2N=20$ to $2N=38$. The remarkable phenotypic and karyotypic variability renders the subgenus *Chromaphyosemion* a highly suitable model for evolutionary studies.

Only six species are unambiguously recognized: *A. alpha* (Huber, 1998), *A. bivittatum* (Loennberg, 1895), *A. riggenbachi* (Ahl, 1924), *A. bitaeniatum* (Ahl, 1924), *A. kouamense* (Legros, 1999), and *A. loennbergii*. Recently, two new species have been described based on coloration and genetic differentiation (mtDNA analyses), *A. melanogaster* (Legros et al., 2005), formerly *A.* sp. No7, and *A. punctulatum* (Legros et al., 2005), formerly *A.* sp. No4. Finally, some species have been considered by one or several authors as dubious or in need of revision: *A. multicolor* (Brüning, 1929), *A. pappenheimi*, *A. poliaki* (Amiet, 1991), *A. splendopleure*, and *A. volcanum*.

Poor knowledge of the *Chromaphyosemion* taxonomy and phylogenetic relationships considerably hamper inferences and evolutionary studies that could be made on this particular group of species. Mitochondrial DNA sequences have already been successfully used to assess phylogenetic relationships in fishes including Cyprinodontidae (Duvernell and Turner, 1998; Hrbek and Meyer, 2003; Hrbek et al., 2005; Lüssen et al., 2003; Murphy and Collier, 1999). In the present study, we analyzed most of the known species and forms of *Chromaphyosemion* to obtain genetic data in order to (1) clarify the taxonomic status of the different species, forms, and populations, (2) demonstrate the monophyletic status of *Chromaphyosemion*, (3) give insight into the phylogeography of the group, and (4) determine their chromosomal evolutionary trend.

2. Materials and methods

2.1. Fish samples and DNA extraction

Samples were collected in Cameroon (fishing and export permit number 0020/ASE/MINEPIA/DIRPEC/SDARA) using deep nets in January 2005 from 61 natural populations. The fish were anaesthetized with phenoxyethanol and then preserved in 90% alcohol. Additional specimens either wild catch or aquarium strains were obtained from hobbyists and included in the analyses. Localities of collection and taxonomic identification of samples are provided in Fig. 1 and Table 1.

Voucher specimens for natural populations were deposited in the Royal Museum of Central Africa in Tervuren (Belgium) and registered under number 2005-16-P-01 to 2005-16-P-104 and 2005-11-P-01 to 2005-11-P-35.

2.2. DNA amplification and sequencing

Total DNA was extracted from muscle tissues preserved in alcohol using GenElute mammalian GENOMIC DNA Miniprep Kit from Sigma. Amplification protocols were identical for the mtDNA D-loop, 12S rRNA and cytochrome oxidase I (COI) region fragments: 35 cycles beginning with 3 min at 93 °C for initial denaturation followed by cycles of 30 s at 93 °C, 30 s at 52 °C for annealing, and 30 s at 72 °C for extension, with a final 5 min extension step at 72 °C. Primers used for the amplification of the D-loop region were ACCCCTAGCTCCCAAAGCTA (forward) and CCTGAAGTAGGAACCAGATG (reverse) while TTTTTGATCCCGCTGGGGGA (forward) and CCAG AGAATAGAGGGAATCAGTG (reverse) were used to amplify COI and CAAACTGGGATTAGATACCCC (forward) and AGGGTGACGGGCGGTGTGT (reverse) were used to amplify 12S rRNA. Primers used for COI and 12S RNA were adapted from Murphy et al. (1999a,b). The primers used enabled the amplification of three different fragments 430 bp (305–309 bp sequenced) for D-loop, 530 bp (459 bp sequenced) for COI, and 400 bp (325 or 326 bp sequenced) for 12S rRNA. These fragments were purified with the ExoSAP-IT kit (Amersham Biosciences) and sequenced with the original primers using Big Dye Terminator reaction Mix (Applied Biosystems). Sequencing reactions were electrophoresed on an ABI 3130 XL automated sequencer (Applied Biosystems). Chromatograms were visually checked and sequences were aligned manually using BioEdit. According to the phylogenetic relationships of African Killifishes in the Genera *Aphyosemion* and *Fundulopanchax* proposed by Murphy and Collier (1999), *Aphyosemion ahli* (Myers, 1933) from Sole (ABC 05/16, corresponding to number 10 on the map) was used as an outgroup. Two specimens of each wild population were sequenced (when adequate individuals were available).

Fig. 1. Map of West Africa, showing approximate distribution ranges of the 12 species of the subgenus *Chromaphyosemion* (grey zone in the small map) with sampling locations. White circles, locations for wild samples collected for this study; black circles, locations for aquarium strain specimens. Even if species ranges overlap, the different species are not sympatric except in five locations (not shown on the map). The isolated population of *A. aff splendopleure* Ekom falls (not studied here) is indicated by a star. The question mark indicates a zone where *Chromaphyosemion* are likely to be present but where fish have never been collected because of the absence of road. The hypothesized refuge zone was indicated in black.

When different haplotypes were observed between two specimens from the same location, then, two additional specimens (when available) were sequenced.

2.3. Sequence analysis

Saturation was analyzed by plotting the absolute number of transitions and transversions against patristic distance values. The aligned sequences were analyzed independently with maximum parsimony (MP), maximum likelihood (ML), distance method (DM), and Bayesian inference analysis (BI). For ML, DM, and BI analyses, the Akaike Information Criterion (AIC) approach was used to find the best model of evolution that fit our data using the program ModelTest 3.7 (Posada and Crandall, 1998). ML test was carried out with the PHYML program (Guindon and Gascuel, 2003), MP with Phylip 3.57 (Felsenstein, 1993), DM with PAUP 4.0 (Swoford, 2001) using the Neighbor-joining method (NJ; Saitou and Nei, 1987), and BI with MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Bootstrap analysis (500 replicates) was used (for ML, MP, and DM) to assess the relative robustness of branches (Felsenstein, 1985). Consensus trees were obtained using PHYLIP 3.57 (Felsenstein, 1993).

3. Results

3.1. Sequence variation

A total of 160 specimens representing 88 sampled populations belonging to 18 species or forms have been

Table 1
Population number (No), origin, locality, coordinates, species identification, previous determination (PrDet), and voucher numbers

No	Origin	Locality	Coordinates	Species	Pr.Det	Voucher number
1	AS	Ibadan, 47 km of Lagos	7.400°N, 3.900°E	BIT		
2	AS	Umudike	5.310°N, 7.250°E	BIT		
3	AS	Kwa River Falls Plantation	5.070°N, 8.200°E	BIV		
4	AS	Biafra	4.750°N, 8.920°E	BIV		
5	AS	Funge	4.764°N, 8.907°E	BIV		
6	ABC 05/12	Funge	4.764°N, 8.907°E	BIV		2005-16-P-19-20
7	ABC 05/13	Ekondo Nene	4.690°N, 8.966°E	VOL	SPP	2005-16-P-21-22
8	ABC 05/14	Kumba	4.654°N, 9.506°E	VOL	SPP	2005-16-P-23-24
9	ABC 05/15	Two Wata	4.694°N, 9.791°E	VOL	SPP	2005-16-P-25-26
10	ABC 05/16	Sole	4.602°N, 9.814°E	VOL	SPP	2005-16-P-27-28
11	AS	Lykoko	4.400°N, 9.330°E	VOL	SPP	
12	ABC 05/10	Muyuka	4.380°N, 9.439°E	VOL	SPP	2005-16-P-17-18
13	ABC 05/08	Mile 29	4.250°N, 9.360°E	VOL	PLKaff	2005-16-P-15-16
14	AS	Ekona CBL 01/22	4.340°N, 9.561°E	PLK		
15	ABC 05/07	3 km North Buea cross road	4.254°N, 9.207°E	PLK		2005-16-P-13-14
16	AS	Monea	4.200°N, 9.300°E	PLK		
17	AS	Bolifamba	4.150°N, 9.300°E	PLK		
18	AS	CBL 01/23	4.138°N, 9.395°E	PLK		
19	ABC 05/06	North of Moliwe	4.182°N, 9.308°E	VOL	SPP	2005-16-P-11-12
20	AS	Rio Consul GEMHS 00/43	3.993°N, 9.053°E	SPP	SPBK	
21	AS	Bimbia Camp	3.854°N, 9.184°E	SPP	SPBC	
22	AS	Entrance to Tiko CBL 01/25	4.298°N, 9.474°E	SPP		
23	ABC 05/05	7.5 km East of Tiko	4.185°N, 9.469°E	VOL	SPP	2005-16-P-9-10
24	AS	Nkwo 97/1	4.516°N, 9.966°E	RIG		
25	ABC 05/18	13 km South Edea-Yabassi cross road	4.373°N, 10.018°E	RIG		2005-16-P-29-30
26	ABC 05/19	Nkouli-Ngnock	4.183°N, 10.050°E	RIG		2005-16-P-31
27	AS	Bonepoupa-Yabassi km 20 C 89/18	4.371°N, 10.195°E	RIG		
28	ABC 05/01	North of Pouma	3.895°N, 10.556°E	LOE		2005-16-P-1-2
29	ABC 05/02	17 km West of Pouma	3.823°N, 10.380°E	LOE		2005-16-P-3-4
30	ABC 05/68	Song Ndong	3.823°N, 10.244°E	LOE		2005-16-P-102-103
31	ABC 05/67	West of Song Ndong	3.800°N, 10.216°E	LOE		2005-16-P-100-101
32	ABC 05/66	East of Batombe	3.809°N, 10.201°E	LOE		2005-16-P-99
33	ABC 05/65	Batombe	3.826°N, 10.168°E	LOE		2005-16-P-97-98
34	ABC 05/03	Song Ndong	3.673°N, 10.257°E	LOE		2005-16-P-5-6
35	AS	Edea-Yaounde km 18 C 89/31	3.490°N, 10.150°E	LOE		
36	AS	Edea-Yaounde km 18 CSK 95/28	3.816°N, 10.246°E	LOE		
37	AS	Nkakanzok CBL 01/13	3.958°N, 10.452°E	LOE		
38	ABC 05/64	East of Edea	3.773°N, 10.163°E	LOE		2005-16-P-96
39	ABC 05/04	North of Edea	3.840°N, 10.087°E	SPP		2005-16-P-7-8
40	ABC 05/21	Songueland	3.789°N, 10.071°E	SPP		2005-16-P-33-34
41	AS	Sipe CBL 01/15	3.795°N, 10.213°E	SPP	SPK	
42	ABC 05/63	Koukoue	3.962°N, 10.104°E	SPP	SPK	2005-16-P-94-95
43	AS	Apou C 89/30	3.380°N, 10.070°E	LOE		
44	ABC 05/20	Ndorbe River	3.786°N, 9.908°E	SPP		2005-16-P-32
45	AS	Ndog Bong CBL 01/10	4.007°N, 10.000°E	SPP		
46	AS	Benengue CBL 01/11	3.819°N, 10.017°E	SPP		
47	ABC 05/62	North of Bonguen	3.525°N, 10.110°E	SPP		2005-16-P-92-93
48	ABC 05/61	Bivouba	3.349°N, 10.098°E	LOE		2005-16-P-90-91
49	ABC 05/60	Ebea	3.182°N, 10.028°E	LOE		2005-16-P-88-89
50	ABC 05/59	Bipaga I	3.108°N, 9.991°E	SPP		
51	ABC 05/70	Mpolgue	3.030°N, 9.972°E	LOE		
52	ABC 05/38	Makouré II	3.048°N, 10.164°E	LOE		2005-16-P-60-61
53	ABC 05/32	Bissiang	2.988°N, 10.000°E	SPP + LOE		2005-16-P-51-52
54	ABC 05/33	Bissiang	2.986°N, 9.995°E	LOE		2005-16-P-53-54
55	ABC 05/34	East of Bissiang	2.933°N, 10.012°E	LOE		2005-16-P-55
56	ABC 05/35	Bidou I	3.044°N, 10.113°E	LOE		2005-16-P-56-57
57	ABC 05/36	Bifoum	3.074°N, 10.389°E	LOE	PAP?	2005-16-P-58-59
58	AS	Bidjouka BLLMC05/25	3.139°N, 10.595°E	LOE	PAP?	
59	ABC 05/31	Akom II	2.813°N, 10.568°E	LOE		2005-16-P-49-50
60	ABC 05/30	Assok I	2.887°N, 10.458°E	LOE		2005-16-P-47-48
61	ABC 05/29	Aso I	2.789°N, 10.453°E	LOE		2005-16-P-45-46
62	ABC 05/28	Fenda	2.817°N, 10.381°E	LUGcf	SP6	2005-16-P-43-44
63	ABC 05/27	Elon	2.807°N, 10.234°E	LOE		2005-16-P-41-42

(*continued on next page*)

Table 1 (*continued*)

No	Origin	Locality	Coordinates	Species	Pr.Det	Voucher number
64	ABC 05/26	Adjap I	2.816°N, 10.164°E	LOE		2005-16-P-39-40
65	ABC 05/25	Afanouan	2.833°N, 10.095°E	LOE		2005-16-P-37-38
66	ABC 05/24	Nko olong	2.853°N, 10.033°E	MEL		2005-16-P-35-36
67	ABC 05/23	West of Naha	2.885°N, 9.970°E	MEL		2005-11-P-06-07
68	ABC 05/22	West of Nagadjo	2.900°N, 9.951°E	MEL		2005-11-P-01-05
69	ABC 05/39	Ebome	2.899°N, 9.907°E	MEL		2005-16-P-62-63
70	ABC 05/42	Mbode	2.749°N, 9.877°E	SPP	SPM	2005-16-P-64-65
71	ABC 05/43	North of Mboro	2.734°N, 9.878°E	SPP	SPM	2005-16-P-66-67
72	ABC 05/58	700 m North of Lolabe River	2.687°N, 9.857°E	MEL		2005-16-P-86-87
73	ABC 05/46	Near Ebodje	2.582°N, 9.848°E	SPP	SPL	2005-16-P-69-70
74	ABC 05/57	Mamelles region	2.492°N, 9.907°E	SPP	SPL	2005-16-P-85
75	ABC 05/47	Malaba	2.497°N, 9.836°E	SPP	SPL	2005-16-P-71
76	ABC 05/48	Afan Essokie II	2.464°N, 9.841°E	SPP	SPL	2005-16-P-72-73
77	ABC 05/49	Etonde Fang	2.427°N, 9.849°E	PUN		2005-16-P-74-75
78	ABC 05/50	Pondo road (Campo)	2.371°N, 9.855°E	PUN		2005-16-P-76-77
79	ABC 05/51	Bibabimvoto	2.362°N, 9.912°E	PUN		2005-11-P-23-25
80	ABC 05/56	Akak	2.376°N, 9.964°E	PUN		2005-16-P-83-84
81	ABC 05/52	Afan Essokie	2.381°N, 9.979°E	LUG		2005-16-P-105-106
82	AS	Afan Essokie HLM 99/28	2.395°N, 9.992°E	LUG		
83	ABC 05/53	East of Afan Essokie	2.382°N, 9.992°E	LUG		2005-16-P-78
84	ABC 05/54	N'koleon	2.396°N, 10.043°E	LUG		2005-16-P-79-80
85	ABC 05/55	Gate of the Campo Parc	2.372°N, 10.104°E	LUG		2005-16-P-81-82
86	AS	North Ndyiacom GEMHS 00/32	2.149°N, 10.005°E	SPRM		
87	AS	27.8 km NE Kougouleu LEC 93/24	0.250°N, 10.040°E	KOU		
88	AS	17,1 km to Cap Esterias LEC 93/26	0.566°N, 9.383°E	ALP		

AS, aquarium strain; ABC 05/.., fish collected during a filed trip in January 2005 by J.F. Agnèse, R. Brummett, and P. Caminade, the last number refer to the station number during this collection trip; identically CBL 01/.., C89/.., GEMHS 2000/.., CSK 95/.., BLLMC 05/.., HLM 99/.., and LEC 93/.. refer to other field trips. Details concerning all these collections can be found at http://www.killifish.f9.co.uk/Killifish/Killifish%20Website/Code_Library/Code_Index.htm, or at http://www.chromaphyosemion.com or at http://chromaphyosemion.killi.org/foreigners/index.html. ALP, *A. alpha*; BIT, *A. bitaeniatum*; BIV, *A. bivittatum*; KOU, *A. kouamense*; LUG, *A. lugens*; LUGcf, *A. cf lugens*; RIG, *A. riggenbachi*; LOE, *A. loennbergii*; MEL, *A. melanogaster*; PAP, *A. pappenheimi*; PLK, *A. poliaki*; PLKaff, *A. aff poliaki*; SPP, *A. splendopleure*; SPBK, *A.* sp. Bioko; SPBC, *A.* sp. Bimbia Camp; SPK, *A.* sp. Koukoue; SPL, *A.* sp. Likado; SPM, *A.* sp. Mboro; SP6, *A.* sp. No6; SPRM, undescribed species from Rio Muni; VOL, *A. volcanum*. All voucher numbers refer to fish deposited at the Royal Museum of Central Africa, Tervuren, Belgium.

successfully analyzed. A 459-bp fragment of the COI gene was consistently sequenced. The *Chromaphyosemion* dataset contained 163 variable sites, 131 of which were parsimony informative. The most variable sites were found in third codon position (147, i.e., 90%), 4 (2%) in second codon positions, and 12 (8%) in first codon positions. These variations led to 10, non-parsimony informative, amino acid substitutions. The D-loop fragments sequenced were variable in size ranging from 305 to 309 bp due to several indels. A total of 82 putative mutations were scored from which 51 were parsimony informative. One indel was also observed in the 325- to 326-bp long 12S rRNA fragments in which 53 putative mutations were scored (30 were parsimony informative). All the new sequences have been deposited in GenBank under Accession Nos. DQ267261–DQ267418 for COI, DQ278264–DQ278418 and DQ286834 for 12S rRNA, and DQ284591–DQ284749 for D-loop.

Sequences of the three genes were aligned (D-loop aligned sequences were registered in the EMBL database: ALIGN_000981) and merged resulting in 102 different haplotypes of 1153 bp (total length after alignment and including indels). Empirical attempts were made to detect putative saturation within *Chromaphyosemion* sequences. The observed numbers of transitions were plotted against the observed numbers of transversions for all pairs of sequences. The observed numbers of transitions or transversions were also plotted against all pairwise distances (*P* distances). All plots indicated relatively linear relationships between substitution rates and genetic distances (not shown). This suggested that transitions and transversions were not saturated.

3.2. Monophyletic status of the Chromaphyosemion subgenus

In order to assess the monophyletic status of *Chromaphyosemion*, 12 partial sequences of the 12S rRNA gene (325 bp) representing all the 12 *Chromaphyosemion* species commonly considered as valid were aligned and compared with other *Aphyosemion* and *Fundulopanchax* sequences obtained from GenBank (from analyses of Murphy and Collier, 1999). The *Aphyosemion* species represented all defined species groups in this genus (Scheel, 1990): *calliurum* group (CAL), *cameronense* group (CAM), *coeleste* group (COL), *elegans* group (ELE), *exiguum* group (EXI), *georgiae* group (GEO), *striatum* group (STR), and one species remaining ungrouped (UG). *Aphyosemion hera* Huber, 1998, not studied in Murphy and Collier (1999), was included because this species seems to be close to *Chromaphyosemion* species when taking into account meristic data (Huber, 1998). Only two *Fundulopanchax*

species were retained because *Fundulopanchax* is a monophyletic genus and sister to *Aphyosemion* (Murphy and Collier, 1999). These two species have been introduced in the analyses in order to root the trees.

Table 2 summarizes the pairwise divergence values (Kimura 2 genetic distances; Kimura, 1980) among all species of Killifish studied. Values between *Fundulopanchax* species and *Aphyosemion* species were ranged between 0.1009 and 0.2680 (mean = 0.1649) while distances between *Aphyosemion* species (including species from the subgenus *Chromaphyosemion*) were ranged from 0.0031 to 0.1256 (mean = 0.0783). Inside the subgenus *Chromaphyosemion*, pairwise divergences never exceeded 0.0540 (mean = 0.0 255).

The General Time Reversible model with among site heterogeneity (GTR + I + G) was selected as the best fit for our data using ModelTest (Posada and Crandall, 1998). Rate matrix parameters were $R(a) = 1551.2255$, $R(b) = 4210.1299$, $R(c) = 3014.7166$, $R(d) = 201.8017$, and $R(e) = 13949.0020$. Among site variation was approximated with the gamma distribution shape parameter $\alpha = 0.5439$. These parameters were used for subsequent phylogenetic analyses. The analyses based on MP, ML, DM, and BI produced congruent topologies. Fig. 2 shows the consensus tree obtained with the three different methods. The two *Fundulopanchax* species clustered together as previously described in Murphy and Collier (1999), and were used to root the trees. As earlier observed the species groups within *Aphyosemion* were generally strongly supported while the relationships between the groups were weaker (Murphy and Collier, 1999). Species from the CAL group: *A. celiae*, *A. calliurum*, *A. ahli*, and *A. australe* were clustered together with the *Chromaphyosemion* species but *Chromaphyosemion* species appeared clearly as a monophyletic assemblage (Bootstrap values—bv—ranged from 67 to 75%). *A. hera* was related to *A. ogoense* and *A. louessense* (striatum group) and not to any *Chromaphyosemion* species. Meristic similarities between *Chromaphyosemion* species and *A. hera* are then only due to convergence and have no phylogenetic significance.

Species phylogenetic relationships within *Chromaphyosemion* could not be resolved in this analysis but *A. alpha*, *A. lugens*, and *A. bitaeniatum* appeared to occupy a basal position inside the *Chromaphyosemion* cluster. This is congruent with Murphy and Collier (1999) observations where *A. alpha* (LEC 93/26 but erroneously referred to as LEC 93/27 in their article) occupied a basal position in the BIV (= *Chromaphyosemion*) group compared with *A. bivittatum* and *A. volcanum*. To further resolve relationships within *Chromaphyosemion*, additional sequence data were necessary.

3.3. Species characterization

The three sequence fragments (D-loop, COI, and 12S rRNA) were combined because they were linked on a single mtDNA molecule and because no incongruence existed between topologies obtained with each of the three sequences (topologies not shown). The highest genetic divergences (*P* distance, excluding indels) were observed, as expected, between the outgroup species *Aphyosemion ahli* and the *Chromaphyosemion* species: *P* distances range from 0.0972 to 0.1579 between these two groups while pairwise *P* distances between *Chromaphyosemion* species never exceeded 0.1134.

The Tamura–Nei model (Tamura and Nei, 1993) with among site heterogeneity (TN + I + G) was selected as the best fit for our data using ModelTest (Posada and Crandall, 1998). Rate matrix parameters were $R(a) = 1.0000$, $R(b) = 10.4125$, $R(c) = 1.0000$, $R(d) = 1.0000$, $R(e) = 14.2876$, and $R(f) = 1.0000$. Among site variation was approximated with the gamma distribution shape parameter $\alpha = 0.6753$. These parameters were used for subsequent phylogenetic analyses. The analyses based on MP, ML, DM, and BI produced congruent topologies. Fig. 3 shows the consensus tree obtained with the three different methods.

Seven species were clearly genetically defined: *A. alpha* (bv ranged from 83 to 100%), *A. bitaeniatum* (98–100%), *A. bivittatum* (100%), *A. melanogaster* (99–100%), *A. riggenbachi* (96–100%), *A. punctulatum* (100%), and *A. loennbergii* (82–100%). *A. kouamense* appeared separated from all other species. *A.* sp. Rio Muni (GEMHS 00/32) was clustered with *A. melanogaster* (these two species can be easily separated on a color pattern basis). *A. volcanum*, *A. poliaki* and *A. splendopleure* were grouped together (bv between 54 and 80%). The first two species were clustered together with a high bootstrap value of 100%. In this cluster, all samples belonging to *A. poliaki* were grouped together (bv between 46 and 71%).

Several populations with ambiguous taxonomic status were analyzed in this study. *A. cf. lugens* (62) was grouped together with *A. lugens* (bv varied from 95 to 100%). These two species have extremely similar phenotypes. It is very likely that the population of *A. cf. lugens* (also called *A.* sp. No6) belong to *A. lugens*. *Aphyosemion aff. splendopleure* CBL 01/10, CBL 01/11 (45, 46), *A.* sp. Sipe (41), *A.* sp. Koukoue (42), *A.* sp. Bioko GEMHS 00/43 (20), and *A.* sp. Bimbia Camp (21) are clearly clustered with all *A. splendopleure* populations and should be considered as belonging to this species. Samples 70, 71 on one hand and 73, 74, 75, and 76 on the other hand correspond to what was referred as to *A.* sp. Mboro and *A.* sp. Likado, respectively. These two forms should also be considered as belonging to *A. splendopleure*.

Aphyosemion aff. poliaki ABC 05/8 (13) present at "Mile 29" is clustered with *A. volcanum* populations. Amiet (1991) when describing *A. poliaki* left this population with an undetermined taxonomic status because its color pattern was quite different from the other *A. poliaki* populations pattern. Moreover, it is not impossible that at "Mile 29" two different species or forms exist. Amiet (1991) noted that pictures of *Chromaphyosemion* captured at "Mile 29" at different times looked different. The present results demonstrated that the "Mile 29" population studied belongs to *A. volcanum* and not to *A. poliaki*. In this study,

Table 2
Pairwise divergence values (Kimura 2 genetic distances) between all species studied

	DEL	WAL	EXU	BUA	CYA	LAB	MIM	MAC	CAM	OCC	AUR	CIT	COE	LAM	CHR	COG	DEC	ELE	MLP	STR	GAB
WAL	.0883																				
EXU	.1452	.1369																			
BUA	.1375	.1372	.0217																		
CYA	.1383	.1420	.0927	.0821																	
LAB	.1297	.1364	.0883	.0988	.0989																
MIM	.1446	.1477	.1023	.1131	.1066	.0413															
MAC	.1487	.1477	.0990	.1133	.1033	.0381	.0187														
CAM	.1452	.1443	.1027	.1030	.1071	.0317	.0250	.0124													
OCC	.1456	.1450	.1131	.1063	.1099	.0675	.0811	.0745	.0610												
AUR	.1611	.1490	.1098	.1135	.1101	.0476	.0711	.0579	.0545	.0250											
CIT	.1526	.1482	.1207	.1245	.1134	.0642	.0781	.0714	.0646	.0443	.0347										
COE	.1301	.1296	.0890	.0962	.0893	.0446	.0480	.0416	.0447	.0541	.0379	.0543									
LAM	.1370	.1328	.0713	.0919	.0920	.0281	.0577	.0479	.0513	.0809	.0577	.0743	.0446								
CHR	.1370	.1401	.0781	.0919	.0920	.0313	.0610	.0578	.0546	.0843	.0677	.0776	.0578	.0155							
COG	.1367	.1361	.0816	.1025	.0957	.0314	.0611	.0513	.0547	.0845	.0611	.0778	.0513	.0093	.0124						
DEC	.1367	.1435	.0885	.1025	.0957	.0314	.0611	.0579	.0547	.0777	.0611	.0778	.0579	.0155	.0124	.0062					
ELE	.1330	.1398	.0850	.0990	.0957	.0282	.0578	.0546	.0514	.0811	.0644	.0744	.0546	.0124	.0093	.0031	.0031				
MLP	.1407	.1438	.0781	.0919	.0955	.0281	.0577	.0545	.0513	.0809	.0643	.0743	.0545	.0124	.0093	.0093	.0093	.0062			
STR	.1381	.1487	.1176	.1215	.1068	.0923	.0925	.0892	.0964	.0997	.0964	.1032	.0823	.0714	.0782	.0784	.0784	.0818	.0816		
GAB	.1542	.1649	.1215	.1253	.1183	.0929	.1072	.1039	.0966	.1034	.1072	.1034	.1034	.0719	.0719	.0789	.0720	.0754	.0753	.0251	
OGO	.1446	.1329	.0885	.0956	.0788	.0917	.1063	.1030	.1067	.1207	.0993	.1135	.0925	.0852	.0921	.0923	.0923	.0958	.0956	.0917	.0954
LOU	.1483	.1293	.0881	.0986	.0751	.0883	.1093	.0990	.1027	.1166	.0993	.1025	.0995	.0781	.0815	.0816	.0816	.0850	.0849	.0952	.0954
HER	.1603	.1634	.1251	.1253	.1109	.1176	.1251	.1253	.1220	.1217	.1256	.1145	.1145	.1176	.1212	.1251	.1251	.1251	.1248	.0956	.1067
EXD	.1180	.1325	.0850	.0956	.0822	.0781	.0923	.0890	.0927	.1030	.0997	.0991	.0753	.0714	.0781	.0783	.0851	.0817	.0815	.0713	.0748
PRI	.1177	.1249	.0921	.0958	.0855	.0848	.0921	.0888	.0925	.0993	.0960	.0989	.0717	.0780	.0848	.0849	.0918	.0884	.0882	.0611	.0818
ALH	.1120	.1009	.0960	.0927	.0959	.0645	.0890	.0823	.0790	.1032	.0925	.1064	.0792	.0715	.0783	.0819	.0853	.0819	.0817	.0854	.0964
CAL	.1161	.1156	.1069	.1179	.0998	.0647	.0964	.0896	.0863	.1032	.0925	.1067	.0896	.0785	.0785	.0750	.0716	.0750	.0820	.1036	.1079
AUS	.1193	.1188	.0995	.0927	.0920	.0709	.0886	.0820	.0787	.0923	.0886	.1024	.0789	.0712	.0780	.0815	.0849	.0815	.0814	.0681	.0787
CEL	.1123	.1011	.0962	.0929	.0891	.0610	.0892	.0825	.0792	.0852	.0816	.0953	.0719	.0747	.0747	.0783	.0817	.0783	.0781	.0999	.1112
ALP	.1294	.1325	.1058	.0990	.0819	.0914	.1058	.0990	.1027	.1131	.1027	.1122	.0960	.0880	.0914	.0916	.0916	.0950	.0948	.0956	.1138
LUG	.1297	.1364	.1060	.0993	.0955	.0776	.0986	.0919	.0886	.0919	.0886	.0985	.0854	.0810	.0776	.0846	.0846	.0812	.0810	.0915	.0956
BIT	.1149	.1404	.0885	.0886	.0887	.0812	.0988	.0956	.0958	.1095	.1027	.1238	.0856	.0744	.0812	.0848	.0882	.0848	.0846	.1030	.1143
MEL	.1221	.1325	.0885	.0921	.0819	.0812	.0919	.0852	.0850	.0952	.0919	.1122	.0784	.0711	.0812	.0814	.0882	.0848	.0846	.0886	.0958
BIV	.1182	.1286	.0849	.0885	.0749	.0776	.0849	.0782	.0852	.0954	.0921	.1124	.0751	.0676	.0776	.0778	.0846	.0812	.0810	.0850	.0995
RIG	.1291	.1322	.0952	.0988	.0886	.0878	.1021	.0954	.0990	.1023	.0990	.1266	.0888	.0776	.0878	.0880	.0880	.0914	.0912	.0919	.1030
KOU	.1219	.1286	.0917	.0919	.0851	.0844	.0986	.0919	.0956	.0988	.0956	.1089	.0785	.0743	.0844	.0846	.0914	.0880	.0878	.0885	.0995
PLK	.1369	.1326	.1024	.0922	.0955	.0881	.1059	.0991	.0959	.1026	.1029	.1306	.0926	.0813	.0915	.0917	.0952	.0952	.0950	.0991	.1104
VOL	.1182	.1213	.0849	.0816	.0817	.0743	.0917	.0850	.0818	.0919	.0921	.1160	.0785	.0676	.0776	.0778	.0846	.0812	.0810	.0919	.1030
SPP	.1257	.1288	.0919	.0886	.0887	.0812	.0988	.0921	.0888	.0990	.0993	.1233	.0856	.0744	.0846	.0848	.0916	.0882	.0880	.0956	.1067
LOE	.1219	.1249	.0883	.0850	.0851	.0776	.0952	.0885	.0852	.0954	.0956	.1195	.0820	.0709	.0810	.0812	.0880	.0846	.0844	.0919	.1030
PUN	.1219	.1249	.0883	.0850	.0851	.0776	.0952	.0885	.0852	.0954	.0956	.1195	.0820	.0709	.0810	.0812	.0880	.0846	.0844	.0919	.1030

	OGO	LOU	HER	AXD	PRI	ALH	CAL	AUS	CEL	**ALP**	**LUG**	**BIT**	**MEL**	**BIV**	**RIG**	**KOU**	**PLK**	**VOL**	**SPP**	**LOE**	
LOU	.0250																				
HER	.0751	.0820																			
EXD	.0443	.0508	.0820																		
PRI	.0542	.0542	.0852	.0124																	
ALH	.0815	.0883	.1171	.0849	.0746																

CAL	.0890	.0925	.1176	.0999	.0962	.0283																			
AUS	.0883	.0883	.1100	.0747	.0645	.0218	.0479																		
CEL	.0923	.0958	.1174	.0959	.0853	.0155	.0251	.0379																	
ALP	.0847	.0776	.1281	.0776	.0741	.0711	.0821	.0677	.0715																
LUG	.0986	.0845	.1207	.0914	.0810	.0543	.0751	.0443	.0546	**.0540**															
BIT	.0988	.0984	.1317	.0848	.0846	.0577	.0821	.0511	.0648	**.0476**	**.0412**														
MEL	.0881	.0946	.1174	.0709	.0741	.0610	.0821	.0479	.0648	**.0506**	**.0410**	**.0251**													
BIV	.0811	.0909	.1135	.0674	.0706	.0609	.0820	.0478	.0646	**.0473**	**.0409**	**.0282**	**.0062**												
RIG	.0913	.0944	.1243	.0775	.0807	.0676	.0820	.0543	.0714	**.0505**	**.0474**	**.0314**	**.0124**	**.0155**											
KOU	.0948	.0978	.1171	.0741	.0773	.0642	.0854	.0510	.0680	**.0473**	**.0377**	**.0282**	**.0093**	**.0124**	**.0155**										
PLK	.0916	.0981	.1137	.0777	.0809	.0644	.0891	.0545	.0682	**.0539**	**.0508**	**.0379**	**.0187**	**.0218**	**.0218**	**.0218**									
VOL	.0845	.0909	.1135	.0674	.0706	.0576	.0785	.0445	.0613	**.0473**	**.0409**	**.0282**	**.0093**	**.0062**	**.0155**	**.0124**	**.0155**								
SPP	.0915	.0980	.1174	.0743	.0775	.0610	.0821	.0479	.0648	**.0539**	**.0442**	**.0282**	**.0093**	**.0124**	**.0155**	**.0124**	**.0155**	**.0062**							
LOE	.0879	.0944	.1135	.0708	.0740	.0576	.0785	.0445	.0613	**.0505**	**.0409**	**.0250**	**.0062**	**.0093**	**.0124**	**.0093**	**.0124**	**.0031**	**.0031**						
PUN	.0709	.0810	.0812	.0880	.0846	.0844	.0919	.1030	.0879	**.0944**	**.1135**	**.0708**	**.0740**	**.0576**	**.0785**	**.0445**	**.0613**	**.0505**	**.0409**	**.0250**					

DEL, *Fundulopanchax deltaense*; WAL, *Fp. walkeri*; EXU, *Aphyosemion exiguum*; CYA, *A. cyanostictum*; LAB, *A. labarrei*; MIM, *A. mimbon*; MAC, *A. maculatum*; CAM, *A. cameronense*; OCC, *A. occelatum*; AUR, *A. aureum*; CIT, *A. citrineipinnis*; COE, *A. coeleste*; LAM, *A. lamberti*; CHR, *A. christyi*; COG, *A. cognatum*; DEC, *A. decorsei*; ELE, *A. elegans*; MLP, *A. melanopteron*; STR, *A. striatum*; GAB, *A. gabunense*; OGO, *A. ogoense*; LOU, *A. louessense*; HER, *A. hera*; EXD, *A. exigoideum*; PRI, *A. primigenium*; ALH, *A. ahli*; CAL, *A. calliurum*; AUS, *A. australe*; CEL, *A. celiae*; ALP, *A. (Chrom.) alpha*; LUG, *A. (Chrom.) lugens*; BIT, *A. (Chrom.) bitaeniatum*; MEL, *A. (Chrom.) melanogaster*; BIV, *A. (Chrom.) bivittatum*; RIG, *A. (Chrom.) riggenbachi*; KOU, *A. (Chrom.) kouamense*; PLK, *A. (Chrom.) poliaki*; VOL, *A. (Chrom.) volcanum*; SPP, *A. (Chrom.) splendopleure*; LOE, *A. (Chrom.) loennbergii*; PUN, *A. (Chrom.) punctulatum*. Abbreviations for species of the subgenus *Chromaphyosemion* and distance between these species are in bold format.

only one population could not be unambiguously identified to a known species: *A.* sp. Rio Muni GEMHS 00/32 (86). Specimens studied clustered with *A. melanogaster* but can be easily distinguished from this species by color pattern. It is then very likely that *A.* sp. Rio Muni GEMHS 00/32 represents an undescribed species.

3.4. Phylogenetic relationships within Chromaphyosemion

The phylogenetic networks (Fig. 3) have been rooted with *A. ahli* following previous observations (Murphy and Collier, 1999) and the present study. Results observed using all the gene partitions were congruent with what was obtained with the 12S rRNA fragment alone (Fig. 2). *A. alpha* and *A. lugens* appeared to be close to the root, followed by *A. bitaeniatum*. All the other species were clustered together but this assemblage was poorly supported (bv ranged from 33 to 63%). Nevertheless, in this poorly defined group, it is possible to identify some clusters. *A.* sp. Rio Muni was related to *A. melanogaster*; *A. volcanum*, *A. poliaki*, *A. splendopleure*, *A. punctulatum*, and *A. loennbergii* formed a monophyletic group (bv ranged from 93 to 100%). In this group, on one hand *A. punctulatum* was related to *A. loennbergii* and on the other hand *A. splendopleure* was related to *A. volcanum* and *A. poliaki*. These two last species were clustered together (bv = 100%) in one paraphyletic group. Phylogenetic relationships of the other species (*A. melanogaster*, *A. riggenbachi*, *A. kouamense* and *A. bivittatum*, and *A.* sp. Rio Muni) could not be completely resolved. *A. melanogaster* and *A.* sp. Rio Muni were nevertheless often grouped together in the analyses.

4. Discussion

4.1. What is a species in the subgenus Chromaphyosemion?

Until recently, the main criteria used to distinguish between *Chromaphyosemion* species were coloration patterns. These criteria gave good results in some cases such as *A. bitaeniatum*, *A. riggenbachi*, and *A. bivittatum*. In many other cases, slight differences or intermediate phenotypes were extremely confusing and led to the creation of various undescribed forms. Recently (Legros et al., 2005), genetic characterization was used together with coloration patterns to described two new species, *A. melanogaster* (formerly *A.* sp. No7) and *A. punctulatum* (formerly *A.* sp. No4). The present study confirmed the power of genetic tools to assess the taxonomic status of the various forms. Several undetermined forms were unambiguously attributed to some species: *A.* sp. Mboro (70,71), *A.* sp. Likado (73–76), *A.* sp. Bimbia Camp (21), *A.* sp. Bioko Island (20), *A.* sp. Sipe (41), *A.* sp. Koukoue (42) were assigned to *A. splendopleure*; *A. cf. lugens* (*A.* sp. No6) could be considered as belonging to *A. lugens*, *A.* sp. Rio Muni (86) very likely corresponds to a undescribed species. Inversely, there is no evidence of *A. pappenheimi* in our sample. Two populations could have

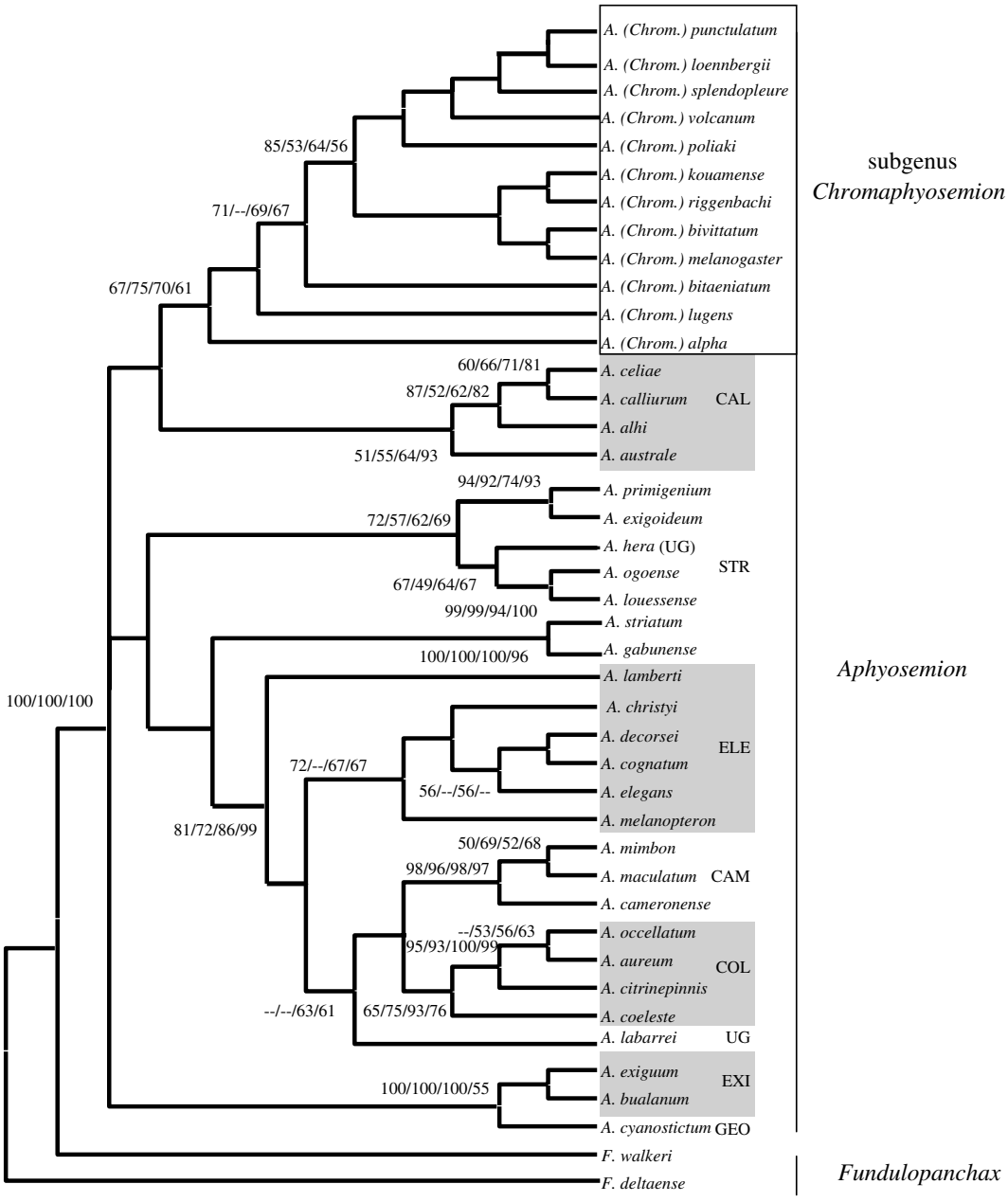

Fig. 2. Consensus tree based on distance method, maximum likelihood, maximum parsimony, and Bayesian inference methods. Numbers above the branches are percentages of bootstrap values based on 500 replicates for each method (except for Bayesian inferences where the numbers represent the percentage of trees with best likelihood scores in which the particular node was found). Values below 50% are not shown. *Fundulopanchax* species (a monophyletic genus according to Murphy and Collier (1999)) were used to root the trees. *Chromaphyosemion* species formed a monophyletic group (bootstrap values ranged from 67 to 75%). *Aphyosemion* (except *A. hera*) and *Fundulopanchax* sequences used are the same than those used in Murphy and Collier (1999) and are available in GenBank. CAL, *calliurum* group; CAM, *cameronense* group; COL, *coeleste* group; ELE, *elegans* group; EXI, *exiguum* group; GEO, *georgiae* group; STR, *striatum* group; UG, ungrouped species.

been assigned to this species (57, 58) taking into account their geographical position, near Bipindi, *Terra typica* of *A. pappenheimi*. These two populations can be considered as belonging to *A. loennbergii*. This is congruent with some authors' viewpoint that considered *A. pappenheimi* as a junior synonym of *A. loennbergii* (Huber, 1981; Wildekamp, 1993). The specific status of some other doubtful species was confirmed: *A. volcanum*, *A. splendopleure*. It is noteworthy that this last species exhibits the highest degree of phenotypic variability in the subgenus. One can also observe that this species is parapatric to eight *Chromaphyosemion* species (three/four of the described species) and that its range is highly fragmented (Bioko island, Likado region, Mboro region, the main range from Kribi to Tiko, and probably Ekom falls). Populations of *A. splendopleure* will need more investigations to elucidate what is responsible of this high phenotypic diversity.

Only the species status of *A. poliaki* (14–18) still remains unclear. There are two alternative hypotheses: *A. poliaki* is a valid species or *A. poliaki* only represents an ecopheno-

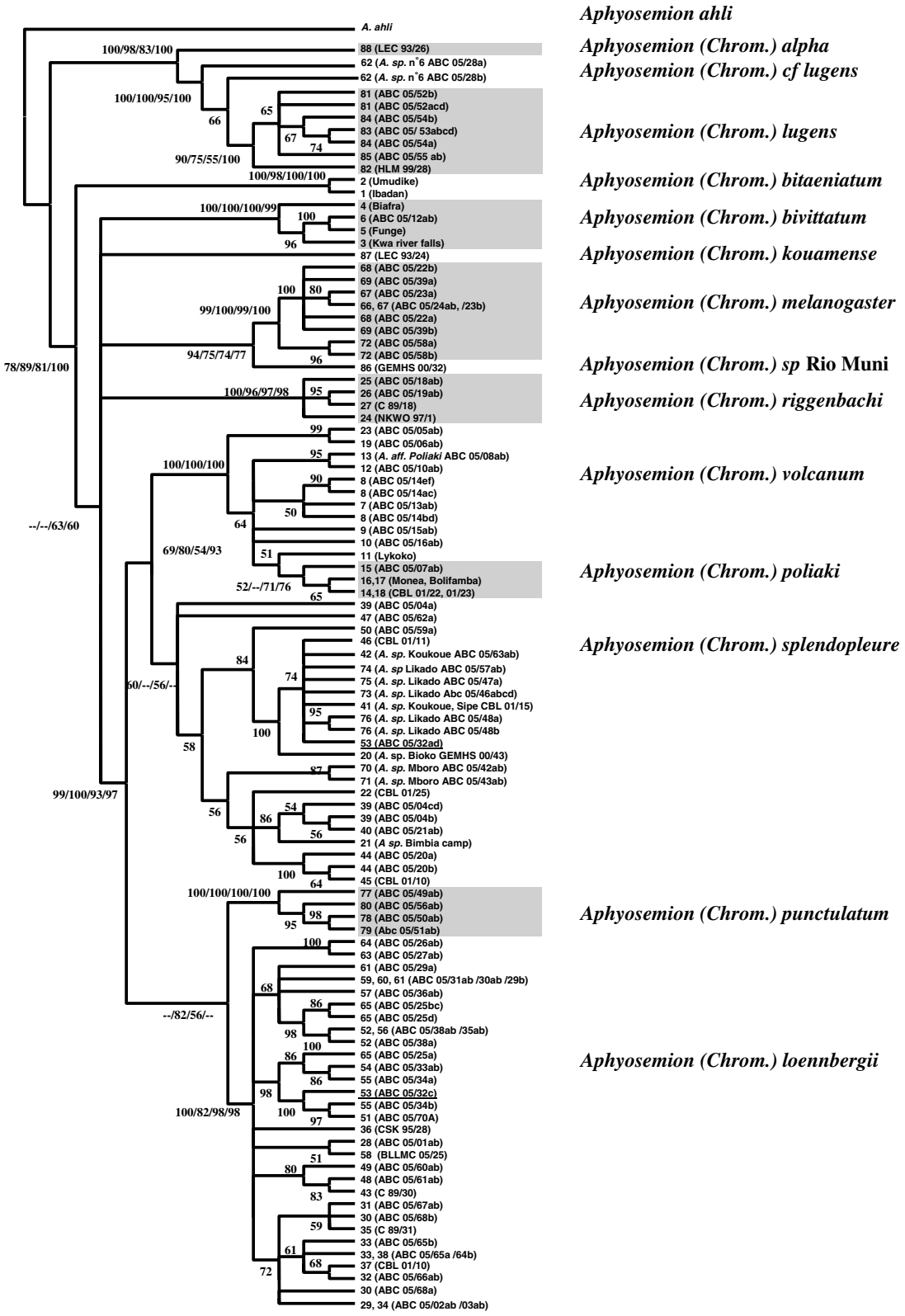

Fig. 3. Consensus tree based on distance method, maximum likelihood, maximum parsimony, and Bayesian inference methods. Numbers above or below the branches are percentages of bootstrap values based on 500 replicates for each method (except for Bayesian inferences where the numbers represent the percentage of trees with best likelihood scores in which the particular node was found). Only bootstrap values with maximum parsimony method are given for intraspecies nodes. Values below 50% are not shown. *Aphyosemion ahli* was used to root the different networks. Numbers correspond to sample location on the map (Fig. 1). When more than one specimen were sequenced from the same location, they have been noted a, b, c, etc. References of specimens collected at location 53 where two different species have been identified are underlined.

type of *A. volcanum*. If *A. poliaki* is a valid species, then *A. volcanum* is paraphyletic (a similar situation has been documented (Agnèse and Teugels, 2001) for the catfish *Clarias gariepinus* and the *Bathyclarias* species flock from Lake Malawi). The present results do not allow choosing between any of these hypotheses. *A. poliaki* is the only *Chromaphyosemion* species that lives in an "uncommon" environment due to Mount Cameroon: high altitude, water with a pH > 7.2 and a temperature often <23 °C, streams with strong current, often outside of the forest with a dark soil (lava). Under these conditions, fishes could have evolved to develop a taller body and a darker coloration than fishes of the coastal plains. These important phenotypic differences do not imply strong genetic differentiations according to our observations. The ecophenotype hypothesis is then applicable. To assess that question, more populations of *A. poliaki* and *A. volcanum* should be investigated using both nuclear and mitochondrial markers with particular attention to the "Mile 29" (13) population. This population has been considered as phenotypically intermediate between *A. poliaki* and *A. volcanum* by Amiet (1991). In our analyses, specimens from Mile 29 unambiguously belong to *A. volcanum*. Indeed, the river flowing at Mile 29 could represent an intermediate biotope between conditions observed on Mount Cameroon (biotopes of *A. poliaki*) and those found in the plain (where *A. volcanum* is found). Nevertheless, phenotypically intermediate populations are not uncommon in *Chromaphyosemion* (Legros, 2000; Sonnenberg, 2000). Another example is provided by *A.* sp. Sipe (41) and *A.* sp. Koukoue (42) that have been grouped under the Koukoue phenotype (Legros, 2000). This phenotype is intermediate between *A. splendopleure* and *A. loennbergii*. In our analyses, these two populations were grouped with the other *A. splendopleure* samples. Interestingly, populations with the Koukoue phenotype (41, 42) are found in a region where both species (*A. splendopleure* and *A. loennbergii*) are present. This situation is somewhat identical to the Mile 29 one, but in this case there is no obvious ecological differentiation between biotopes where *A. splendopleure* and *A. loennbergii* are found. How widespread are these phenotypically intermediate populations? Are they mainly distributed in contact zones between species? What are they originated from? These are questions that we hope to address in the future.

4.2. Need of taxonomic redescription

Aphyosemion splendopleure is undoubtedly a true species and was first described by Brüning (1929), based on specimens from the Tiko area. Meinken (1930) gave a more complete description and deposited one lectotype and one paralectotype (one male and one female) from Tiko area in the Berlin Museum. We know now that in the Tiko area, three *Chromaphyosemion* species are present, *A. splendopleure*, *A. volcanum*, and *A. poliaki*. This situation was unknown to Brüning and Meinken and it seems justifiable to now ask whether the specimens used to describe *A. splendopleure* really belonged to that species. It is unlikely that the observation of preserved lectotype of the Berlin Museum will help to solve the problem because most of the color patterns observed in live specimens disappear when fish are preserved in alcohol and because these species resemble one another. Another alternative would be to carefully look at the description of the live fish given by Brüning (1929). This author described a fish with purple blue to emerald green sides. This description eliminates *A. poliaki* as a possible candidate species (*A. poliaki* has a dark color livery tending toward brown, violet or bronze). There is nothing in the original description about the color of the caudal peduncule that is pinkish in *A. volcanum*. It is then not possible to exclude the hypothesis that the *A. splendopleure* lectotype from Berlin could be a specimen of *A. volcanum*. Consequently, a redescription of *A. splendopleure* based on the present knowledge of the group seems to be necessary.

4.3. Species distribution

Having now clarified most of the taxonomy of *Chromaphyosemion* it is possible to consider the different present factors that could explain the distribution of species. Usually, for a fish species, one major factor can be attributed to the drainage structure. In many cases, it is difficult for fishes to move from one basin to another and species range borders correspond to the drainage limits. Obviously, *Chromaphyosemion* species ranges do not correspond to river basins. For example, there are three species present in the Ntem drainage *A. lugens*, *A. splendopleure*, and *A. punctulatum*. The Sanaga drainage is also shared by three species *A. splendopleure*, *A. loennbergii*, and *A. riggenbachi*. Large rivers could also have made strong barriers to species expansion because *Chromaphyosemion* are never found in deep water environments (even in the surface layer) or where a strong current exists. Nevertheless, it is easy to observe that large rivers have been crossed many times by different species: *A. riggenbachi*, *A. loennbergii*, and *A. splendopleure* crossed the Sanaga River, *A. bitaeniatum* crossed the Niger River. However, Scheel (1968) observed in *A. bitaeniatum* a karyotypic differentiation between populations West of the Niger River and populations East of the River. This observation suggests that large rivers even if not impassable can reduce gene flow. Altitude is another factor often evoked to explain species distribution. *Chromaphyosemion* are never present at an altitude higher than 400 m except *A. poliaki* found up to 600 m. Elevation increases the range between diurnal and nocturnal temperature and also the strength of the water stream. At high altitude, *Chromaphyosemion* are replaced by other rivulins in the same biotopes like species of the subgenus *Kathetys* in Cameroon (Amiet, 1987; Scheel, 1974).

Vegetation could be another factor affecting species distribution. *Chromaphyosemion* are present in forest biotopes, although they still occupy rivers and streams in deforested areas in Benin or Togo. Fishes are able to survive in these

conditions as long as some aquatic vegetation subsists. We can therefore say that forest cover seems to be not necessary to *Chromaphyosemion*. The influence of soil composition, volcanic, sedimentary, and basement complex has been suggested by Scheel (1974) but this hypothesis has been falsified many times. For example, *A. poliaki* is only found on Volcanic soil but *A. volcanum* and *A. splendopleure* are also present on such soil (in Bioko Island for the later). *A. riggenbachi*, present on basement complex soil, is also present on sedimentary soils (Nkapa population). *A. splendopleure* is found in sedimentary, volcanic or basement complex soils.

The final present factor that could explain species distribution is species competition. Outside the *Chromaphyosemion* species range, rivers and streams are occupied by *Aphyosemion* species of the subgenus *Paraphyosemion* (*A. gardneri* (Boulenger, 1991)) in the North, and by species of the subgenus *Kathetys* Eastwards in Cameroon and in Rio Muni. Scheel (1974) considered these species as ecological counterparts of *Chromaphyosemion* species. Inside the *Chromaphyosemion* species range, it is remarkable to observe that species have only parapatric distributions. If two different *Chromaphyosemion* species can be present in a same drainage, they are rarely found in the same location (they occupy different water courses from the same drainage). Four putative cases of sympatry have been observed but have only been reported in "field trip reports" available in hobbyists websites (http://chromaphyosemion.killi.org, http://www.chromaphyosemion.com). Two were observed in the Somakak region between *A. riggenbachi* and *A. loennbergii*, another between *A. riggenbachi* and *A. splendopleure* in the Kopongo region. Another case has been observed in the Kribi–Bipindi region between *A. cf. lugens* (*A.* sp. No6) and *A. loennbergii* (KEK 98/12, Toko area). In the present study, a fifth case is reported between *A. loennbergii* and *A. splendopleure* at Bissiang, ABC 05/32 (53). No natural hybridization event has ever been suspected.

Taking into account that two or more different species are often present in the same drainage, but never in the same water course, these cases of sympatry can be considered as extremely rare and indicate that a strong competition should occur between these species. This has already been hypothesized by Amiet (1987) who observed such species distributions that he called "intricate parapatry." He was even able to observe the replacement of a species by another (in the *cameronense* group) that took place over a space of only a year.

It is then likely that the major present factor affecting the species distribution is interspecific competition. This idea is reinforced by the fact that all *Chromaphyosemion* species inhabit the same biota (except *A. poliaki*) and are morphologically very similar. Species mainly differ by male coloration. These colorations are known to be very important for mating success (Haesler and Seehausen, 2005) and lead to the opportunity of sexual selection that could explain the absence of hybridization. If the present relationships between *Chromaphyosemion* species can be driven by species competition, it can be admitted that historical factors played a major role in species distribution.

4.4. Evolutionary history of Chromaphyosemion species

One would consider *Chromaphyosemion* species being so morphologically close to have evolved very recently in geological time scale. We may compare, for example, with Cichlid fishes from Lake Victoria, which exhibit an important morphological and ethological differentiation and originated probably only few ten of thousand years ago (Verheyen et al., 2003). These extremely morphologically divergent Cichlid species are so genetically close that in many cases, it is not possible to unambiguously determine the species status of a specimen using any genetic tool (Verheyen et al., 2003). Results observed here indicate that even conspecific *Chromaphyosemion* populations are often genetically differentiated. In the absence of any geological data to calibrate a molecular clock it is not possible to precisely determine a time frame for the speciation events. Nevertheless, if we consider that differentiation rate of mtDNA to range between 0.5 and 3% per million years (Echelle et al., 2005; Hrbek and Meyer, 2003) then, the origin of *Chromaphyosemion* species can be estimated to have occurred between 4 and 24 million years ago.

The haplotypes closest to the root (Fig. 2) are those observed in *A. alpha* and *A. lugens* (including *A. cf. lugens*). These species are present in the northern part of Gabon and in the southern part of Cameroon. This region can be considered as the cradle of the subgenus. Nevertheless, this hypothesis has to be confirmed because *A. alpha* and *A. lugens* appeared to be the sister group of all other *Chromaphyosemion* species in Fig. 3 when only *A. ahli* was used to root the tree. From that region, *Chromaphyosemion* probably experienced a large expansion phase that brought species up to the present Togo where *A. bitaeniatum* the next species in the tree is present. More recently, the monophyletic assemblage composed of *A. punctulatum*, *A. loennbergii*, *A. splendopleure*, *A. volcanum*, and *A. poliaki* very likely originated in Cameroon. *A. punctulatum* and *A. loennbergii* are present in the South part while the three other species are present in the North part. These two monophyletic groups could have originated by vicariance. *A. volcanum* and *A. poliaki* have differentiated very recently in the *Chromaphyosemion* history because *A. poliaki* haplotypes are not completely differentiated from the haplotypes of *A. volcanum* (this group is paraphyletic). *Aphyosemion poliaki* could have originated from populations of *A. volcanum* that have been able to adapt to Mount Cameroon biotopes. All *Chromaphyosemion* species are lowland fish, thus the most parsimonious explanation would be a lowland to mountain colonization. Since sexual selection is probably very strong in *Chromaphyosemion*, the simplest hypothesis to explain speciation is to consider vicariance events. During dry phases of the climate, conspecific populations could have been separated in different relict populations.

During that separation time, male colorations could have randomly diverged from their common type and when these populations entered again in contact, reinforcement may have occurred preventing inbreeding. For the last 800,000 years, Africa has experienced cold–dry and warm–humid periods (Maley, 1996a). African forest was very fragmented and only survived in a series of relatively small refuges spread across the original forest area. Some refuge zones have been hypothesized in Cameroon–Gabon region during the late quaternary dry periods (Maley, 1996). Most of these refuges were mountain forest refuges because with altitude, humidity still persisted. It is unlikely that *Chromaphyosemion* survived in these refuges because they do not seem to be able to live at high altitude. *Chromaphyosemion* could mainly have been able to survive at low altitude. If such refuge zones existed, they very likely exhibit at present a high degree of endemism. When we look at the present distribution of species and populations, it can be seen that the region that stretches from 2° and 3° North contains at least six species (*A. splendopleure*, *A. loennbergii*, *A. melanogaster*, *A. punctulatum*, *A. lugens*, and one undescribed species *A.* sp. Rio Muni) that represents half of the *Chromaphyosemion* species. As a consequence, this region could have been a refuge zone for *Chromaphyosemion* during the last dry period and perhaps throughout the different dry phases that the region experienced in the last 800,000 years. Successions of dry and humid phases were numerous and led to many regression extension phases of species range. This explains why some species like *A. splendopleure* have at present a discontinuous range that alternates along the south coast with *A. melanogaster*, or the isolated population of *A. aff. splendopleure* Ekom falls Northerly to the present range of *A. riggenbachi* (Fig. 1).

4.5. Chromosomal evolution in Chromaphyosemion

Following Scheel (1972, 1974, 1990), Aplocheiloid karyotypes have evolved via pericentric inversions and centric fusions. A metacentric chromosome can be transformed into an acrocentric one through pericentric inversions. Two acrocentric chromosomes could undergo centric fusion and thus reduce the total number of chromosomes. If this hypothesis is correct, basal taxa should have higher chromosome numbers and more acrocentric elements, while derived taxa would have lower haploid numbers with symmetrical (metacentric) chromosomes. Until recently, descriptions of *Chromaphyosemion* karyotypes only consisted to the number, size, and shape of chromosomes (Scheel, 1972, 1974, 1990). Völker et al. (2005) were the first to use banding techniques (4′,6-diamidino-2-phenylindole, Chromomycin A_3, C banding, $AgNO_3$ staining). Even if some species still need to be investigated, it should now be possible to determine if there is an evolutionary trend in *Chromaphyosemion* karyotype evolution. In Fig. 4, haploid numbers of chromosomes (Scheel, 1990; Völker et al., 2005) have been plotted against the percentage of bi-armed

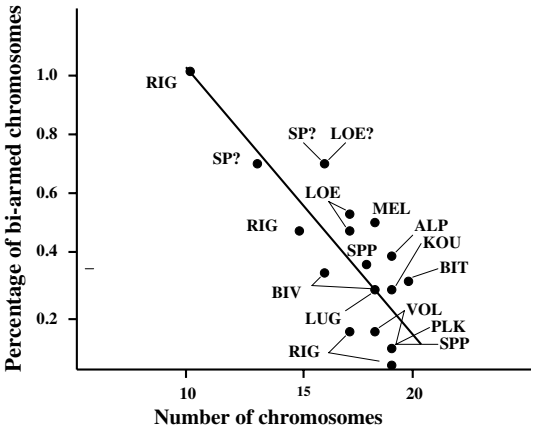

Fig. 4. Scatter plot of the number of chromosomes versus the percentage of bi-armed chromosomes for the 17 *Chromaphyosemion* karyotypes that have been studied (Scheel, 1990; Völker et al., 2005). Species identifications for Scheel (1974, 1990) karyotypes do not necessarily follow this author as taxonomy has changed since 1990. In most of the cases, it was possible to precisely determine what species Scheel (1974, 1990) did karyotype. When it was not possible to unambiguously determine the species, data are presented with a question mark. ALP, *A. alpha*; BIT, *A. bitaeniatum*; BIV, *A. bivittatum*; KOU, *A. kouamense*; LUG, *A. lugens*; RIG, *A. riggenbachi*; LOE, *A. loennbergii*; LOE?, doubtful status (*A. splendopleure* ?); MEL, *A. melanogaster*; PLK, *A. poliaki*; SPP, *A. splendopleure*; SP?, undescribed species from Rio Muni (not necessarily identical to GEMHS 00/32); VOL, *A. volcanum*. The equation of the trend line is $y = -0.091x + 1.9091$. There is a statistically significant relationship (Spearman test) between these two variables.

chromosomes (all but acrocentric chromosomes) for *A. alpha* (haploid number $N = 19$; number of arms 26), *A. bitaeniatum* (20; 26), *A. bivittatum* (16–18; 21–23), *A. loennbergii* (17–18 (perhaps 19); 25–27), *A. kouamense* (19; 24), *A. lugens* (18; 24), *A. melanogaster* (18; 27), *A. poliaki* (19; 20), *A. riggenbachi* (10–19; 19–22), *A. splendopleure* (19; 20), *A.* sp. from Rio Muni (13–17; 22–25), and *A. volcanum* (18–19; 20). The 17 haploid chromosome numbers ranged from 10 (*A. riggenbachi*) to 20 (*A. bitaeniatum*) but 15 values ranged between 15 and 20. Proportions of bi-armed chromosomes varied from 100% (*A. riggenbachi*) to 69% (*A.* sp. from Rio Muni). The equation of the trend line was $y = -0.091x + 1.9091$ ($R^2 = 0.6497$). A Spearman rank test indicated that there was a negative correlation between the number of chromosomes and the percentage of bi-armed chromosomes ($Rs = -0.570$). Consequently, Scheel's (1990) hypothesis about karyotype evolution in this group of species is congruent with these observations. Nevertheless, it will be difficult to use karyotype data to infer *Chromaphyosemion* phyletic relationships because ancestral species and derived ones cannot be separated using their karyotype description. Moreover, some species exhibited a high degree of karyotypic variability like *A. riggenbachi* ($N = 10$–19; number of metacentic = 0–10) or *A. loennbergii* ($N = 16$–18 (perhaps 19); number of metacentric = 7–11). Taking into account this important variability, karyotypic evolution in *Chromaphyosemion* should be studied at the intraspecific level and should be regarded as a complex mechanism.

Acknowledgments

This work was supported by L'Institut de Recherche pour le Développement (IRD). Special thanks to P. Caminade, R. Brummett for their help during the fish collection trip in Cameroon. We thank M. Chauche, Dr. Baba Malloum Ousman, Dr. Jacob Ayuk-Taken, Dr. Kr. Zephyrin Felixe Kamanga, Dr. F. Rivière, Dr. F. Simard, J.G. Kayoum, all the I.R.D. staff in Cameroon, and the Ministère de la pêche du Cameroun for their assistance in collecting wild fish in Cameroon. We are grateful to C. Montgelard, S. Lavoué, E. Desmarais, R. Belkhir, J. Dutheil, J. Maley, D. Nyingi, J.J. Troubat, and R. Labia for their help during the preparation of the manuscript. Many thanks to the following hobbyist for supplying various added specimens, either wild-caught or aquarium strains: O. Buisson, P. Lambert, C. Lambert, J.Y. Melin, M. Bogaert, F. Malumbres, G.L. Huijgevoort, J. Sanjuàn, A. Cerfontaine, and W. Eberl. We are indebted to international killifish organizations for their past and continuing preservation of these fishes.

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