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On the stability of dispensable constituents of the eukaryotic genome: Stability of coding sequences versus truly hypervariable sequences in a clonal vertebrate, the amazon molly, *Poecilia formosa*

(DNA fingerprinting/androgen-induced masculinization/gynogenesis/simple repeat sequences)

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ABSTRACT **In clonal unisexual vertebrates, the genes specifying the males become dispensable. To study the fate of such genes the gynogenetic all-female fish *Poecilia formosa* was treated with androgens. Phenotypic males were obtained that exhibited the complete set of male characteristics of closely related gonochoristic species, including body proportions, pigmentation, the extremely complex insemination apparatus of poeciliid fish, sexual behavior, and spermatogenesis. The apparent stability of such genic structures, including those involved in androgen regulation, is contrasted by high instability of noncoding sequences. Frequent mutations, their clonal transmission, and at least two truly hypervariable loci leading to individual differences between these otherwise clonal organisms were detected by DNA fingerprinting. These observations substantiate the concept that also in "ameiotic" vertebrates certain compartments of the genome are more prone to mutational alterations than others.**

Clonal organisms occur in natural populations of multicellular animals at all levels of organismic evolution, but within vertebrates they are confined to teleost fish, amphibians, and reptiles (1). They exist as unisexual lineages that exclude effective genetic recombination. In such animals the genetic information specifying the other sex has apparently become dispensable. These organisms are therefore uniquely suited to investigate questions centering on problems of "dispensable" genes (2). Moreover, the existence of naturally occurring clonal animals poses questions about their origin and abilities to evolve in the absence of meiotic recombination.

The amazon molly, *Poecilia formosa*, is an all-female poeciliid fish that inhabits freshwater streams and brackish coastal lagoons over a broad geographic range from southeastern Texas to northeastern Mexico. The reproductive mechanism in *P. formosa* is gynogenesis, a modified form of parthenogenesis. Sperm do not participate in syngamy but are required to trigger the onset of embryogenesis in diploid eggs, containing only the maternal genetic information (3, 4). Except for rare cases (5) the offspring are clonal replicates of their mothers (6–8). In natural populations sperm is contributed by males of two closely related bisexual species that occur sympatric with *P. formosa*. In the northern range this is *Poecilia latipinna* (5), whereas in the mexican habitats it is *Poecilia mexicana* (10). Biochemical and cytological data (8) supported the initial hypothesis (11) that the amazon molly is a hybrid species between *P. latipinna* and *P. mexicana*. The question arose if the genes determining male sex, phenotype, and behavior are absent or not functional in *P. formosa*. Preliminary studies (12–15) reported that treatment with androgenic steroids induces several phenotypic changes that resembled the male characteristics of related species. Androgen-induced masculinization, therefore, appeared to be a useful approach to investigate if the genes instrumental in determining the male phenotype and behavior that became dispensable in *P. formosa* are present and functional.

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MATERIAL AND METHODS

Experimental Animals. Two different stocks of *P. formosa* were analyzed. Laboratory lines were derived from single females and propagated in population stocks of 40–100 individuals. The generation time was ≈4–6 months. For the apomictic breeding ornamental "black mollies" (MMmm) were used. Line 1 is derived from a field collection [C. P. Haskins (1953) Brownsville, TX] handed to us in 1983. Line 2 arrived in our laboratory in 1986 and was given to us from a broodstock separated from line 1 before 1983. *P. latipinna* is an aquarium stock derived from fish of an introduced wild population. *P. mexicana mexicana* (PSO) was from the Rio Tacotalpa system. For comparative analysis of meristic and gonopodial structures, fixed-type material of the gonochoristic species from original habitats was used.

Hormone Treatment and Behavioral Tests. Newborn *P. formosa* were treated as described (16) until male secondary sex characteristics had developed. Pregnant females were treated once for 24 hr immediately before siring offspring. For behavioral tests juvenile *P. formosa* were treated as described (17). After four to six treatments in all of these fish the anal fin was transformed, indicating the change to phenotypic males. Test fish were placed into a divided 25-liter aquarium. After acclimatization for 24 hr the opaque partition was removed and the behavior of the fish was observed for 15 min. Male sexual behavior recorded was "following," "nipping," and "copulatory movements" (18). We tested masculinized *P. formosa*, *P. mexicana*, and *P. latipinna* males with conspecific females. Females were in nonattractive status (19). Each male was tested twice. For statistics, see ref. 20. All *P* values are two-tailed.

DNA Fingerprinting. This was performed as described (21). Probes were used $(GGAT)_4$, $(GACA)_4$, $(GATA)_4$, $(GAA)_6$, and $(CA)_8$.

RESULTS

For an investigation of the inducibility of male phenotypic characteristics in *P. formosa*, 30 specimens treated prepartum, as neonates, and as young fish were analyzed. All animals clearly developed the male phenotype (Fig. 1) but

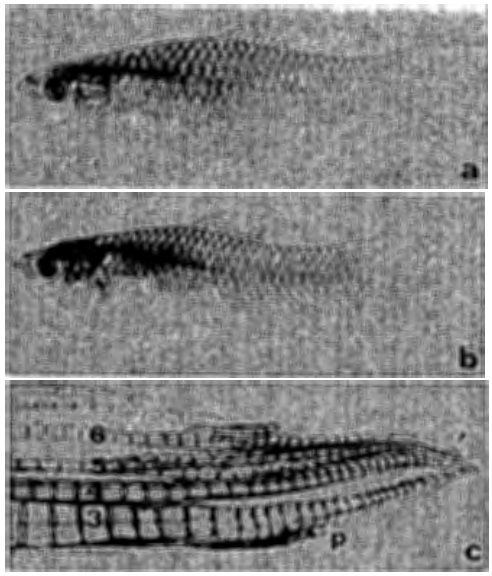

FIG. 1. *P. formosa*, untreated (*a*) and treated (*b*). Note the yellow pigmentation pattern in the tail fin. (*c*) Gonopodial structure of masculinized *P. formosa*. p, Gonopodial palp; arrowhead, membraneous hook on ray 5p; rays 3–6 are numbered.

with some individual variation in the degree of masculinization of specific characteristics. Morphometric analysis revealed that the phenotypic males of *P. formosa* developed the typical body proportions of males of closely related gonochoristic species (data not shown). A typical male-specific xanthophore pigmentation pattern like that known from sexually active males of *P. latipinna* was observed after reaching a size of 2–3 cm. Most strikingly, the pelvic and anal fins transformed into the male insemination apparatus of the live-bearing poeciliid fish. The rays of the pelvic fins elongated in the typical way. The anal fin rays formed a gonopodium exhibiting all the minute bony elements seen in fertile males of gonochoristic species (Fig. 1*c*, Table 1). At the basis of the gonopodium the baseosts developed, which are small skeletal elements that warrant the lateral movements of the male anal fin during copulation. The internal anal fin rays constituted the suspensorium, flat bony structures the male-specific muscles insert to move the gonopodium. In histological sections these muscles were seen also in the treated *P. formosa* (Fig. 2*a*). Gonopodial movements were observed frequently, demonstrating full functionality of the male structures.

The ovary has, in contrast to the testis due to unilateral development of the primordium, a single lumen that opens into the gonopore. Treated animals frequently developed a bilateral gonad with symmetric ducts that converged into a common ductus to the gonopore. The gonads consisted mostly of degenerating follicles; however, interspersed clusters of early stages of male germ-cell development were also seen—e.g., Sertoli cells with spermatogonia. In two individuals that received prepartum treatment all stages of spermatogenesis, including mature spermatozoa arranged in the typical bundles (spermatozeugmata) of poeciliid fish, were found (Fig. 2*b*). Here the gonad was nearly completely male.

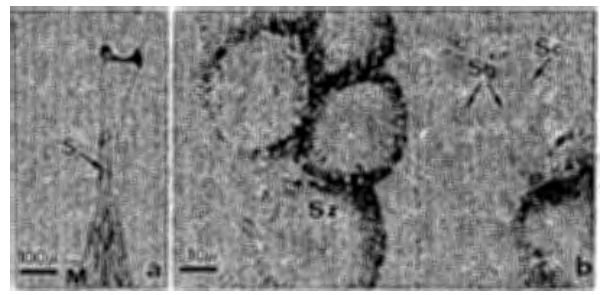

FIG. 2. Histological section through gonopodium suspensorial apparatus (*a*) and gonad (*b*) of a phenotypic male of *P. formosa*. M, suspensorial muscles; S, bony elements of suspensorium; Sz, spermatozeugma with spermatozoa arranged in peripheral bundles; Sc, Sertoli cell; Sg, spermatogonia.

The behavior known from males of *P. mexicana* and *P. latipinna* (18) was also present in hormone-treated phenotypic males of *P. formosa* except for "courtship display." Copulatory movements were rare in masculinized *P. formosa* (Fig. 3); however, "gonopodial swinging" was frequently observed. We found no significant statistical differences between the two bisexual species (Mann–Whitney U test, not significant; $n = 19$) for each type of behavior. For following, no significant difference was found between *P. latipinna* and masculinized *P. formosa* (not significant; $n = 17$), whereas the difference was significant between *P. mexicana* and *P. formosa* ($P < 0.05$; $n = 20$). For nipping and copulatory movements, all differences were significant (*P. mexicana* vs. phenotypic males, $P < 0.005$; *P. latipinna* vs. phenotypic males, $P < 0.05$).

The observation that treatment with androgen of *P. formosa* leads to phenotypic males shows that most, if not all, genes specifying the male, including its behavior, remained intact, although they are obviously dispensable. To determine if this species has an extremely stable genome, DNA fingerprinting was employed. By analyzing siblings of a single brood it was found that all individuals display almost identical fingerprint patterns. This is consistent with earlier findings (22, 23) and could be expected due to the apomictic breeding system. However, with the $(GATA)_4$ probe a truly hypervariable locus was detected, which gives rise to variable restriction fragment lengths (Fig. 4*a*). With the $(GAA)_6$ probe in *Hin*fI digests independent of the clonal origin, two bands are present or not in both lines (Fig. 4*b*). This points to a second category of truly hypervariable loci. The discrete bands obtained with DNA samples from pooled adult organs indicate that the differing fragments are due to mutations that occurred during early post-"zygotic" stages.

When individuals from the same line were compared over several generations, a very similar overall fingerprint pattern was obtained with subtle differences. They indicate muta-

Table 1. Gonopodial structures in male *P. mexicana*, *P. latipinna*, *P. mexicana*/*P. latipinna* F_1 hybrid, and masculinized *P. formosa*

Gonopodial structure	*P. mexicana*	*P. latipinna*	*P. mexicana*/*P. latipinna* F_1 hybrid	Masculinized *P. formosa*
Membranous hook of ray 3	Small	Large	Small	Intermediate
Gonopodial palp on ray 3	Well developed	Well developed	Well developed	Weakly developed
Spine length on ray 3 in relation to basal elements	Longer	Shorter	Longer	Slightly longer
Retrorse segment of ray 5p	Stout	Slender	Slender	Intermediate
Tip of ray 6	Not curved	Strongly curved ventrally	Curved ventrally	Curved ventrally
Serrae segments	Long, slender	Short, stout	Long, slender	Intermediate

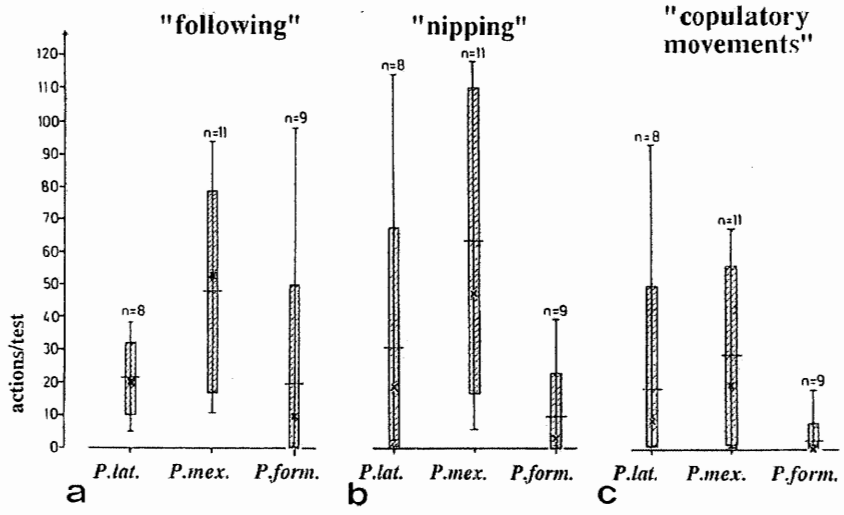

FIG. 3. Descriptive statistics for following (*a*), nipping (*b*), and copulatory movements (*c*), giving mean (—) ± 1 SD (hatched), median (×), and range (|) (for *P* values see text).

tions that occur in simple repeats (Fig. 4*b*). Such mutations are seen in succeeding generations and therefore are clonally transmitted and stable. They can be clearly distinguished from the truly hypervariable loci, which were also seen in this analysis. Comparison of both *P. formosa* lines revealed closely related fingerprints, with a maximum band sharing of, for example, ≈95% in the $(GAA)_6$/*Hae* III combination.

DISCUSSION

Using androgenic steroid we were able to induce an almost complete male phenotype and behavior in the all-female fish *P. formosa*. This shows that most, if not all, structural genes specifying the male remained functional, although they were not expressed over a distinct period since the origin of the unisexual species. In addition, also those genes involved in regulation of male-specifically expressed genes—e.g., androgen receptor, sequences instrumental in androgen regulation, appear fully functional.

The overall sexual activity was lower in masculinized *P. formosa*. It is, however, surprising that the repertoire of male sexual behavior in this all-female species is more or less complete and is displayed in a typical manner. In the case of following, even the activity level of one of the gonochoristic species is reached. The fact that courtship display is missing does not necessarily mean that hormone-induced phenotypic males are unable to show this. It is possible that a certain size of males is required as in *P. latipinna* (19, 24) that is not reached in treated animals. In addition, *P. formosa* as a species hybrid might lack the appropriate genetic information. F_1 hybrids of *P. latipinna* and *P. mexicana* also did not show this behavioral element (ref. 25 and unpublished data).

In extremely rare cases phenotypic males of *P. formosa* of obscure origin have been collected from natural habitats or occurred spontaneously in laboratory stocks (see ref. 25). Hormone induction in laboratory animals of some male characteristics has been reported (12–15), but mostly only to very limited extent. Turner and Steeves (15) reported on a single fish that developed ovotestis with a preponderance of development toward the male sex. Our study shows that indeed most, if not all, male characters including male germ cells can be completely induced in all-female *P. formosa*. Thus the spontaneously occurring males in natural populations may be the result of some (non)physiological stimulus that changed the sexual development of the otherwise all-female fish.

The phenotypic males also provide some indications as to the hybrid origin of *P. formosa*. Gonopodial structures are taxonomically highly informative. All elements were developed in masculinized *P. formosa* as in *P. mexicana*/*P. latipinna* F_1 hybrid males (Table 1). In addition, none of the meristic characteristics found in *P. formosa* contradicts the hypothesis that *P. formosa* is a hybrid species (Table 2).

Using DNA fingerprinting two *truly* hypervariable loci were detected. In the gynogenetic fish these loci, which vary by differing restriction fragment lengths even between the siblings of one brood, lend support to the notion that these otherwise clonal organisms are not genetically identical *sensu stricto*. The nature and meaning of truly hypervariable loci are discussed in the context of obligatory rearrangements, as examplified by a paradigmatic locus in the chicken that defies the Mendelian rules (32).

Various different clones of *P. formosa* exist, even within one habitat (6, 7, 22). Our studies present critical evidence that these clones can arise simply by spontaneous mutation. The data also show that once such a mutation is present in the germ line, it is transmitted clonally within populations. The occurrence of mutations in multilocus fingerprints shows that the genomes of *P. formosa* are principally not prone to less mutations than other vertebrate genomes. Clonally reproducing organisms lack a means of eliminating deleterious mutations. In the absence of recombination, the number of deleterious genes in a parthenogenetic lineage can only increase (33). In the long run, the fitness of a unisexual population is expected to decline. Unisexuality would therefore not be favored in evolution (34). An escape from being "ratcheted" out of existence would be if permanently new clones of *P. formosa* were generated *de novo* via species hybridization. This would also explain the persistence of the full set of male-specific genes due to an extremely short evolutionary history. The finding that clonal variation and clonal inheritance of acquired mutations occur weakens the argument that the naturally occurring clones of *P. formosa* may indicate such multiple hybridization events. In addition, the presumed founder species for *P. formosa* are sympatric only in a very limited area of the range of *P. formosa* (35). A typical marker chromosome, so far found only in *P. formosa*, is present in fish from Texas as well as from the Rio Soto la Marina (8), indicative of a single common ancestor.

Is *P. formosa* then an extremely fast colonizer? This explanation would be the single one in favor of a very short evolutionary history plus a very wide range of this species.

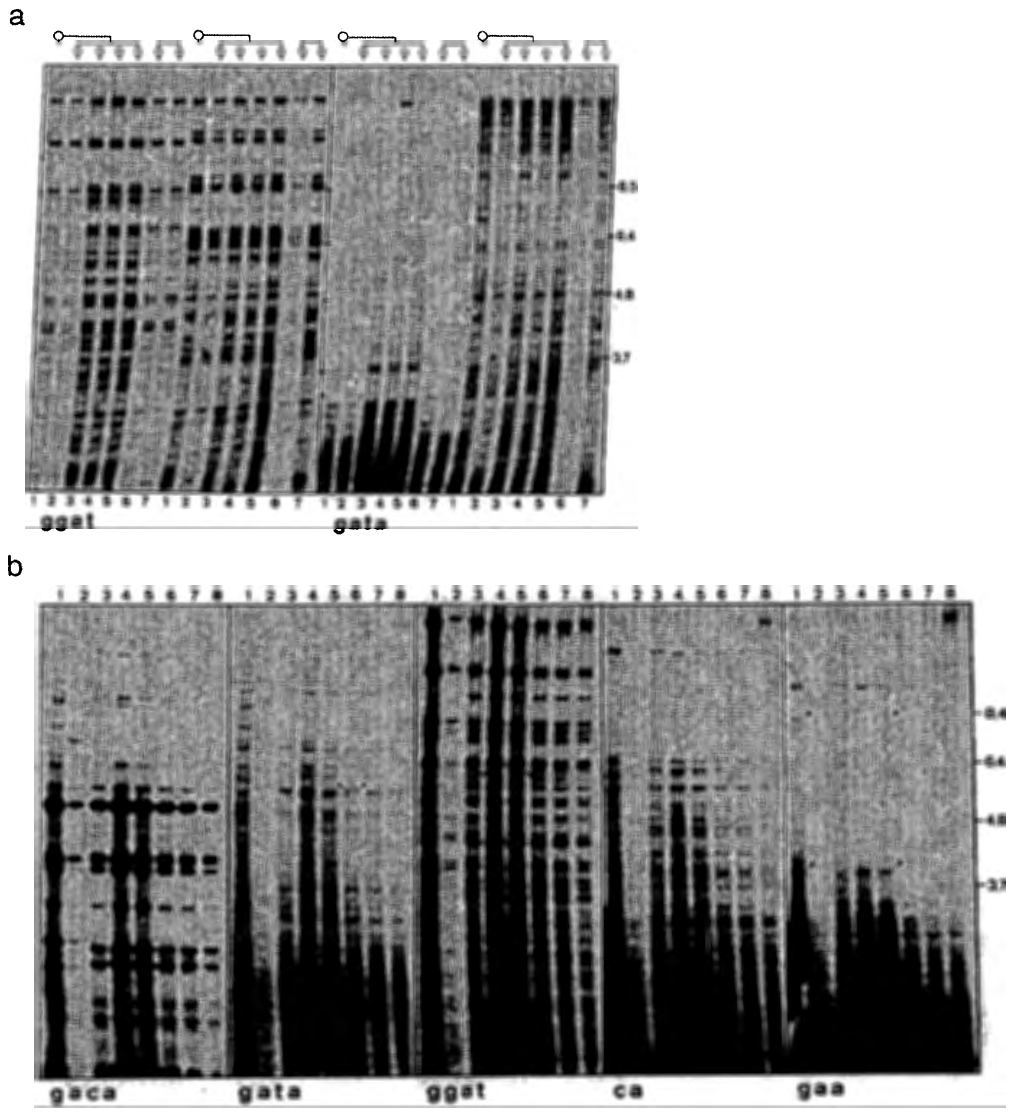

FIG. 4. DNA fingerprint analysis of *P. formosa*. (*a*) Siblings (lanes 2–5) from one brood (mother, lanes 1) and from an independent sibship (lanes 6 and 7; mother was a sister to the female in lanes 1) were analyzed for hypervariable loci. With the $(GATA)_4$ probe in *Hin*fI (left) and *Hae* III (right) digests a single fragment (arrowheads) was observed that differed in length. The high molecular weight band in lane 4 of the $(GATA)_4$ hybridization is due to "hidden" partial digestion. Sizes are given in kilobase pairs. (*b*) Individuals from different generations of two lines of *P. formosa* (lanes 1 and 2, line 2; lanes 3–8, line 1) were analyzed for mutations in the fingerprints (*Hin*fI). DNA was extracted from fish taken in December 1988 (lanes 1 and 6–8), February 1989 (lanes 4 and 5), December 1989 (lanes 2), and October 1990 (lanes 3). A high degree of band sharing is still visible in both lines, which is higher than that observed for different clones in a natural habitat (22). Clonal mutations are indicated by arrowheads. A hypervariable locus that is independently apparent in both separate lines is indicated by arrowheads in the $(GAA)_6$ hybridization.

During the past 60 years of intensive studies of *P. formosa*, no extension of its natural zoogeographic range has been noted. From distributional evidence (35), *P. formosa* should have arisen in the Late Pleistocene. Taking into account the more recent origin of *P. formosa* of 10,000 years (equivalent to 30,000 generations) and 40 male-specific genes [androgen receptor, 1; behavior, 24 (36); body proportions, 2; pigmentation, 1; elongation of pelvic fin rays, 1; gonopodium, 2; suspensor, 1; suspensorial muscles, 1; baseosts, 1; testis development, 1; spermatogenesis, 5; counting unknown polygenic characters with the exception of spermatogenesis with a minimal number of 2 genes, and monogenic and unknown inheritance with 1], with an average gene size (including androgen response regulatory elements within a functional promoter) of 1000 base pairs (bp), this would account for 4×10^4 bp that undergo mutation. At a mutation rate of 1×10^8 per base pair per year (37), one would expect ≈1–10 mutations. Of these, only a fraction would interfere with the normal function of the affected gene. In mammals the average deleterious mutation rate per locus per generation is in the order of 10^{-5} (38).

Thus, can one expect impaired functionality of male-specifically expressed genes? Studying enzyme loci in fish tetraploid species, Ohno (2) determined the half-life time for functionality of duplicated dispensable alleles as 47 million years. A similar conclusion was reached by Allendorf (39). If these numbers also apply to the dispensable genes expressed only in "males," the complete inducibility and functionality of a large number of such coding sequences in the all-female *P. formosa* would have been predicted.

Table 2. Meristic characteristics (MC) of *P. formosa* and its candidate parental species

MC	*P. latipinna*		*P. mexicana limatouri*		*P. mexicana/P. latipinna*	*P. formosa*	
	Elements*	Ref(s).	Elements*	Ref(s).	F_1 hybrid elements	Elements*	Ref.
DFR	12–14	26	9 (9–10)	29	12	11	26
	12–16	27	9 (8–10)	30		12 (11–12)	29
	13 (12–14)	28				11 (10–12)	28
	14 (14–15)		9 (9–10)			11.5 (11–12)	
AFR	9	26	9 (8–9)	28, 31	9	9	26
	9		9			9 (8–9)	
CFR			18 (16–21)	30			
BRC	18 (17–19)		16 (14–17)	29	16	17 (16–18)	
			17			17 (16–18)	
LAT	27 (26–28)		27 (25–29)	30	27	27 (26–28)	
			27 (26–28)				
PSC	10 (9–11)		13 (12–14)	29, 30	13	12 (11–12)	30
			17 (15–19)			13 (12–13)	
SCP	16	26, 27	18 (16–18)	28, 31	17	17 (16–18)	
	16		18				
ITE	Unicuspid		Unicuspid		Unicuspid	Unicuspid	
SUP	Absent		Present		ND	Present or absent	
GAP	21		21		ND	23	
IND	22 (22–23)		12–14	29	15	17–19	29
			9 (8–11)			15.5 (15–17)	

AFR, anal fin rays; BRC, branched caudal rays; CFR, caudal fin rays; DFR, dorsal fin rays; GAP, distance between middle of the anus to the anal fin base in standard length; IND, index: (DFR + BCR) − PSC; ITE, inner teeth; LAT, lateral scale row; PSC, predorsal scales determined according to ref. 9; SCP, scales around caudal peduncle; SUP, supraorbital pores 1–2a; ND, not determined. The median or range is given; if both are given, the range is in parentheses.

*Either the number of elements developed or a description is given.

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