Original Article

The Karyotype of *Holoaden luederwaldti* (Anura, Strabomantidae), with Report of Natural Triploidy

(cytogenetics polyploidy / Terrarana / chromosome / Amphibia / FISH)

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Abstract. The genus *Holoaden* includes three species described so far, but the only published cytogenetic data is from *Holoaden bradei*, with the karyotype 2n = 18, based on conventional staining. In the present paper we report, for the first time, data on chromosomes of *H. luederwaldti*, which presented 2n = 18 and a case of natural triploidy, with 2n = 3x = 27. In this sample, another karyotypic variation was observed due to the occurrence of two types of chromosome 8, which present submetacentric or subtelocentric morphologies. Homomorphic subtelocentric or heteromorphic condition was observed among the diploid specimens, whereas the triploid had one submetacentric and two subtelocentric chromosomes 8. In all specimens, Ag-NOR was located in the long arms of chromosomes 8, at the interstitial region when subtelocentric, or in the proximal region when submetacentric, confirmed by fluorescent *in situ* hybridization with the HM123 probe. The C bands showed centromeric distribution and distribution at Ag-NOR site. The centromeric heterochromatin was fluorescent with DAPI staining, whereas the Ag-NOR displayed bright fluorescence with CMA_3. Fluorescent *in situ* hybridization using a telomeric probe labelled exclusively the telomere regions. Although the same 2n = 18 chromosome numbers have been observed in *H. luederwaldti* and *H. bradei*, some differences in both karyotypes can be visualized, mainly with regard to the morphology of the last chromosome pairs.

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Abbreviations: Ag-NOR – silver-stained nucleolar organizing region, BrdU – 5-bromo-2'-deoxyuridine, CMA_3 – chromomycin A_3, DAPI – 4',6-diamidino-2-phenylindole, FISH – fluorescent *in situ* hybridization.

Introduction

In recent years, several revisions mainly based on molecular sequence data have dealt with the systematics and taxonomy of amphibians, as performed by Faivovich et al. (2005), Frost et al. (2006), Grant et al. (2006) and Hedges et al. (2008). According to the last authors, the genus *Holoaden* Miranda-Ribeiro, 1920, formerly recognized as belonging to Brachycephalidae, was assigned to a new family, Strabomantidae, subfamily Holoadeninae. At that time only two species were described from this genus, *H. bradei* and *H. luederwaldti*, both endemic from the Atlantic Forest in south-eastern Brazil, but recently Pombal Jr et al. (2008) reported a new species, *Holoaden pholeter*, from a mountain rainforest area, also in south-eastern Brazil. According to these last authors, a superficial examination of one specimen from Salesópolis, São Paulo (SP), described as *H. luederwaldti*, performed in the MZUSP (Museu de Zoologia da Universidade de São Paulo) indicated that it might be a new undescribed species. At present, the only cytogenetic information obtained from this genus is based on conventional staining, and refers to the karyotype of *H. bradei* with 2n = 18 (Lucca et al., 1974). With regard to the 44 remaining species of Holoadeninae, chromosome data are also very scarce and only *Barycholos ternetzi* (2n = 22) was analysed, with conventional and differential staining (Campos et al., 2008; Siqueira Jr et al., 2009).

Polyploidy has been previously reported in lower vertebrates, including anuran species belonging to different families with distinct levels of ploidy (Beçak et al., 1966; Bogart, 1967; Bogart and Wasserman, 1972; Tymowska, 1991; Kasahara and Haddad, 1996; Schmid et al., 2003; Vieira et al., 2006; among others). Nevertheless, as regards triploidy, few cases in Amphibia from South America have been described so far, as exemplified by the result of the natural hybridization between the diploid *Phyllomedusa distincta* and the tetraploid *Phyllomedusa tetraploidea* (Batistic, 1989; Haddad et al., 1994). On the other hand, numerous amphibian species presenting natural triploidy have been described in Europe and North America, such as salamanders

(Borkin et al., 1996; Litvinchuk et al., 1998; 2001) and one anuran of the *Bufo viridis* group (Borkin et al., 2007).

In the current paper, we present, for first time, chromosomal data of *H. luederwaldti* based on standard Giemsa staining, silver-stained nucleolar organizer regions (Ag-NOR), C banding, base-specific fluorochrome staining and fluorescent *in situ* hybridization (FISH) techniques. The karyotypic variation related to heteromorphism observed in the chromosome pair 8 and the occurrence of natural triploidy are discussed.

Material and Methods

We carried out cytogenetic analyses in five males and two females of *Holoaden luederwaldti* collected in Atlantic Forest in the municipality of Campos do Jordão, SP, altitude ca. 1800 m a.s.l. (22°42'17.9''S, 45°28'15.4''W). The animals were collected using pitfall traps consisting of buckets that are buried into the ground of the forest, making it possible for the animal to fall inside them. The permission for collecting was provided by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, Process Number 14031-1). All the studied specimens were deposited in the Coleção de Anfíbios of the Departamento de Zoologia (CFBH), Instituto de Biociências, UNESP, Rio Claro, São Paulo, Brazil.

Chromosome spreads were obtained from direct cytological preparations from bone marrow, liver, and testes, according to Baldissera Jr et al. (1993), or from intestinal epithelium, according to Schmid (1978), with adaptations. Briefly, the animals received intraperitoneal injection of aqueous solution of colchicine (0.01 ml/g body weight) 0.01% per 12 h (Baldissera Jr et al., 1993) or 1% per 4 h (Schmid, 1978), and before euthanasia they were deeply anaesthetized with commercial Lidocaine 5% pomade. The protocols were in accordance with the Ethical Committee for Animal Use of this Institution (CEUA-IB-UNESP-CRC).

Conventional staining was performed with Giemsa diluted in phosphate-buffered saline, pH 6.8. Ag-NOR and C-banding techniques were performed according to the procedures of Howell and Black (1980) and Sumner (1972), respectively. Fluorochrome staining using AT-specific 4',6-diamidino-2-phenylindole (DAPI) and GC-specific chromomycin A_3 (CMA_3) followed the method described by Christian et al. (1998). FISH was performed according to Viegas-Péquignot (1992), using the rDNA probe HM123 (Meunier-Rotival et al. 1979) or according to Pinkel et al. (1986) using a telomeric probe. Bi-armed chromosomes were classified as metacentric, submetacentric and subtelocentric, and uni-armed ones as telocentric, according to the nomenclature suggested by Green and Sessions (1991, 2007).

Results

Six of the seven specimens of *H. luederwaldti* showed 2n = 18 chromosomes and FN = 34 (Fig. 1) with the following karyotypic constitution: one large pair (pair 1), six pairs that gradually vary in length from large to medium size (pairs 2–7) and, of course, two small ones (pairs 8–9). Pairs 1, 7 and 9 were metacentric, pairs 2–5 submetacentric, and pair 6 telocentric. The chromosomes 8 presented two distinct morphologies, both were either subtelocentric in three diploid specimens, or heteromorphic with subtelocentric/submetacentric combination in the remaining ones (Fig. 1a, b; Table 1). Meiotic cells showed nine bivalents in diplotene/metaphase I and nine chromosomes in metaphase II (Fig. 2a, b).

One remaining male with overall normal phenotype of the sample exhibited 2n = 3x = 27 karyotype (Fig. 3), with the same chromosomal morphology as that found in diploid specimens. In this triploid, the triad 8 was composed by two subtelocentrics and one submetacentric chromosome. Cytological preparations from the testes showed some unidentified meiotic phases (Fig. 2c).

Prominent secondary constriction was visualised in the submetacentric 8, at the proximal long arms. In the

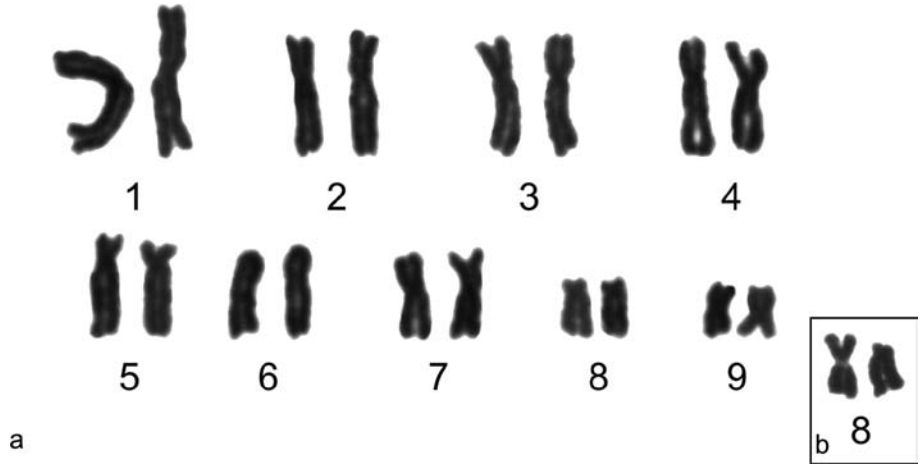

Fig. 1. Standard stained karyotype of a *Holoaden luederwaldti* male with 2n = 2x = 18. Pair 8 is homomorphic with two subtelocentric chromosomes (a). Inset: heteromorphic pair 8 from a female with submetacentric and subtelocentric chromosomes (b). Secondary constriction is visualized at the proximal long arms of submetacentric 8.

Table 1. Morphometric analysis of the chromosomes of Holoaden luederwaldti

		Chromosome pair									
		1	2	3	4	5	6	7	8a	8b	9
Holoaden luederwaldti	RL*	17.57	14.04	12.70	12.16	11.83	10.87	9.29	5.91	-	5.63
	CR	1.37 ±0.07	1.78 ±0.02	2.04 ±0.11	2.05 ±0.06	2.75 ±0.09	12.71 ±0.92	1.54 ±0.04	3.30 ±0.11	1.75 ±0.04	1.22 ±0.03
	CI	0.424 ±0.012	0.361 ±0.002	0.330 ±0.011	0.328 ±0.007	0.267 ±0.007	0.074 ±0.005	0.393 ±0.006	0.233 ±0.006	0.345 ±0.016	0.450 ±0.007
	CT	m	sm	sm	sm	sm	T	m	st	sm	m

* To determine the RL values the secondary constriction observed in the chromosomes 8b was not considered.
RL = relative length, CR = centromeric ratio, CI = centromeric index, CT = chromosome type: m = metacentric, sm = submetacentric, t = telocentric, st = subtelocentric. Data are presented as mean ± standard error.

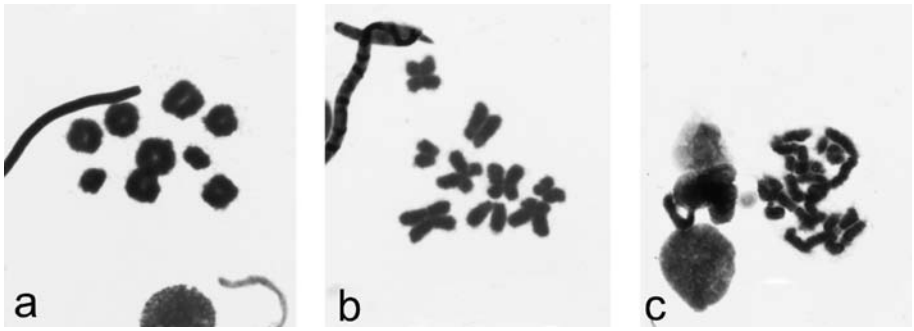

Fig. 2. Standard staining of meiotic cells of *Holoaden luederwaldti*, (a) diplotene/metaphase I with 9 bivalents, (b) metaphase II with 9 chromosomes, both from the male with 2n = 2x = 18, and (c) non-identified meiotic phases from a male with 2n = 3x = 27.

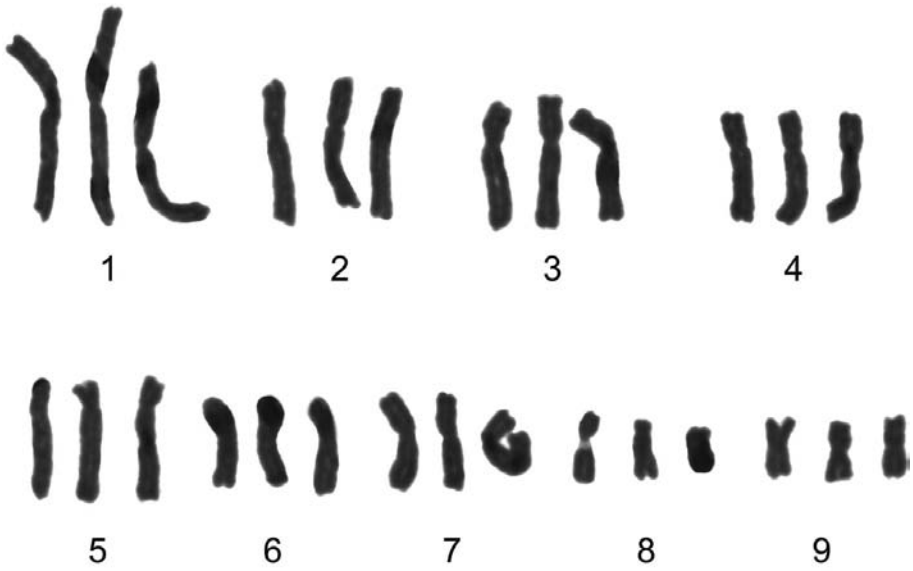

Fig. 3. Standard stained karyotype of the *Holoaden luederwaldti* male with 2n = 3x = 27. Note one submetacentric and two subtelocentric chromosomes in the triad 8. Secondary constriction is visualized at proximal long arms of submetacentric 8.

subtelocentric type it was absent in general, but a subtle secondary constriction could be noticed in some metaphases at interstitial long arms. In all specimens of *H. luederwaldti*, Ag-NOR was located on chromosomes 8, in the site coincident to the secondary constriction. In the subtelocentric 8, the Ag-NOR was tiny, smaller than that observed in the submetacentric (Fig. 4a, b, c). The FISH technique with rDNA probe confirmed this region as a true NOR site in both chromosome types (Fig. 4d).

With the C-band technique, heterochromatin distribution predominantly at the centromeric region was noted, but the Ag-NOR site was also positively banded

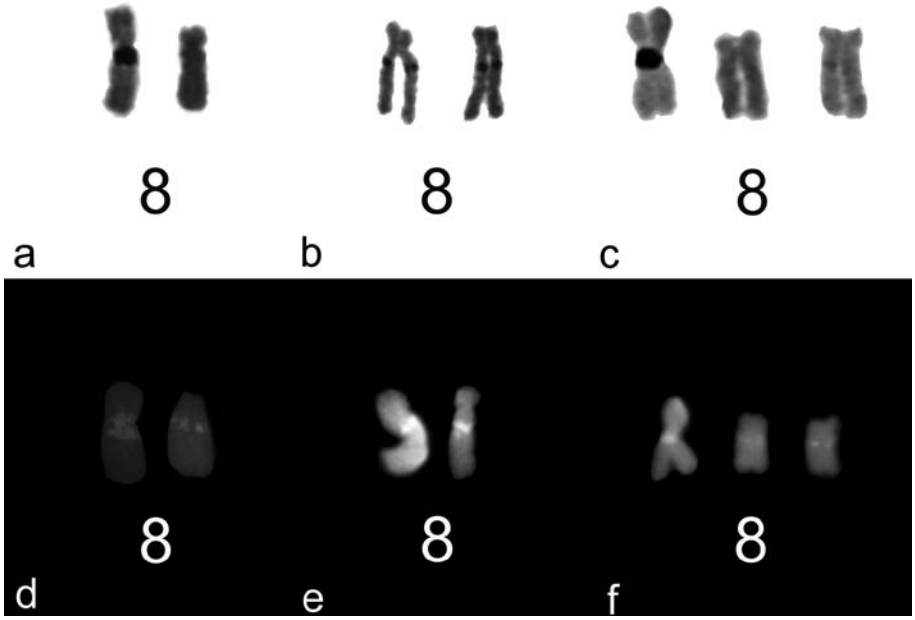

Fig. 4. Chromosomes 8 of *Holoaden luederwaldti*: (a), (b), and (c) silver impregnation; (d) FISH with an rDNA probe; (e) and (f) with CMA_3 staining. Note that the submetacentric 8 showed large Ag-NOR (a, c), FISH signal (d), and bright CMA_3 site, while in the subtelocentric 8 the same labelling is weak (a, b, c, d, e, and f).

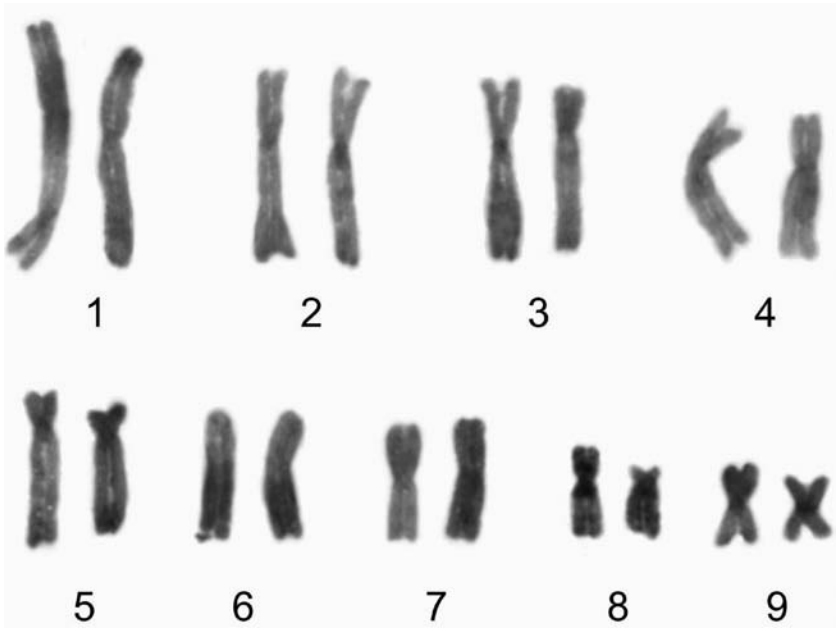

Fig. 5. C-banded metaphase of *Holoaden luederwaldti* with 2n = 2x = 18. Note that the heteromorphic pair 8 shows C banding in both chromosomes at the same site of Ag-NOR.

(Fig. 5). With DAPI staining the centromere of all chromosomes was slightly bright fluorescent, and with CMA_3, only the site of the Ag-NOR was labelled, in both morphological types of chromosome 8 (Fig. 4e, f). FISH using the telomeric probe labelled exclusively the telomeric regions (Fig. 6a, b).

Discussion

Contrarily to what is observed in the majority of anurans, which in general exhibit very conservative karyotypes, the small sample of *H. luederwaldti* analysed here showed karyotypic variations. One of them involved the chromosomes of pair 8, which had two distinct morphologies, most probably ascribed to a structural rearrangement, such as pericentric inversion. This heteromorphism is not related to the occurrence of cytologically differentiated sex-chromosomes, because it was observed in both sexes. The two chromosome types also differed in their length, the submetacentric being longer than the subtelocentric one, probably due to the extent of the nucleolar organizer regions, which was confirmed

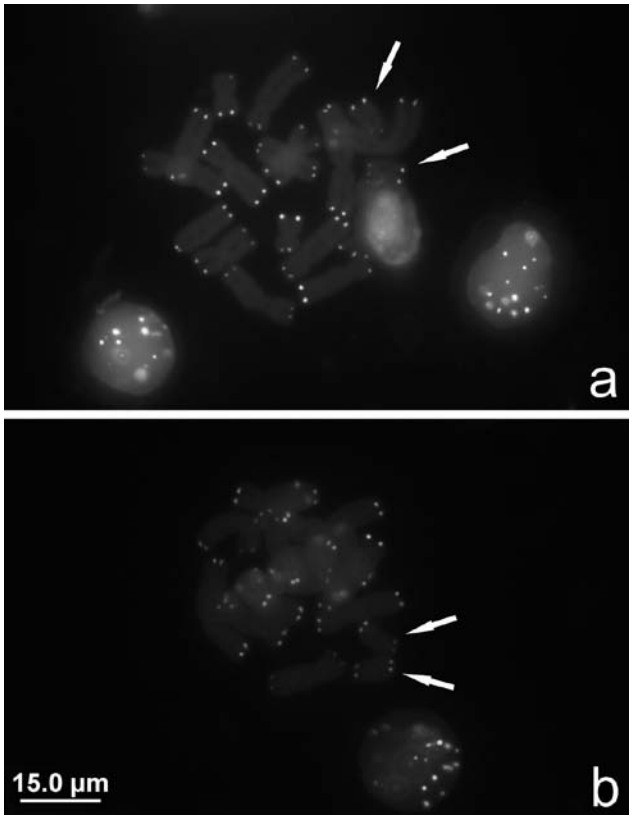

Fig. 6. Metaphases of *Holoaden luederwaldti* with 2n = 2x = 18 after FISH with a telomeric probe: (a) homomorphic pair 8 (arrows); (b) heteromorphic pair 8 (arrows)

by the sizes of the secondary constriction, Ag-NOR and rDNA fluorescent labelling. Moreover, as expected, the telomeric probe hybridized in the telomere, with no vestige of interstitial bands that could suggest the occurrence of pericentric inversion in one of the chromosomes 8.

Holoaden luederwaldti and *H. bradei* (Lucca et al., 1974) share the same diploid number (2n = 18) and very similar karyotypes, differing in the fact that in the latter species all chromosomes have been described as metacentric. Part of the differences seem to be due to the nomenclature adopted, as well as to the ordering of the pairs in the karyograms, but small differences in the arm ratio of some chromosome are not ruled out. Nevertheless, some discrepancies may be pointed out in the karyotypes of both species. One of them concerns the pair 6, which is undoubtedly telocentric in *H. luederwaldti* and bi-armed in *H. bradei*, this morphological difference ascribed, most probably, to a pericentric inversion. Another relevant difference between both karyotypes is the size of the pairs 8 and 9, which are clearly smaller then pair 7 in *H. luederwaldti*, whereas in *H. bradei* this distinct demarcation is not observed.

A chromosome marker bearing secondary constriction was not described in *H. bradei*, but in the karyogram presented by Lucca et al. (1974), possibly a secondary constriction was seen at interstitial long arms of one homologue of the pair 9. In this case, the pair would be homologous to the pair 8 of *H. luederwaldti*.

Certainly, analyses of more specimens of *H. luederwaldti* are necessary in order to better evaluate the variability with regard to the morphology of the pair 8, since the combination submetacentric/submetacentric was not observed in the present sample, as a result of crossings between individuals bearing a heteromorphic pair. Furthermore, the BrdU treatment for replication banding patterns would be useful to confirm pericentric inversion as the mechanism of the chromosome rearrangement. This technique would also be of use to verify whether the morphological difference between chromosomes 6 of *H. luederwaldti* and *H. bradei* is, in fact, due to a pericentric inversion. Nevertheless, karyotype comparisons could not be carried out because *H. bradei* has not been found in the nature since 1976, being considered as "critically endangered" in the "Livro Vermelho da Fauna Brasileira Ameaçada de Extinção" according to Machado et al. (2008).

As usually observed for anurans, in *H. luederwaldti* the Ag-NOR was found in a single chromosome pair, with a marked difference in the labelling size, when chromosomes 8 presented distinct morphologies. Heteromorphism in the Ag-NOR size is common, and may be explained by differential gene activity or amount of the repetitive rDNA sequences. In the case of *H. luederwaldti* the second alternative was confirmed, since after the FISH technique done with the HM123 probe, the fluorescent signal was stronger in the chromosome bearing large Ag-NOR. An intriguing fact is that the large NOR was always found in submetacentrics 8, whereas the small one was associated to the subtelocentrics. This finding seems not to be fortuitous, although our sample was not very large. In this case, the difference in the amount of rDNA might be a result of the chromosome rearrangement, which altered their morphologies. At first sight, the NOR was not included in the inverted segment, since it was located at the same site in the long arms of both chromosome types.

Holoaden luederwaldti has a relatively low amount of heterochromatin, exhibiting slightly stained C-bands, predominantly in the centromeric region of all chromosomes. This heterochromatin is clearly AT-rich, because it appeared with brilliant fluorescence after DAPI staining. With CMA_3 only the site of the NOR presented fluorescent labelling due to their GC-richness.

The subfamily Holoadeninae comprises 47 species distributed in six genera, one of them *Holoaden* (Frost, 2011). Nevertheless, only *Barycholos ternetzi* was previously karyotyped, showing 2n = 22 and FN = 38 (Campos et al., 2008; Siqueira Jr et al., 2009). Although *H. luederwaldti* has a smaller diploid number than *B. ternetzi*, it is important to notice that the first five chromosome pairs of these two species are morphologically very similar, probable homologous. The marked karyotype difference is ascribed to the remaining four pairs in *H. luederwaldti* and the six in *B. ternetzi*, so that, with the exception of pair 9 of both species, all other chromosomes have discrepant morphologies. Most probably, structural rearrangement as chromo-

some fusions might be responsible for the reduction of the 2n in *H. luederwaldti*, while inversions would have changed the morphologies of some chromosome pairs. Moreover, the pair 11 of *B. ternetzi*, which includes Ag-NOR-bearing chromosomes, might be homologous with the chromosome pair 8 of *H. luederwaldti*, assuming the possibility of a pericentric inversion, altering the rDNA site. Certainly, cytogenetic data of other representatives of Holoadeninae will provide a better understanding of the chromosome evolution in this group. It is also important to determine the karyotype constitution of the new species *H. pholeter* (Pombal Jr et al., 2008), as well as of a presumptive unknown species collected in Salesópolis, SP, according to these last authors and described as *H. luederwaldti* by Heyer et al. (1990).

In our sample of seven individuals, one of the males showed polyploidy, with 2n = 3x = 27 chromosomes. This individual was collected along with the remaining specimens, and no morphological difference was recognized among them. The polyploidy condition was noticed only after the cytogenetic analysis. Several mechanisms are responsible for the origin of a triploid individual, such as the fusion of a diploid female gamete with normal haploid spermatozoa. Non-reduced oocytes may be formed, among others mechanisms, by meiotic non-disjunction, by retention of the secondary polar body, occurring spontaneously or under diverse factors, such as shock of temperature. The triploidy in the specimen of *H. luederwaldti* might have originated by one of these mechanisms, similarly to what has been reported in fishes collected in the same geographical region of the animals of our sample. According to Garcia et al. (2003), the origin of triploidy in the fish *Rhamdia* sp. was explained by a drastic temperature decrease, due to hailstorm, very frequent in that locality. The triploidy in *H. luederwaldti* could also be the result of natural hybridization between specimens with diploid and tetraploid constitution. For this reason and in view of the small sample studied here, analyses of more specimens of *H. luederwaldti* are indicated in order to verify the occurrence and frequency of other triploid individuals in the same locality or, optionally, specimens with tetraploid karyotype.

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