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Natural Hybridization Between Diploid and Tetraploid Species of Leaf-frogs, Genus *Phyllomedusa* (Amphibia)

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ABSTRACT. — **Sympatric populations of the leaf-frogs *Phyllomedusa distincta* (2n = 26) and *P. tetraploidea* (4n = 52) were studied in southeastern Brazil. In a region of sympatry, seven of 15 leaf-frogs were triploid hybrids (3n = 39). The advertisement calls of both species are similar, and may not function adequately as a premating isolation mechanism. Triploids apparently exhibit low fertility or sterility, supporting the assertion that diploid and tetraploid populations are valid species. We suggest that *P. tetraploidea* originated by autopolyploidy of *P. distincta*, based on the following evidence: (1) the indistinguishable vocalizations of diploid and tetraploid species, and (2) the geographic distribution of species in the *Phyllomedusa burmeisteri* group.**

Speciation is generally considered a process, not an event (Templeton, 1981). Nevertheless, polyploidy is an event that may generate instantaneous speciation without transitional forms ("bad species" of Templeton, 1989). Polyploidization may have played an important role in the speciation and evolution of anurans; it occurs independently in several genera and families (Bogart, 1980; Mahony and Robinson, 1980; Schmid et al., 1985; King, 1990; Tymowska, 1991). There are few reports of triploidy in anurans, although triploids are the most common ploypoid class in amphibians (Bogart and Wasserman, 1972). Triploid anurans have been considered to be autotriploids (Green et al., 1984), allotriploids between diploid species (Green and Delisle, 1985), or hybrids between diploid and tetraploid populations (Ruiz et al., 1980).

The advertisement call of anurans is a powerful ethological barrier that can prevent mistakes in pair formation (e.g., Blair, 1958; Martof, 1961; Littlejohn and Loftus-Hills, 1968; Telford and Passmore, 1981). Vocalizations are considered a primary reproductive isolating mechanism between related diploid and polyploid anuran species occurring in sympatry (Gerhardt, 1974; Ralin, 1977; Bogart, 1980; Mahony and Robinson, 1980).

In the *Phyllomedusa burmeisteri* species group, most taxa are diploid (Batistic, 1989). However, *P. tetraploidea* is a tetraploid species (4n = 52) assigned to the *burmeisteri* group (Pombal and Haddad, 1992). *Phyllomedusa tetraploidea*, here considered as a full species (see Pombal and Haddad, 1992), was considered as a *P. burmeisteri* tetraploid population by Beçak et al. (1970) and Batistic et al. (1975). Here we present field evidence of the production of viable triploid hybrids through natural hybridization between the diploid species *Phyllomedusa distincta* and the tetraploid species *Phyllomedusa tetraploidea*. We discuss possible causes of hybridization and the origin of the tetraploid species.

MATERIAL AND METHODS

Field work was conducted in a pond located at a forest edge, in the region of Ribeirão Branco, São Paulo State, southeastern Brazil, in January 1988, February 1989, and December 1992. We collected egg clutches and counted the number of developing and non-developing eggs. We also recorded vocalizations of *Phyllomedusa distincta*, *P. tetraploidea*, and putative triploid hybrids of these species. The specimens whose calls were recorded were placed in individual plastic bags, and maintained alive until karyotypic analysis in the laboratory.

Vocalizations were recorded with a Uher 4000 Monitor and with a Sony TCM 12 tape recorder. The recorded tapes were analyzed in a Voice Identification Series 700 sound spectrograph, with a wide band filter (300 Hz). In the laboratory we reproduced the original recorded tapes at 19 cm/sec and played back the vocalizations at low speed (1.2 cm/sec) to count the number of pulses in the advertisement calls.

Cytogenetic analysis was performed with two samples of reproductively active adults found at the study site; the first with nine and the second with six specimens, collected in January 1988 and February 1989, respectively. Two hours before death the animals received an intraperitoneal injection of colchicine 1% (0.1 ml per 10 g of body weight). Animals were killed by etherization. Intestine, spleen, and testis were removed and cut into small pieces, immersed in cold distilled water for 15 min and fixed in

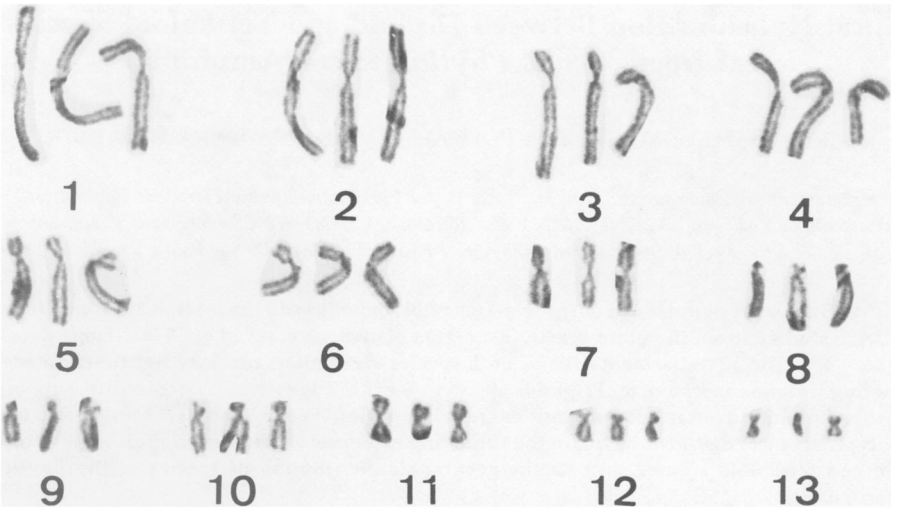

FIG. 1.—Karyotype of a *Phyllomedusa* triploid hybrid from Ribeirão Branco, São Paulo, southeastern Brazil, showing 3n = 39 chromosomes.

acetic acid 50%. The slides were obtained following the squash technique described in Beçak et al. (1969), but using small intestine fragments. The coverslips were removed after immersion of the slides in dry ice plus ethanol, with the aid of a razor blade. Slides were stained with Giemsa 2% in phosphate buffer for about five min. The best metaphases spreads were photographed under a Zeiss photomicroscope, magnified, and the karyotypes were arranged according to the decreasing size of the homologues.

RESULTS

Hybrid Karyotype.—We observed seven triploid (six males; one female), four diploid (all males), and four tetraploid (all males) animals. The 39 chromosomes of the hybrid somatic tissue cells (Fig. 1) may be arranged in 13 groups of three elements each. Groups 7 and 11 are metacentric, group 8 is telocentric, and the remainder are submetacentric. A slight variation in size was observed among the chromosomes in several groups. In some groups there were two large and one small chromosomes, and in others there were one larger and two smaller. Since differences between the length of homologous chromosomes of *P. distincta* and *P. tetraploidea* are small (Batistic, 1989), it is unclear whether the difference in size of the chromosomes is due to their origin (different parental species) or to variations caused by different degrees of a random distension of the chromosomes. In the meiotic metaphase there are univalents, bivalents, and trivalents.

Vocalizations.—Males of both *P. distincta* and *P. tetraploidea* occupied identical reproductive sites, calling side by side on leaves and branches of vegetation at the pond. The advertisement calls of diploid, triploid, and tetraploid individuals are indistinguishable to the human ear. The physical similarities of these vocalizations are qualitatively confirmed by the sonagrams (Fig. 2). The advertisement calls of all analyzed individuals are composed of pulses generally grouped in pairs, ranging from about 700 Hz to 2.5 kHz in frequency. The emphasized frequencies range from about 700 Hz to 1.8 kHz. The calls of *P. distincta* have 5.1–9.7 pulses ($\bar{x} \pm$ SD: 7.4 ± 1.45, N = 4 males); the calls of *P. tetraploidea* have 7.4–9.9 pulses (8.9 ± 1.19, N = 4 males); and the calls of triploid hybrids have 7.3–9.3 pulses (8.5 ± 0.8, N = 5 males). The duration of the advertisement calls is variable according to the number of pulses in the vocalization.

Morphology.—*Phyllomedusa distincta* and *P. tetraploidea* are distinguished mainly by their different thigh color patterns (Pombal and Haddad, 1992). We observed two thigh color patterns, one attributable to *P. distincta*, the other to *P. tetraploidea*. Triploid hybrids generally exhibited an intermediate thigh color pattern. However, some triploids were indistinguishable when compared to one of the parentals.

The SVL of male *P. tetraploidea* (58.16 ± 3.23, N = 45, range = 48.7 to 63.8 mm) was similar to the SVL of triploid males (58.74 ± 2.00, N = 5, range = 55.2 to 60.1 mm); males of *P. distincta* were smaller (52.26 ± 4.14, N = 67, range = 46.6 to 66.0 mm).

Mating and Egg Clutches.—We observed three

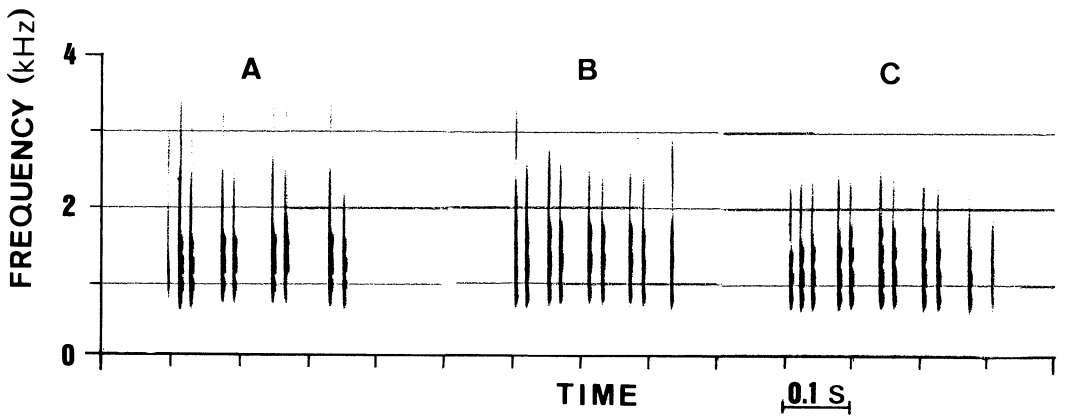

FIG. 2.—Sonagrams of the advertisement calls of *Phyllomedusa* from Ribeirão Branco, São Paulo, southeastern Brazil; (A) *Phyllomedusa distincta*, (B) triploid hybrid, and (C) *Phyllomedusa tetraploidea*.

amplectant pairs: one formed by a male with *P. tetraploidea* thigh color pattern and a female with *P. distincta* thigh color pattern; a second formed by a male with intermediate thigh color pattern and a female with *P. distincta* thigh color pattern; and a third formed by a *P. distincta* male and a female with intermediate thigh color pattern. This latter pair was analyzed in the laboratory; the male was diploid (2n = 26), and the female was triploid (3n = 39). This female was dissected and contained 12 apparently mature ovarian eggs. One female with an intermediate thigh color pattern, found unpaired, contained five apparently mature ovarian eggs.

The eggs of both *Phyllomedusa* species are placed on leaves hanging over water bodies. *Phyllomedusa tetraploidea* lays 151 to 332 eggs (227.3 ± 76.44, N = 4; Pombal and Haddad, 1992), and *P. distincta* lays 200 to 228 eggs (214 ± 19.80, N = 2). The eggs of both species are similar in size, color, and arrangement inside the leaf nest. Nine egg clutches, observed at the study site, were apparently deposited by triploids or by conspecific and heterospecific pairs. Six egg clutches had egg numbers similar (131, 146, 180, 195, 224, and 333 eggs) to those of both parental species. The clutches with 131 and 146 eggs were maintained in the laboratory; only 10 and 35 embryos, respectively, survived to birth. Three egg clutches had a low number of eggs; one clutch with 11 eggs had three developing embryos, one with 16 eggs had eight developing embryos, and one with 19 eggs had one developing embryo.

Geographical Distribution.—The only species in the *P. burmeisteri* group known to occur sympatrically with *P. tetraploidea* is *P. distincta*. Diploid (*P. distincta*) and tetraploid (*P. tetraploidea*) populations occur in sympatry in three localities in Brazil (Fig. 3): Guapiara (24°11'S, 48°32'W) and Ribeirão Branco (24°13'S, 48°46'W), two nearby municipalities in the São Paulo State, and Bituruna municipality (26°8'S, 51°33'W) in the Paraná State. It is possible that a large contact zone occurs in the interior of São Paulo and Paraná States in Brazil. However, we only have evidence of triploid hybrids in Ribeirão Branco.

DISCUSSION

The distinction between allopolyploidy and autopolyploidy in natural populations is difficult (Bogart and Wasserman, 1972). However, most of the polyploid species of anurans described are considered autopolyploids of recent origin (Tymowska, 1991). We suggest that *P. distincta* may be the ancestral species that gave rise (probably by autopolyploidy) to *P. tetraploidea*, based on the following evidence: (1) the vocalizations of both species are similar; (2) the present distribution of *P. burmeisteri* group representatives is consistent with a recent origin for *P. tetraploidea* (see below); today, only *P. distincta*, a member of the *burmeisteri* group, occurs sympatrically with *P. tetraploidea* (Pombal and Haddad, 1992). Furthermore, the trivalents in the meiotic metaphase I of the triploid hybrids indicate chromosomic homologies between *P. distincta* and *P. tetraploidea*.

The multivalent association may be a temporal indicator of polyploidy. Recently evolved autopolyploids often show multivalents (Bogart, 1980; Schmid et al., 1985; Tymowska, 1991). The origin of *P. tetraploidea* apparently is recent, based on circumstantial evidence: the tetravalents present in the meiotic metaphase I and the small number of changes of the karyotype. The tetravalents present in the meiotic metaphase I of *P. tetraploidea* may be a result of an autopolyploid origin, as multivalents are regarded as a result of chromosomic homologies

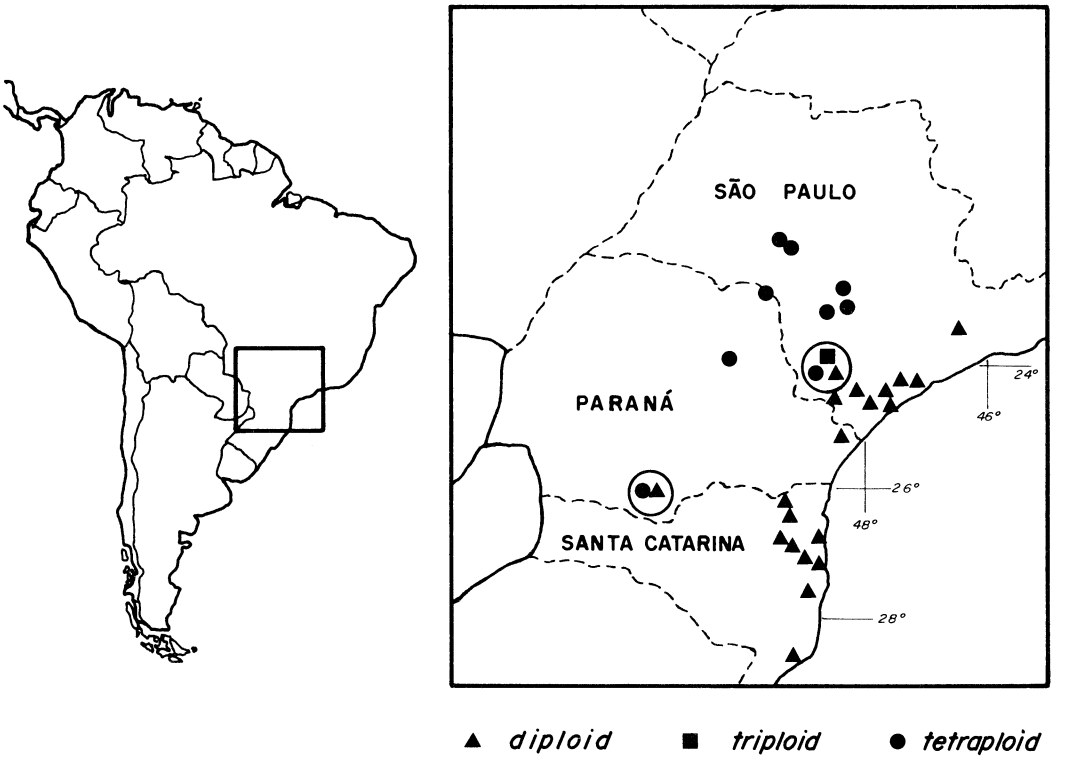

FIG. 3.—Geographic distribution of *Phyllomedusa distincta* (diploid) and *P. tetraploidea* (tetraploid) in Brazil. Large circles indicate sympatry; two nearby localities in the São Paulo State (Guapiara and Ribeirão Branco) are indicated by the upper large circle; the lower large circle indicates Bituruna in the Paraná State.

(Beçak et al., 1967; Bogart, 1980; Schmid et al., 1985; Tymowska, 1991). However, multivalents do not necessarily reflect the absolute homology of the genomes involved, but indicate that the genomes are closely related and that they have retained the ability to pair (King, 1990). The Nucleolar Organizer Region (NOR) position in *P. tetraploidea* is different from all other studied species in the *P. burmeisteri* group (Batistic, 1989). One possible interpretation for the NOR position in *P. tetraploidea* is chromosomal restructuring, originating after polyploidization. Nevertheless, detailed genetic studies of the different *P. distincta* and *P. tetraploidea* populations are necessary to confirm the autopolyploid origin of the later species and its time of origin.

Bogart (1980) stated that increased cell size in polyploid anurans would alter male vocalizations as well as the female's reception. Mable and Bogart (1991) indicated that call characteristics may be simply a by-product of polyploidy. These statements were not supported by the diploid, triploid, and tetraploid *Phyllomedusa* studied here, that exhibit similar vocalizations.

Hybrid sterility (postzygotic isolation) is considered a major contributor to reproductive isolation during the speciation process (Coyne and Orr, 1989). The egg clutches observed in the study site may be regarded as indirect evidence of triploid hybrid sterility or low fertility. When the egg clutch has an abnormally low number of eggs it probably was produced by a triploid female. This view is supported by the low number of mature ovarian eggs observed in the triploid females (5–12). When the egg clutch has the usual number of eggs (130–330), but no fertilized eggs, or low number of fertilized eggs, the pair probably was formed by a *Phyllomedusa tetraploidea* or *P. distincta* female and a triploid male, unable to fertilize most of the eggs.

Sympatric speciation is possible through polyploidy (Grant and Grant, 1989). Distributional evidence suggests that *P. distincta* may represent the diploid stock allowing sympatric speciation of *P. tetraploidea*. Successive tetraploidies within *P. distincta* range may have been eliminated by the absence of premating isolation mechanisms. The apparent success and establishment of *P. tetraploidea* breeding popula-

tions may thus be a consequence of tetraploidy originating at the western distributional border of *P. distincta*, with an expansion in areas not exploited by the latter species. This region exhibits a more pronounced seasonality, with a drier season, when compared to the coastal humid region (Setzer, 1966), the typical distributional range for the *P. burmeisteri* species group (Pombal and Haddad, 1992).

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APPENDIX 1

List of Specimens Examined

Specimens used in karyotypic analysis are deposited in the Seção de Genética Animal, Instituto Butantan, São Paulo, Brazil (GA-IB). Preserved eggs and embryos are deposited in the Museu de História Natural, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil (ZUEC).

The distributional data and measurements of snout-vent length (SVL) were obtained by analysis of preserved specimens from the following collections in Brazil. Adolpho Lutz collection (AL), deposited in the Museu Nacional do Rio de Janeiro; Departamento de Zoologia, Universidade Estadual Paulista, Campus de São José do Rio Preto, São Paulo (DZSJRP); Eugenio Izecksohn collection (EI), deposited in the Universidade Federal Rural do Rio de Janeiro, Itaguaí, Rio de Janeiro; Seção de Genética Animal do Instituto Butantan, São Paulo (GA-IB); Jorge Jim collection (JJ), deposited in the Universidade Estadual Paulista, Campus de Botucatu, São Paulo; Museu Nacional do Rio de Janeiro, Rio de Janeiro (MNRJ); Museu de Zoologia da USP, São Paulo (MZUSP); Werner C. A. Bokermann collection (WCAB), São Paulo; Museu de História Natural, Universidade Estadual de Campinas, São Paulo (ZUEC).

Phyllomedusa distincta.—Bituruna, Paraná: MNRJ 3719. Guaraqueçaba, Paraná: MNRJ 10090–10093. Barra Velha, Santa Catarina: (Batistic, 1989). Brusque, Santa Catarina: MNRJ 10077. Corupá, Santa Catarina: AL 1742 (paratype); MNRJ 4728–4731 (paratypes). Florianópolis, Santa Catarina: (Batistic, 1989). Joinvile, Santa Catarina: (Batistic, 1989). Novo Horizonte, Santa Catarina: MZUSP 35035–35057. Parabeiraba, Santa Catarina: MZUSP 55934, 55935. Penha, Santa Catarina: ZUEC 773. Porto Belo, Santa Catarina: MZUSP 66286. Rio Vermelho, Santa Catarina: MNRJ 4719–4722 (paratypes); WCAB 5093. Santa Luzia, Santa Catarina: MNRJ 10079. São Bento do Sul, Santa Catarina: AL 775 (paratype); MNRJ 4723–4727 (paratypes); WCAB 508. São José, Santa Catarina: ZUEC 4810, 4920, 4950. Timbó, Santa Catarina: MZUSP 64720. Tubarão, Santa Catarina: ZUEC 4940, 4941. Eldorado, São Paulo: MNRJ 4732–4743. Ferraz de Vasconcelos, São Paulo: MZUSP 32103, 32104. Guapiara, São Paulo: (Batistic, 1989). Iguape, São Paulo: MZUSP 12743, 12744. Iporanga, São Paulo: MZUSP 51664. Jacupiranga, São Paulo: DZSJRP 5013; JJ 6938–6941. Miracatu, São Paulo: WCAB 6700, 31740, 31742, 31747, 45145–45148, 45218; ZUEC 4984–4987, 4989, 4990. Pedro de Toledo, São Paulo: MNRJ 10081–10083. Ribeirão Branco, São Paulo: GA-IB 6145, 6168, 6261, 6269.

Phyllomedusa tetraploidea.—Bituruna, Paraná: AL 10071–10074; MNRJ 3857. Caviuna, Paraná: MZUSP 12741. Monte Alegre, Paraná: MZUSP 32067. Botucatu, São Paulo: GA-IB 503, 504, 2410, 2963, 3596, 3597, 4039; MNRJ 1094, 1095. Gália, São Paulo: ZUEC 7581–7584. Garça, São Paulo: WCAB 13674; DZSJRP 1340, 1689, 1690. Guapiara, São Paulo: GA-IB 4898, 4912. Ingá, São Paulo: MZUSP 22476. Paranapanema, São Paulo: EI 8202–8206; GA-IB 4469, 4492, 4530, 4537, 4855, 4880, 4911, 5009, 5011, 5013, 5014, 5097; MNRJ 10787, 10788; MZUSP 67085 (paratype); WCAB 49656, 49657 (paratypes); ZUEC 7585–7589 (four paratypes and the holotype, respectively). Pardinho, São Paulo: JJ 6936, 6937. Ribeirão Branco, São Paulo: GA-IB 6177, 6264, 6280, 6283.

Triploid Hybrids.—Ribeirão Branco, São Paulo: GA-IB 6146, 6150, 6155, 6156, 6159, 6172, 6250.