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Aspects of reproduction and sexual dimorphism of *Lygophis flavifrenatus* (Dipsadidae: Xenodontinae)

Fernando Marques Quintela & Daniel Loebmann

Laboratório de Vertebrados, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Av. Itália s/n, 96203-900, Rio Grande, RS, Brazil.
(fmquintela@yahoo.com.br; contato@danielloebmann.com)

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ABSTRACT. *Lygophis flavifrenatus* Cope, 1862 is a terrestrial dispsadid distributed in subtropical Brazil, Paraguay, Uruguay, and Argentina. Herein, we present data on sexual dimorphism and reproductive biology of this very poorly studied species. A total of 108 specimens (55 females, 50 males, three hatchlings with unidentified sex) from Brazilian states of Rio Grande do Sul, Santa Catarina and Mato Grosso do Sul were analyzed. Sexual dimorphism by size, body dimensions (tail length, head length, head width) and a number of ventral and subcaudal scales is not well marked in the species. Mature females attain the greater size and have a higher number of ventral scales than mature males. Mature males, in turn, presented longer tail, longer and wider head and a higher number of subcaudal scales. Meanwhile, marked overlaps were observed in ranges of all body dimensions proportions and a number of ventral and subcaudal scales. Females attained sexual maturity at a greater size than males. The smallest mature female showed snout-vent length (SVL) = 402 mm while the smallest mature male presented SVL = 285 mm. Females presented a seasonal reproductive cycle, with advanced stages (advanced development of secondary follicles and development of eggs) occurring in late winter and spring. Hatchlings were found in late summer. It is supposed that only one clutch is laid per cycle, considering that the great majority of secondary follicles found in females carrying eggs were irregular or lamellar shaped (possibly atresic). Clutch sizes varied from 6 to 12 eggs (mean = 8.2) and presented a tendency for positive correlation with progenitors SVL. The present study adds new information to the knowledge about the natural history of snakes from subtropical Neotropics.

KEYWORDS. Natural history, Neotropical region, Squamata, subtropical domain.

RESUMO. Aspectos da reprodução e dimorfismo sexual de *Lygophis flavifrenatus* (Dipsadidae: Xenodontinae). *Lygophis flavifrenatus* Cope, 1862 é um dipsadídeo terrestre distribuído pelo Brasil subtropical, Paraguai, Uruguai e Argentina. Apresentamos aqui dados sobre dimorfismo sexual e biologia reprodutiva desta espécie muito pouca estudada. Um total de 108 espécimes (55 fêmeas, 50 machos, três filhotes com sexo não identificado) procedentes dos estados brasileiros do Rio Grande do Sul, Santa Catarina e Mato Grosso do Sul foram analisados. Dimorfismo sexual por tamanho, dimensões corporais (tamanho da cauda, tamanho da cabeça, largura da cabeça) e número de escamas ventrais e subcaudais não é bem marcado na espécie. Fêmeas maduras atingem tamanhos maiores e possuem maior número de escamas ventrais do que os machos maduros. Machos maduros, por sua vez, apresentaram maior cauda, maior comprimento e largura de cabeça e maior número de escamas subcaudais. Entretanto, marcada sobreposição foi observada para os intervalos de todas as dimensões corporais e número de escamas ventrais e subcaudais. Fêmeas atingem a maturidade sexual com maior tamanho do que machos. A menor fêmea madura apresentou comprimento rostro-cloacal (SVL) = 402 mm, enquanto que o menor macho maduro apresentou SVL = 285 mm. As fêmeas apresentaram um ciclo reprodutivo sazonal, com os estágios avançados (desenvolvimento avançado de folículos secundários e desenvolvimento de ovos) ocorrendo no final do inverno e primavera. Filhotes recém-eclodidos foram encontrados no fim do verão. É suposto que apenas uma desova ocorra por ciclo, considerando-se que a grande maioria dos folículos secundários encontrados em fêmeas ovadas apresentou forma irregular ou lamelar (possivelmente atrésicos). O tamanho da prole variou de 6 a 12 ovos (média = 8,2) e apresentou uma tendência **à** correlação positiva com o SVL das progenitoras. O presente estudo adiciona novas informações ao conhecimento sobre a história natural de serpentes procedentes do Neotrópico subtropical.

PALAVRAS-CHAVE. História natural, Região Neotropical, Squamata, domínio subtropical.

Information on aspects of natural history of snakes is crucial for understanding on ecological and evolutionary processes of species and communities (Greene, 1993). Among the several aspects of natural history, data on the reproductive biology are especially essential, considering its importance for the adoption of strategies for the species conservation (Shine & Bonnet, 2009; Almeida-Santos *et al.*, 2014). Once scarce, studies on the reproductive aspects of Neotropical snakes have received a substantial increment in the past decades (Almeida-Santos *et al.*, 2014), which included relevant investigations on species or populations occurring in subtropical climatic domains (Hartmann *et al.*, 2004; Aguiar & Di-Bernardo, 2005; Balestrin & Di-Bernardo, 2005; Leite *et al.*, 2009; Zanella & Cechin, 2010; Oliveira *et al.*, 2011; Mesquita *et al.*, 2013; Panzera & Maneyro, 2013; Rebelato *et al.*, 2016; Loebens *et al.*, 2017; Quintela *et al.*, 2017). These studies revealed a predominant pattern of seasonal female reproductive cycle (restricted to the warmer period of the year) and marked sexual dimorphism, where females attain larger body sizes and males have tails comparatively larger.

The genus *Lygophis* currently comprises eight species of small to medium-sized slender dipsadids, which is distributed from Panamá to Central-Eastern Argentina (Uetz & Hošek, 2018). Previously included in genus *Liophis* (currently *Erythrolamprus*), species of *Lygophis* differ from those ones by the presence of longitudinal well-marked dorsal stripes, reduced optic foramen, besides of hemipenial morphology (Zaher *et al.*, 2009). The "Fronted Ground Snake" *Lygophis flavifrenatus* Cope, 1862 is a typical subtropical species, distributed in Brazilian states of São Paulo, Mato Grosso do Sul, Paraná, Santa Catarina, and the Rio Grande do Sul, southeastern Paraguay, Uruguay, and northeastern Argentine (Michaud & Dixon, 1987; Giraudo *et al.*, 2001; Uetz & Hošek, 2018). Typically terrestrial, it can be found mainly in open habitats such as grasslands and swamps. (Quintela & Loebmann, 2009).

Lygophis flavifrenatus has been considered an uncommon species along its distributional range (Smith, 2006; Ghizoni-Jr *et al.*, 2009; Quintela & Loebmann, 2009), which is a possible reason why data on its natural history are extremely scarce. There are few and scattered data on diet (Carreira-Vidal, 2002), litter size (Amaral, 1977; Achaval & Olmos, 2003), morphometrics and folidosis (Michaud & Dixon, 1987; Giraudo, 2001), based on analysis of few specimens. Previous data on reproductive traits of *L. flavifrenatus* are limited to observation on clutch size (Amaral, 1977; Achaval & Olmos, 2003), and *Lygophis anomalus* (Günther, 1858) is the only *Lygophis* species further investigated for reproduction (Panzera & Maneyro, 2013; Sivan *et al.*, 2016). *Lygophis anomalus* females presented seasonal reproductive cycle, clutch size averaging 7.37 eggs, possibly lay multiple clutches during one reproductive season, and reach sexual maturity with a larger size than males (Panzera & Maneyro, 2013). Mature females and males of *L. anomalus* also differ significantly in body size and tail length (Panzera & Maneyro, 2013). In relation to *L. flavifrenatus*, there are no available data on sexual dimorphism and life-history traits such as size at sexual maturity, reproductive cycle, and size of newborns.

In the present study, we accessed and analyzed specimens of *Lygophis flavifrenatus* from Pampa and Atlantic Forest Domains in subtropical Brazil. We provide data on morphometrics, sexual dimorphism, and aspects of reproductive biology, aiming to contribute to the knowledge on this poorly investigated species as well as to add data for the understanding of reproductive/evolutionary patterns occurring in Neotropical snakes. Considering the patterns recurrently found in the aforementioned studies, we expect that *L. flavifrenatus* females will show a seasonal reproductive cycle and larger size than males at sexual maturity. We also expect to evidence the sexual dimorphism in relation to tail length, with males showing tail proportionally longer.

MATERIAL AND METHODS

We examined specimens deposited in the herpetological collections of Universidade Federal do Rio Grande (CHFURG) and Pontifícia Universidade Católica do Rio Grande do Sul (MCP), collected between 1968 and 2016 in 16 municipalities located in the Brazilian states of Mato Grosso do Sul, Santa Catarina and the Rio Grande do Sul (23°07'57"S, 55°11'03"W and 32°32'23"S, 52°32'25"W) (Appendix I). Collection sites are inserted in biomes Atlantic Forest and Pampa (IBGE, 2018). The annual average temperature is 18.6°C and the monthly averages of minimum (June-July) and maximum (January) temperatures are 13.9 and 19.5°C, respectively. Altitude range from 1 to 1,028 m (a.s.l.) and the annual average precipitation range from 1,205 to 2,109 mm (Climate-Data, 2018).

The following data were taken from each specimen (linear measurements in mm): data of collection, locality, sex, condition of sexual maturity, snout-vent length (SVL), tail length (TL), total length (TTL, the sum of SVL and TL), head length (HL) and head width (HW), number of ventral scales (VS), number of subcaudal scales (SS), ratio of TL to SVL (TL/SVL), ratio of HL to SVL (HL/SVL), ratio of HW to SVL (HW/SVL). The sex of each specimen was determined with the verification of the presence or absence of hemipenis via the subcaudal incision. Females were considered sexually mature when showing at least one of the following characteristics: secondary follicles; oviductal eggs or embryos; folded oviducts, indicating recent oviposition (Mesquita *et al.*, 2013). Males were considered sexually mature when presenting coiled and opaque ductus deferens, indicating the presence of sperm (Shine, 1977; Almeida-Santos *et al.*, 2014). The sexual size dimorphism index (SSD) was calculated as the mean SVL of the larger sex divided by the mean SVL of the smaller sex, minus one. Negative values indicate that males are larger than females (Shine, 1994). The existence of significant sexual dimorphism in SVL, TTL, VS, and SS was examined through a Student's *t*-test (significance $p < 0.05$). The existence of significant sexual dimorphism in TL, HL, and HW was examined through a one-way ANCOVA, using SVL as the independent variable (significance $p < 0.05$) (Siqueira *et al.*, 2013). This procedure was adopted with the objective of minimizing the effect of body size on the analyzes. Only mature specimens were used for SVL, TTL, TL, HL and HW comparisons while mature and immature specimens were pooled for VS and SS comparisons. Specimens with damaged venter, tail or head were excluded from analysis.

The following data were taken from females: 1) a total number of ovarian follicles; 2) a total number of secondary follicles (follicles in secondary vitellogenesis); 3) largest diameter of the largest secondary follicle; 4) a total number of oviductal eggs; 5) largest diameter of the largest egg. The initial diameter of secondary follicles was determined based on coloration; primary follicles differ conspicuously from secondary follicles due to their darker yellow coloration (see Almeida-Santos *et al.*, 2014). The largest diameter of secondary follicles and eggs of females was plotted in an annual temporal axis aiming to obtain data on female reproductive cycle (Mesquita *et al.*, 2013; Almeida-Santos *et al.*, 2014). Fecundity was determined by the number of oviductal eggs (real fecundity) and a number of secondary follicles (potential fecundity) (Mesquita *et al.*, 2013). The relationship between female SVL and real fecundity was examined through simple linear regression analysis (Siqueira *et al.*, 2013). Statistical tests were performed in software PAST v.3.20 (Hammer *et al.*, 2001).

RESULTS AND DISCUSSION

A total of 108 specimens (55 females and 50 males) were analyzed. It was not possible to identify the sex of three hatchlings. Although we observed an overlap of values between the sexes, the males presented higher mean values for all body ratios (TL/SVL, HL/SVL and HW/SVL) (Tab. 1). Significant differences between sexes were found for the variables SVL, TTL, TL, HL, VS and SS (Tab. II). Females showed a higher number of ventral scales (VS) while males presented a higher number of subcaudal scales (SS), but overlapped ranges were observed for both of these characters (Tab. II). The positive value of the sexual size dimorphism index (SSD = 0.206) indicated that females are larger than males.

The smallest male with coiled/opaque ductus deferens (mature male) presented SVL = 285 mm. Reproductive males (n = 28) corresponded to 50% of the male sample. The smallest follicle showing characteristics of secondary vitellogenesis presented the largest diameter of 5.93 mm. The smallest female carrying secondary follicles (mature female) presented SVL = 402 mm while the smallest female carrying eggs showed SVL = 409 mm. Reproductive females (n = 23) corresponded to 41.8% of the female sample. The smallest female carrying primary follicles presented SVL = 202 mm. Primary follicles were found in females collected in all seasons. Secondary follicles were found in 13 females collected from May (middle autumn) to December (late spring). Eggs were found in seven females collected in October and November (early and middle spring) (Fig. 1). Two hatchlings (MCP 14350, SVL = 123 mm; MCP 14351, SVL = 100 mm) retained information about their birth in captive, occurred on March 13 (late summer), but lacked information on the date of their mother egg-laying. A third hatchling specimen with a marked umbilical scar and similar size (MCP 6859, SVL = 101) was collected on February 25 (late summer). The potential fecundity varied from 1 to 13 (1 to 5 in female carrying eggs [X = 2.5; n = 5], 3 to 13 in females without eggs [X = 7.4; n = 7]). The real fecundity varied from 6 to 12 (X = 8.2; n = 7). Five of the six females carrying eggs also presented secondary follicles, but the great majority of these follicles were lamellar or irregular shaped, possibly atretic. The result of the simple linear regression analysis (r = 0.74; p = 0.086; n = 7) indicates a tendency for positive correlation between female SVL and real fecundity.

Females of *Lygophis flavifrenatus* attain greater total length than males, as well as the majority of dipsadids investigated so far (e.g. Aguiar & Di-Bernardo, 2005; Balestrin & Di-Bernardo, 2005; López & Giraudo, 2008, Pizzatto *et al.*, 2008; Zanella & Cechin, 2010; Mesquita *et al.*, 2013; Panzera & Maneyro, 2013; Quintela *et al.*, 2017). Body size in females is associated with fecundity so that a larger body can accommodate a larger offspring (Shine, 1994). In addition to total length, significant differences between the sexes of *L. flavifrenatus* were found for all the other characters except head width (Tab. I). Nevertheless, the superimposed values of the tail and head

Tab. I. Values of ratio of tail length to snout-vent length (TL/SVL), ratio of head length to snout-vent length (HL/SVL) and ratio of head width to snout-vent length (HW/SVL) for males and females of *Lygophis flavifrenatus* Cope, 1862 from Brazil. Values are presented as mean ± one standard deviation (range); n = number of analyzed specimens.

Ratio	Females	Males
TL/SVL	0.372 ± 0.025 (0.339 – 0.452) n = 18	0.405 ± 0.016 (0.367 – 0.447) n = 24
HL/SVL	0.037 ± 0.002 (0.033 – 0.041) n = 22	0.040 ± 0.002 (0.036 – 0.044) n = 27
HW/SVL	0.018 ± 0.002 (0.015 – 0.021) n = 22	0.020 ± 0.001 (0.016 – 0.027) n = 27

Tab. II. Summary statistics (mean ± one standard deviation (range); n = number of analyzed specimens) and values of statistical tests for the variables analyzed for sexual dimorphism in *Lygophis flavifrenatus* Cope, 1862 from Brazil. Significant differences are indicated in bold.

Variable	Females	Males	Statistical test	p values
SVL	495.26 ± 61.53 (402 – 627) n = 23	375.34 ± 47.87 (285 – 460) n = 28	$t = 7.813$	**3.683** $^{\text{E-10}}$
TTL	649.77 ± 62.74 (553 – 746) n = 18	520.71 ± 66.99 (402 – 629) n = 24	$t = 6.346$	**1.538** $^{\text{E-07}}$
TL	179.00 ± 18.16 (146 – 206) n = 18	150.16 ± 18.83 (117 – 174) n = 24	$F = 4.532$	**0.039**
HL	18.15 ± 1.77 (14.98 – 21.65) n = 22	15.04 ± 1.59 (12.63 – 18.46) n = 27	$F = 36.73$	**2.349** $^{\text{E-07}}$
HW	8.90 ± 1.05 (7.19 – 10.85) n = 22	7.44 ± 0.87 (5.66 – 9.53) n = 27	$F = 0.023$	0.878
VS	164 ± 4 (155 – 179) n = 55	156 ± 4 (147 – 167) n = 50	$t = 9.214$	**4.0418** $^{\text{E-15}}$
SS	81 ± 3 (75 – 92) n = 46	87 ± 4 (78 – 99) n = 41	$t = 6.081$	**3.2805** $^{\text{E-08}}$

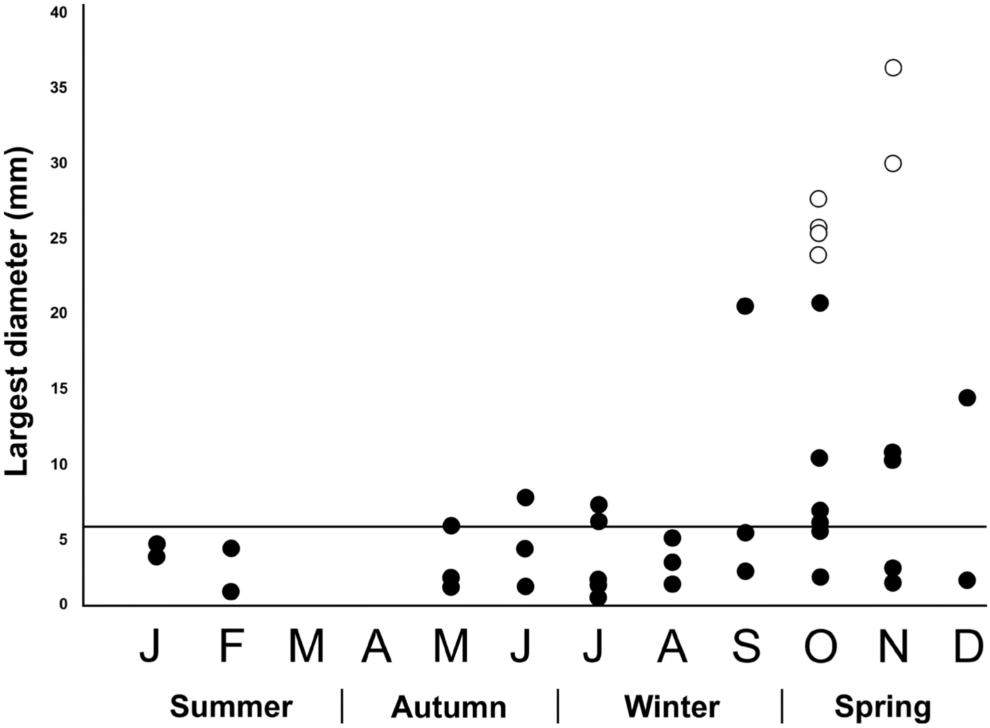

Fig. 1. Monthly variation in largest diameter (in millimeters) of the largest follicles (black circles) and eggs (white circles) of *Lygophis flavifrenatus* Cope, 1862 from Brazil. The horizontal line indicates the size from which follicles were considered as being in secondary vitellogenesis (secondary follicles).

proportions in relation to body size (TL/SVL, HL/SVL, and HW/SVL), as well as the number of ventral and subcaudal scales, makes these characters not absolutely dimorphic. Considering the mean values, males have a comparatively longer tail (TL/SVL) and a larger and wider head (HL/SVL and HW/SVL, respectively; Table 1). However, it is notable that the maximum value for the TL/SVL ratio (0.452) was recorded for a female specimen (Tab. I). Values of TL/SVL ratios calculated from minimum and maximum TL and SVL measures of *L. flavifrenatus* from northeastern Argentina (Giraudo, 2001) were similar between sexes, but males retained the highest proportion (males: 0.340 – 0.366; females: 0.322 – 0.346). Minimum and maximum TL/SVL ratios of Argentine specimens were also lower than those recorded in our sample. Although the limited samples do not allow further comparisons and conclusions, it is possible the existence of geographic variation in this character, as already observed for the sympatric dipsadidae *Erythrolamprus poecilogyrus sublineatus* (Cope, 1860) (Quintela *et al.*, 2017). In relation to head dimensions (HL, HW), females presented larger length and width although significant differences were found only for the first (Tab. II). It is known that sexual dimorphism in size and shape of the head in snakes can be related to combat behavior, divergence in diet or non-adaptative allometry (Meik *et al.*, 2012). Once male-male combat is unknown for *L. flavifrenatus* or any other *Lygophis* species, it is possible that differences in head proportions are related to dietary divergences between sexes (males preying on relatively larger prey), as a mechanism for the reduction of intraspecific competition for food resources, or may even constitute a result of non-adaptive allometry.

The clutch size (real fecundity) herein reported for *L. flavifrenatus* had a broader range than previous observations for the species (8 to 12; Amaral, 1977; Achaval & Olmos, 2003) and was exactly the same found for *L. anomalus* (Panzera & Maneyro, 2013). A positive correlation between female SVL and clutch size was also detected for *L. anomalus* (Panzera & Maneyro, 2013). However, when potential fecundity is considered, a much broader range was observed in *L. anomalus* (1 to 27; Panzera & Maneyro, 2013), emphasizing that the minimum size of secondary follicles was the same for both species (6 mm). Therefore, *L. flavifrenatus* and *L. anomalus* share some similarities concerning the female reproductive biology, which includes the cyclicity pattern, clutch size, relationship between the size of female body and clutch size, and the size of the follicle at the beginning of secondary vitellogenesis. This is not surprising, considering that phylogenetic conservatism in reproductive traits is pronounced in Xenodontinae snakes (Bellini *et al.*, 2017). Moreover, the two species are sympatric in a great part of their distribution (Giraudo, 2001), sharing environmental conditions (climate, habitats) and even feeding habits (Carreira & Maneyro, 2013), which may reinforce such similarities if environmental factors also contribute to shaping the reproductive patterns.

The annual profile of the largest follicles and eggs indicates a seasonal reproductive cycle for *Lygophis flavifrenatus* females. Vitellogenesis begins in autumn and extends until spring when copulation and development of eggs take place. Copulation occurs at the end of winter, considering that eggs were found already in early spring (October). The finding of mature females euthanized in summer (January and February) showing folded oviducts and carrying only primary follicles associated to the information on date of hatchlings birth and collection (late February and March) indicates that egg-laying occurs during middle/late spring and early summer, seeing that period of egg incubation for other xenodontines in the region varied from 55 to 84 days (J. G. Frota, unpubl. data; F. Bonfiglio, unpubl. data; G. M. Funk-Pontes, unpubl. data). Once cold temperatures represent a limiting factor mainly for egg incubation (HUBERT, 1985), the seasonal cycle with the advanced stages restricted to warmer seasons was expected. This pattern corroborates with the female cycle observed for congeneric *Lygophis anomalus* (PANZERA & MANEYRO, 2013), as well as subtropical populations of other dipsadid species (AGUIAR & DI-BERNARDO, 2005; BALESTRIN & DI-BERNARDO, 2005; LÓPEZ & GIRAUDO, 2008; ZANELLA & CECHIN, 2010; OLIVEIRA *et al.*, 2011; MESQUITA *et al.* 2013; REBELATO *et al.* 2016; QUINTELA *et al.*, 2017; J. G. Frota, unpubl. data; F. Bonfiglio, unpubl. data; G. M. Funk-Pontes, unpubl. data). The preponderant occurrence of irregular-shaped secondary follicles (probably atretic) in females carrying eggs also indicates that a single egg-laying take place during a cycle. This finding contrasts *L. anomalus*, supposed to lay multiple clutches during a cycle (PANZERA & MANEYRO, 2013).

This is the first study to bring further information on sexual dimorphism and reproduction of *Lygophis flavifrenatus*, adding to the knowledge on the biology of Neotropical snakes. The new data herein presented support the predominant pattern of female reproductive cycle found so far for dipsadids from subtropical domains of South America. The similarities of female reproduction traits with the congenus *L. anomalus* indicate the phylogenetic conservatism, although environmental driving forces may not be disconsidered. Ongoing studies on morphometrics and histology of male gonads and ductus deferens will soon provide data on male reproductive cycle, bringing an integrative perspective on the reproductive biology of this poorly known species.

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Appendix I. Specimens of *Lygophis flavifrenatus* Cope, 1862 analyzed and vouchered in herpetological collections of Universidade Federal de Rio Grande (CHFURG) and Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul (MCP).

BRAZIL, **Mato Grosso do Sul**: Amambaí (MCP 16357); **Santa Catarina**: Fraiburgo (MCP 16355, 16356), Campo Belo do Sul (MCP 12097), Lages (MCP 18580); **Rio Grande do Sul**: São Francisco de Paula (MCP 7777), Arroio do Sal (MCP 4347, 4384, 5247, 11296), Capão da Canoa (MCP 6759, 6760), Balneário Pinhal (MCP 8586, 8587, 11832, 13122, CHFURG 5405), General Câmara (MCP 4232), Canoas (MCP 110), Porto Alegre (MCP 3182, 3277, 6859, 14350, 14351), Cacequi (MCP 18736), Cachoeira do Sul (MCP 6184), Palmares do Sul (MCP 5248, 10650, 10651, 10998, 11187, 11188, 11196), Tavares (MCP 4219), Lavras do Sul (MCP 18831), São Gabriel (MCP 18832), Rio Grande, Senandes (CHFURG 813, 1029, 1253), Bolaxa (CHFURG 3161), Barra (CHFURG 1694, 1701, 1702, 1835, 1857, 1867, 1859, 1877, 1989, 3082, 3111, 3113, 3330, 3331, 3332, 3437, 4442, 4573, 4697, 4786, 4787, 4815, 4898, 4899, 4900, 4983, 4991, 4996, 4997, 5057, 5058, 5059, 5060, 5064, 5200, 5220, 5223, 5236, 5274, 5282, 5300, 5301, 5308, 5321, 5322, 5335, 5375, 5377, 5381, 5383, 5384, 5385, 5386, 5387, 5388, 5389, 5391, 5392, 5393, 5396, 5402, 5403, 5580, 5581, 5714, 5976), Parque São Pedro (CHFURG 872), Taim (CHFURG 1420).