Biota Neotropica 17(2): e20160225, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
Inventory

The genus *Chamaecrista* Moench in a fragment of the Ecological Station Raso da Catarina, Bahia, Brazil

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CORREIA, C.L.S.B., CONCEIÇÃO, A.S. **The genus *Chamaecrista* Moench in a fragment of the Ecological Station Raso da Catarina, Bahia, Brazil**. Biota Neotropica. 17(2): e20160225. http://dx.doi.org/10.1590/1676-0611-BN-2016-0225

***Abstract*:** *Chamaecrista* has a Pantropical distribution, with some occurrences in Australia and temperate areas, and includes about 330 species, of which 266 occur in the Americas. The genus is represented in Brazil by 256 species, of which 97 are cited for the northeast Region. The Ecological Station Raso da Catarina (ESRC) is one of the largest areas of protected Caatinga and occupies about 105,282.00 ha., delimited by the coordinates 09°39'0.30" to 09°50'98.2" S and 38°26'57.5" to 38°29'32.6" W. The floristic survey of *Chamaecrista* in the ESRC included analysis of specimens collected from March 2010 and October 2011. The analyses were supplemented with dried collections from the following herbaria: ALCB, EAC, HRB, HUEFS and MBM. The genus is represented in the study area by ten taxa. The most representative taxa in the area were *Chamaecrista repens* (Vogel) H.S.Irwin & Barneby var. *multijuga* (Benth.) H.S.Irwin & Barneby, *C. brevicalyx* (Benth.) H.S.Irwin & Barneby var. *brevicalyx, C. belemii* (H.S.Irwin & Barneby) H.S.Irwin & Barneby var. *belemii,* wich are directly related to sandy soils common in the region. The taxonomic treatment includes a key for the identification, descriptions, illustrations, photos, data geographical distribution, reproductive phenology and comments about the taxa.

Keywords: *floristics, taxonomy, semiarid, Caatinga, diversity.*

O gênero *Chamaecrista* Moench em um fragmento da Estação Ecológica Raso da Catarina, Bahia, Brasil

Resumo: *Chamaecrista* possui uma distribuição Pantropical, com algumas ocorrências na Austrália e em áreas temperadas, inclui cerca de 330 espécies, das quais 266 ocorrem nas Américas. O gênero está representado no Brasil por 256 espécies, das quais 97 são citadas para a região nordeste. A Estação Ecológica Raso da Catarina (ECRC) é uma das maiores áreas protegidas de Caatinga e ocupa cerca de 105.282.00 ha., delimitada pelas coordenadas 09°39'0,30" a 09°50'98,2" S e 38°26'57,5" a 38°29'32,6" W. O levantamento florístico de *Chamaecrista* na ECRC incluiu análises de espécimes coletados de março 2010 a outubro de 2011. As análises foram complementadas com coleções dos seguintes herbários: ALCB, EAC, HRB, HUEFS e MBM. O gênero está representado na área de estudo por dez táxons, onde os mais representativos foram: *Chamaecrista repens* (Vogel) H.S.Irwin & Barneby var. *multijuga* (Benth.) H.S.Irwin & Barneby, *C. brevicalyx* (Benth.) H.S.Irwin & Barneby var. *brevicalyx, C. belemii* (H.S.Irwin & Barneby) H.S.Irwin & Barneby var. *belemii,* estando diretamente relacionados com solos arenosos comuns na região. O tratamento taxonômico inclui uma chave para a identificação, descrições, ilustrações, fotografias, dados de distribuição geográfica, fenologia reprodutiva e comentários sobre os táxons.

Palavras-chave: *florística, taxonomia, semiárido, Caatinga, diversidade.*

Introduction

Chamaecrista was established by Moench (1794), segregating it from *Cassia s.l.* (Irwin & Barneby 1982). It is one of the largest genera of the subfamily Caesalpinioideae and the largest of the subtribe Cassiinae (Lewis 2005). It has a Pantropical distribution, with some occurrences in Australia and temperate areas. The genus includes approximately 330 species, of which 266 occur in the Americas (Irwin & Barneby 1982, Lewis 2005).

In Brazil, the genus is represented by about 256 species, of which 97 are cited for the Northeast Region. Bahia is considered to be the state with the third greatest number of taxa of *Chamaecrista* with 91 species. The center of their diversity is in campos rupestres (rocky fields) (Lewis 1987, Conceição et al. 2001, Zappi et al. 2003, Souza & Bortoluzzi 2016). In Caatinga twenty-nine species were recorded for *Chamaecrista* (Queiroz 2009).

Chamaecrista is mainly characterized by presence of two alternate bracteoles on the pedicel of the flowers, convex extrafloral nectaries and spiraled elastically dehiscent fruits. According to Irwin & Barneby (1982), the genus includes six sections. All phylogenetic studies based on morphological and molecular data supported the monophyly of *Chamaecrista* (Conceição et al. 2009, Rando et al. 2016). However, with regard to infrageneric classification, the study of Conceição et al. (2009) showed that only two of the six sections recognized by Irwin & Barneby (1982) are monophyletic. Rando et al. (2016) supports the monophyly of *C.* ser. *Coriaceae,* suggesting *C.* ser. *Flexuosae* as the sister group of this clade.

The more comprehensive taxonomic treatments for *Chamaecrista* was carried by Vogel (1837), Bentham (1870, 1871), Greene (1897), Pollard (1902), Britton & Rose (1930), Irwin & Rogers (1967), Irwin (1964) and Irwin & Barneby (1977, 1978, 1981, 1982), Fernandes & Nunes (2005). The last three studies comprised the most relevant treatments of the genus, with the recognition of sections and series, and the descriptions of new species and infraspecific taxa, including additional information on ecology and geographic distribution.

The main contributions on taxonomy of the referred genus in Brazil are generally about local floristic surveys, publication of new species or re-establishment, which showed the great diversity of the genus to Brazil, such as the works conducted by Harley & Simmons (1986), Lewis (1987), Barneby (1994), Costa (1996), Conceição et al. (2001, 2003), Camargo & Miotto (2004), Hervencio & Queiroz (2004), Conceição (2006), Bortoluzzi & Miotto (2007), Cardoso & Queiroz (2007), Queiroz (2009), Queiroz & Loiola (2009), Rando (2009), Rando & Pirani (2012), Dantas & Silva (2013), Rando et al. (2013a, b), Souza & Silva (2014, 2015), Silva & Souza (2015), Souza et al. (2015), Barbosa et al. (2016), Cota et al. (2016).

Despite the existence of studies about *Chamaecrista* for the semiarid of northeastern Brazil, regional works for the state of Bahia are scarce, especially those that include keys of identification and descriptions. Given the importance of *Chamaecrista* in the Caatinga vegetation, this work had as a goal to carry out the survey of the species of the genus in the Ecological Station Raso da Catarina (ESRC) in order to contribute to knowledge about the flora of the semiarid region of Bahia as well as to support the development of the ESRC management plan.

Material and Methods

The Raso da Catarina Ecoregion comprises 30.800 km^2 and is one of the eight Ecoregions recognized for the Caatinga and includes units of conservation. In the North-south direction it is narrow and elongated. In the North, West and East directions it is limited to the southern hinterland depression. The northeastern portion has limits with the Borborema Plateau and the southern part of the Bahia hinterland, in the Zona da Mata. The Ecoregion is a basin with soils that are very sandy, deep and little fertile. Its relief is very flat, but with canyons in the western part (formed by sandstone outcrops). The altitudes above sea level vary from 400 to 600 m in the southern part (Bahia) and from 350 to 700 m in the northern part (Jatobá basin, Pernambuco). In the southern part (Bahia) most of the soils are composed of sand (deep, excessively drained, acid and very low fertility) and oxisol (deep, well drained, acid and low fertility) whereas in the northern part (Pernambuco) sands soils prevail. There exists little surface water in the region except in the areas of the canyons. The predominant vegetation is the sandy, bushy Caatinga, very dense and less thorny than the Caatinga of crystalline soils (Velloso et al. 2002).

The Ecological Station Raso da Catarina (ESRC, Figure 1) is one of the protected areas of the Raso da Catarina Ecoregion. It is one of the

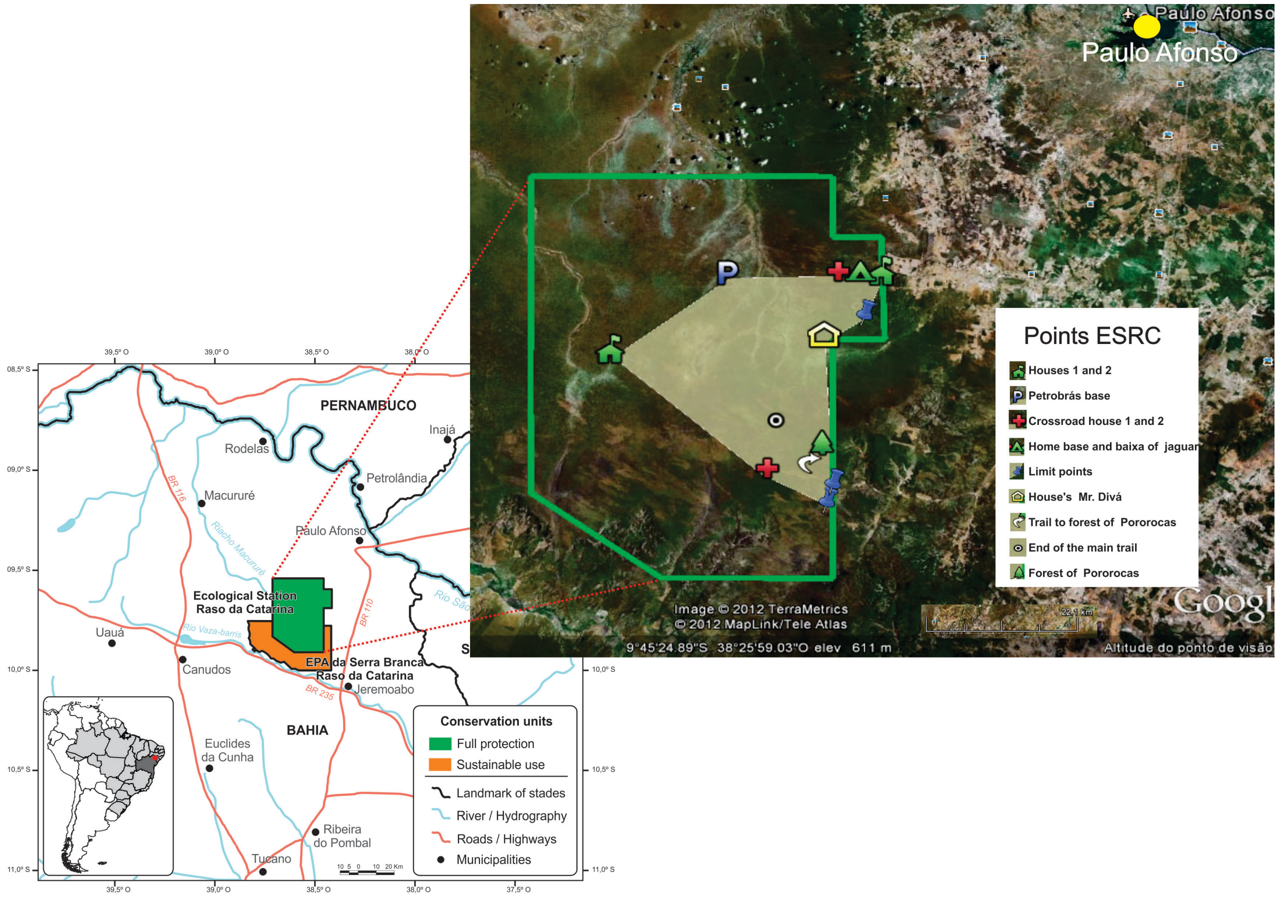

Figure 1. Location of the fragment studied in the Raso da Catarina Ecological Station (Varjão et al. 2013, modified).

largest areas of protected Caatinga and occupies about 105,282.00 ha., delimited by the coordinates 09°39'0.30" to 09°50'98.2" S and 38°26'57.5" to 38°29'32.6" W, limited to the North with the Pankararé aldeia, to the east with the municipalities of Rodelas and Canudos, to the South with the municipality of Jeremoabo and West with the municipalities of Paulo Afonso and Jeremoabo. The climate of the ESRC is semiarid with average rainfalls of 500 mm/year and annual temperature is approximately 23ºC (Szabo et al. 2007). The soils are generally sandy deep and very fertile relief plan with sandstone formations (Velloso et al. 2002).

The study was based on fieldwork carried out in the period between March 2010 and October 2011, besides information complemented by the analysis of species deposited in the herbaria: ALCB, EAC, HRB, HUEFS and MBM, acronyms according to Thiers 2016 (continuously updated). The field collections and observations were performed during random walks exploring most of the study area. Herborization and material processing followed the methodology by Fosberg & Sachet (1965) and Mori et al. (1989), where fertile material was collected with flowers and/or fruit. Observations were made about the distribution of the species and the type of soil (Tricart 1972, Sampaio 1995). The specimens were deposited in the herbarium of the State University of Bahia (HUNEB – Collection Paulo Afonso) and the duplicates were sent to the main herbaria in the state of Bahia.

The identifications were made based mainly on specialized bibliographies (e.g., Irwin & Barneby 1978, 1982 and Queiroz 2009), protologues, photos of type collections and consulting of the collections in the herbaria that were visited. For the taxonomic descriptions, the terminologies proposed by Radford et al. (1974), Irwin & Barneby (1978, 1982), Ribeiro et al. (1999), Harris & Harris (2001) and Gonçalves & Lorenzi (2011) were adopted. The taxonomic treatment includes a key for the identification of taxa, descriptions, illustrations, and data of the geographical distribution and reproductive phenology of the species.

Results and Discussion

Chamaecrista Moench, Meth. pl. hort. Bot. Marburg.: 272, 1794. Lectotype (designated by Irwin, 1964): sect. *Chamaecrista*, *C. nictitans* (L.) Moench.

Herbs, shrubs or subshrubs, erect, prostrate, sarmentose, decumbent or procumbent, little or profusely brancheds; branches cylindrical, rare quadrangular, straight, sometimes tortuous, rare fractiflex. Leaves green, alternating, paripinnate, 2–multifoliolate; extrafloral nectaries present or absent, when present with concave secretory surface, sessile and discoid or stipitate and caliciform, located on the petiole, rarely on the rachis and pairs of leaflets. Inflorescence racemose, elongated, or reduced in fascicles axillary or supra-axillary, with few flowers or reduced to a single flower; bracteoles 2, alternating or subopposite, than in different positions on the pedicel; flowers pentamerous, asymmetric; sepals free, the internal larger the ones; petals free, yellow to orange, heteromorphic, with a differentiated internal abaxial petal (cuculus), falcate, spatulate to suborbicular, bent on the stamens, protecting the androecium, stamens 10, homomorphic, arranged at equal length levels or at different levels, anthers dehiscent by apical pore. Legumes elastically dehiscent, oblong, plan-compressed, with valves chartaceous or coriaceous, spiraled after seed release. Seeds compressed, glossy, rhomboid to pyriform, testa dark brown, pitted, pits aligned in vertical rows.

In the Ecological Station Raso da Catarina ten taxa were recorded for genus. *Chamaecrista repens* (Vogel) H.S.Irwin & Barneby var. *multijuga* (Benth.) H.S.Irwin & Barneby, *C. brevicalyx* (Benth.) H.S.Irwin & Barneby var. *brevicalyx, C. belemii* (H.S.Irwin & Barneby) H.S.Irwin & Barneby var. *belemii,* were taxa predominant in the studied area, being directly related to sandy soils common in the region.

Identification key for the representatives of the genus Chamaecrista

1. Presence of tector trichomes only; extrafloral nectaries present; leaves with 2–21 pairs of leaflets; inflorescences axillary, supra-axillary or reduced to only flower.
    2. Leaves with 2–8 pairs of leaflets; inflorescences axillary, reduced to a single flower.
        3. Leaves with two pairs of leaflets, venation paralelinervous; sepals multistriated.….7. *Chamaecrista ramosa* var. *ramosa*
        3'. Leaves with 4–8 pairs of leaflets, venation palminerveous; sepals striated.
            4. Branches quadrangular, fractiflex; stipules acuminate to aristated; leaflets with cuspidate to spinescent apex ....9. *C. swainsonii*
            4'. Branches cylindrical, erect; stipules lanceolate to deltoid; leaflets with acuminate to cuspidate apex .. ..10. *C. tenuisepala*
    2'. Leaves with 8–21 pairs of leaflets; inflorescences supra-axillary.
        5. Extrafloral nectary sessile to shortly stipitate, discoid to caliciform, head less dilated the stipe, located little above the middle of the petiole; midrib excentric, dividing the leaflet in a ratio of 1:2–2.5 at the base............................... ....8. *C. repens* var. *multijuga*
        5'. Extrafloral nectary shortly stipitate, caliciform, head more dialated than the stipe, located next to the middle of the petiole; midrib little excentric dividing the leaflet in a ratio of 1:1–1.5 at the base …... ....6. *C. nictitans* subsp. *disadena* var. *disadena*

1'. Presence of tector and glandular trichomes; extrafloral nectaries absent; leaves with two pairs of leaflets; inflorescence terminal.
    6. Herbs procumbent to sarmentose; petiole c. 1.5 times longer than the rachis; flower buds acute ....5. *C. carobinha*
    6'. Shrubs to subshrubs erect or herbs to subshrubs procumbent; petiole c. 1.5–7 times longer than the rachis; flower buds rounded.
        7. Petiole c. 7 times longer than the rachis; leaflets glabrous.
            8. Epidermis on the young branches pale to whitish; leaflets ovate to suborbicular, 9–35 mm long.; flowers c. 3 cm. diam................ 4. *C. brevicalyx* var. *brevicalyx*
            8'. Epidermis on the young branches green; leaflets ovate to obovate, 4–11 mm long.; flowers c. 1 cm diam. …....……….................................... 2. *C. amiciella*
        7'. Petiole 1.5–5 longer than the rachis; leaflets glabrous to pilose.
            9. Leaflets ovate to obovate, apex emarginate to rounded; petiole c. 1.5 times longer than the rachis ………...……….............. 3. *C. belemii* var. *belemii*
            9'. Leaflets elliptic to obelliptic, apex acute to emarginate; petiole c. 3–5 times longer than the rachis.……...1. *C. acosmifolia*

1. ***Chamaecrista acosmifolia*** (Benth.) H.S.Irwin & Barneby var. ***acosmifolia***, Mem. New York Bot. Gard. 35(2): 660. 1982. Figures 2a; 3a-k

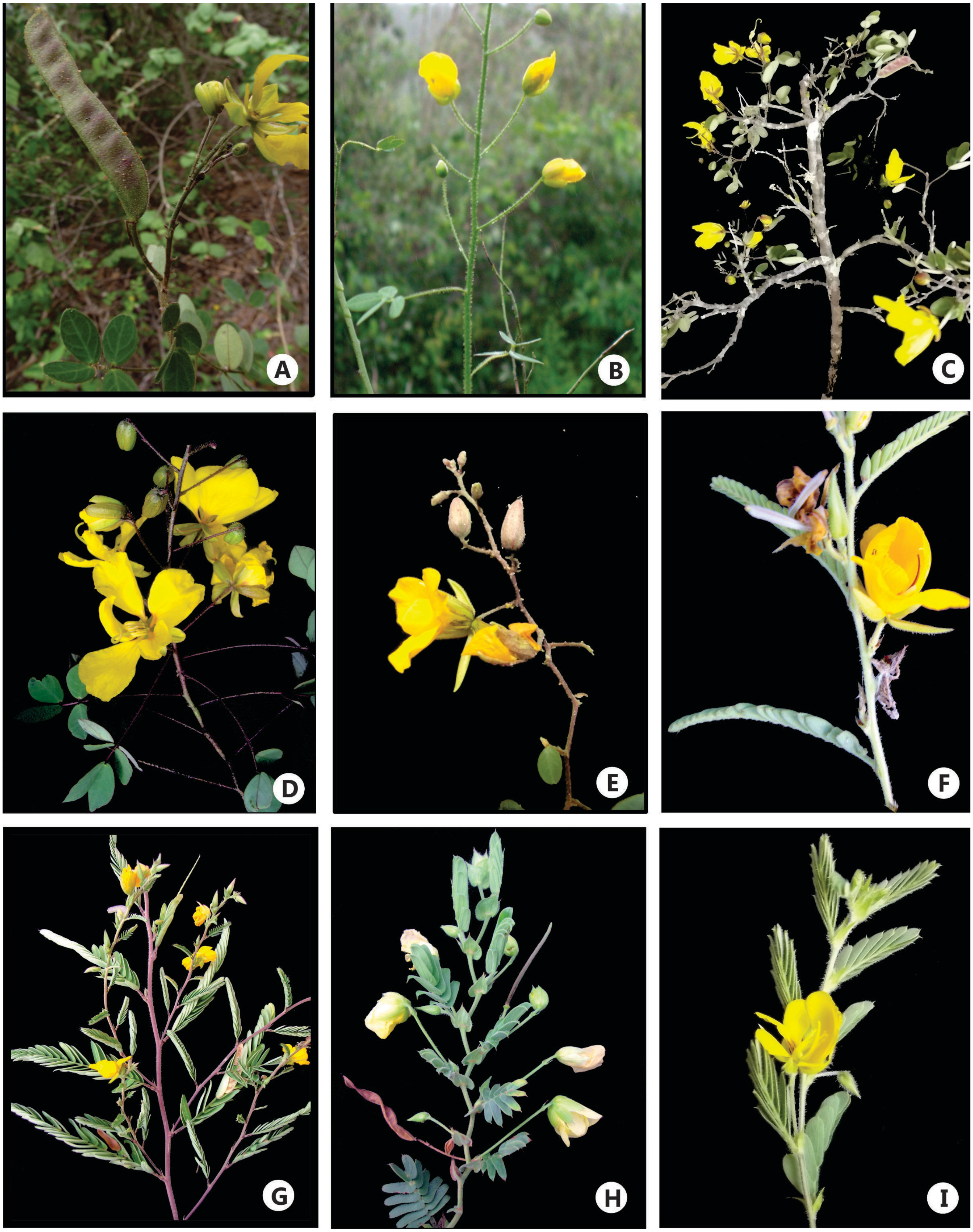

Figure 2. Representative of *Chamaecrista* in the ESRC: a) *Chamaecrista acosmifolia* (Benth.) H.S.Irwin & Barneby var. *acosmifolia*; b) *C. amiciella* (H.S.Irwin & Barneby) H.S.Irwin & Barneby; c) *C. belemii* (H.S.Irwin & Barneby) H.S.Irwin & Barneby var. *belemii*; d) *C. brevicalyx* (Benth.) H.S.Irwin & Barneby var. *brevicalyx*; e) *C. carobinha* (H.S.Irwin & Barneby) H.S.Irwin & Barneby; f) *C. nictitans* Moench subsp. *disadena* (Steud.) H.S.Irwin & Barneby var. *disadena*; g) *C. repens* (Vogel) H.S.Irwin & Barneby var. *multijuga* (Benth.) H.S.Irwin & Barneby; h) *Chamaecrista swainsonii* (Benth) H.S.Irwin & Barneby; i) *C. tenuisepala* (Benth.) H.S.Irwin & Barneby. Photos by C.L.S.B.Correia.

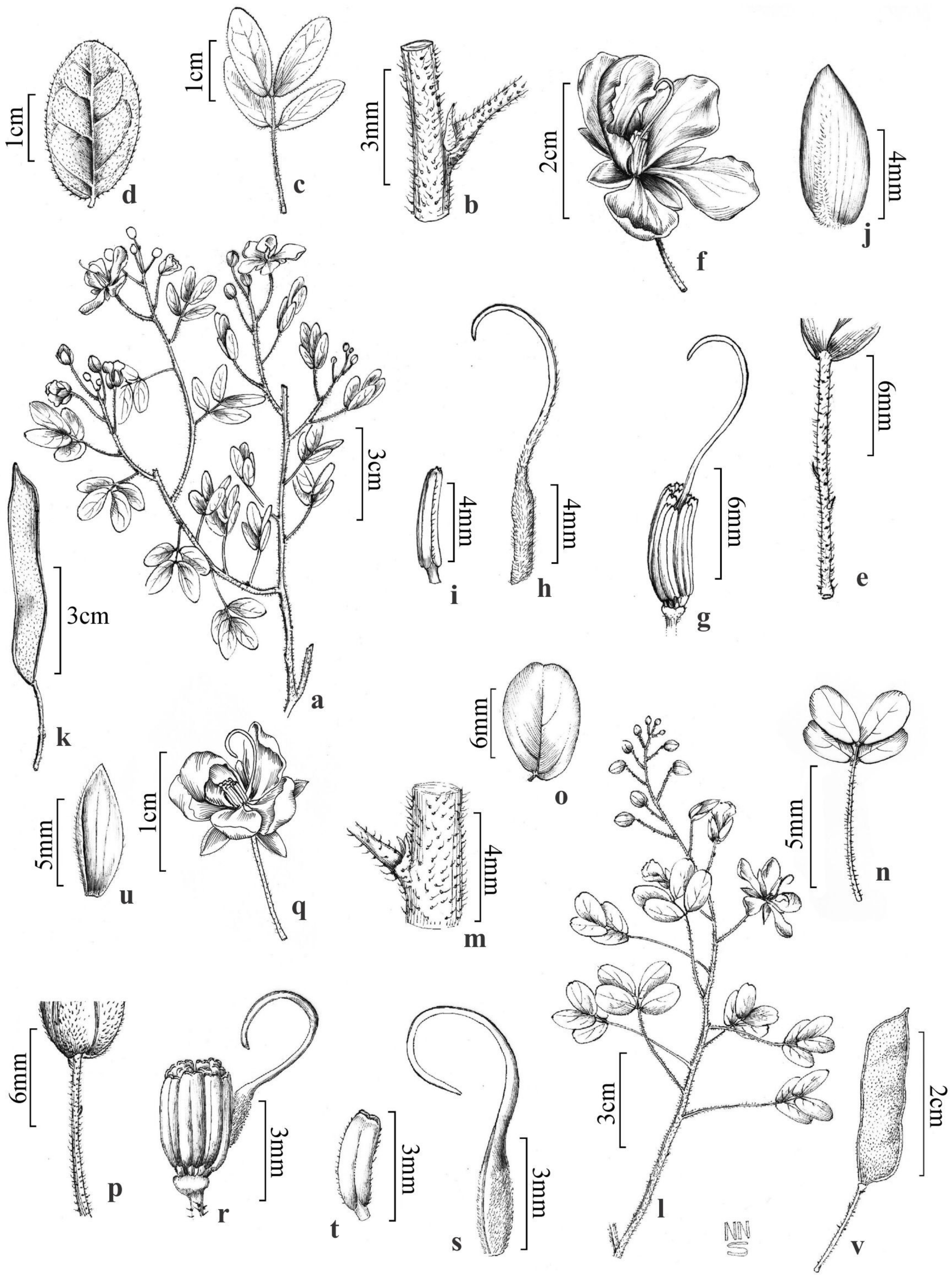

Figure 3. a-k) *Chamaecrista acosmifolia* var. *acosmifolia*: a) flowering branch; b) stipule; c) leaf; d. leaflet; e) pedicel and bracteoles; f) flower; g) androecium and gynoecium; h) pistil; i) stamen; j) sepal; k) fruit. l-v) *Chamaecrista amiciella*: l) flowering branch; m) stipule; n) leaf; o) leaflet; p) pedicel and bracteoles; q) flower; r) androecium and gynoecium; s) pistil; t) stamen; u) sepal; v) fruit. a-k from C.L.S.B. Correia 359; l-v from C.L.S.B. Correia 327.

Shrubs erect, branched until 3 m tall; branches cylindrical,erect, epidermis vinaceous on the young branches, discretely exfoliating and brown on the old branches. Indumentum glabrescent, constituted of trichomes glandular and tector, thin, yellow to dark castaneous, rigid, erect, sparse, c. 1 mm long, distributed on the branches, petioles, inflorescence axis, pedicels, buds and sepals. Stipules vinaceous to brown, filiform, obsolete, 0.5–1 × c. 0.5 mm, caducous. Leaves 2.2–6.1 cm long; pulvinus brown, sparsely pilose, 1–2 mm diam.; petiole 10–22 mm long, c. 3–5 times longer than the rachis; without extrafloral nectaries; rachis 2–7 mm long; leaflets discolorous, 2 pairs, chartaceous, pilose, 15–25 × 7–14 mm, elliptic to obelliptic, apex acute to emarginate, base rounded and asymmetric, venation penninervous. Inflorescences racemose grouped in panicles, terminal, 6–34 flowers; inflorescence axis 5–10 cm long; bracts vinaceous to brown, lanceolate, 1.5–2 × 0.5–1 mm; pedicel 7–24 mm long; bracteoles green vinaceous, lanceolate, 0.5–1 × 0.5 mm, located on the middle of pedicel. Buds green-vinaceous, rounded, 1–5 mm long. Flowers c. 2.5 cm diam.; sepals green glaucous to yellowish-green, ovate to oblong, abaxial surface pilose, striated, 6–9 × 2.5–5.5 mm; petals golden yellow, two external, obovate to orbicular, 12–14 × 8–10 mm, two internal, oblong to obelliptic, 8–15 × 3–9 mm, cuculus falcate, bent around the stamens, 10–13 × 18.5–20 mm; stamens yellow, 3–6 mm long; ovary green glaucous to yellowish, tomentose, 2–6 mm long; style yellow, 8–11 mm long. Legumes oblong, linear, green to green-vinaceous when young, mature brown, 12–64 × 2–9 mm; valves coriaceous, setulose. Seeds ellipsoid when young, quadrangular to rhomboid when mature, brown to vinaceous, glossy, pitted at base, 4–6 × c. 4 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido Baixa da onça, 09°52’21” S and 38º37’88” W, 521 m, 30.XI.2010, fr., C.L.S.B. Correia et al. 310 (HUNEB); Trilha sentido Pedra da janela, 09°39’55” S and 38°28’02” W, 570 m, 30.XII.2010, fl.; fr., C.L.S.B. Correia et al. 359 (HUNEB); Baixa da onça, 09°52’21” S and 38°37’88” W, 521 m, 24.III.2010, fl.; fr., C.L.S.B. Correia et al. 110 (HUNEB).

Chamaecrista acosmifolia is a Brazilian endemic species having been recorded to Minas Gerais, Goiás and Mato Grosso, where it occurs in Caatinga and Cerrado at altitudes of 500-600 m (Irwin & Barneby 1978, Queiroz 2009). Irwin & Barneby (1978) recognized three varieties for the species, two of which occur in the Caatinga: *C. acosmifolia* (Benth.) H.S.Irwin & Barneby var. *euryloba* (H.S.Irwin & Barneby) H.S.Irwin & Barneby, with records only for Bahia, and *C. acosmifolia* (Benth.) H.S.Irwin & Barneby var. *acosmifolia,* distributed in Bahia, Piauí and Maranhão. In the study area, only the *acosmifolia* variety was catalogued; it is rare, flowers and fruits in November, December and March, and occurs in ecotonal areas with rocky soil at altitudes of 521-570 m.

In the study area, the taxon can be recognized by two pairs of elliptic to obelliptic leaflets, but can be confused with *C. belemii* var. *belemii* because both have leaves with two pairs of leaflets and racemose terminal inflorescences. However, the two taxa can be differentiated by size and shape of their leaflets (6–25 × 4–13 mm, ovate to obovate in *C. belemii* var. *belemii vs.* 15–25 × 7–14 mm, elliptic to obelliptic in *C. acosmifolia* var. *acosmifolia*) and size of their fruits (6–38 mm long in *C. belemii* var. *belemii vs.*12–64 mm long in *C. acosmifolia* var. *acosmifolia*).

2. ***Chamaecrista amiciella*** (H.S.Irwin & Barneby) H.S.Irwin & Barneby, Mem. New York Bot. Gard. 35(2): 661. 1982. Figures 2b; 3l-v

Herbs and subshrubs procumbent, little branched until 1 m tall; branches cylindrical, erect or tortuous, epidermis green on the young branches, brown on the older branches. Indumentum glabrescent, constituted of trichomes glandular and tector, thin, yellow to dark brown, rigid, erect, sparse, c. 1 mm long, distributed on the branches, petioles, inflorescence axis, pedicels, buds and sepals. Stipules green to brown, filiform, obsolete, caducous, 0.5–1 × c. 0.5 mm. Leaves 2.2–4.5 cm long; pulvinus brown, sparsely pilose, 1–2 mm diam; petiole 7–28 mm long., c. 7 times longer than the rachis; without extrafloral nectaries; rachis 1–4 mm long; leaflets discolorous, 2 pairs, chartaceous, glabrous, ovate to obovate, 4–11 × 3–7 mm, apex emarginate to retuse, base rounded, venation penninervous. Inflorescences racemose, terminal, 6–34 flowered, inflorescence axis 4.5–6.7 cm long; bracts vinaceous to castaneous, lanceolate, 1.5–2 × 0.5–1 mm; pedicel 6–12 mm long; bracteoles green vinaceous, lanceolate, 0.5–1 × 0.5 mm, located on the upper region of the pedicel. Buds green to green-vinaceous, rounded, 1–7 mm long. Flowers c. 1 cm diam.; sepals green glaucous to green-yellowish, ovate to oblong, abaxial surface pilose, striated, 4–6 × 3–5.5 mm; petals yellow, two external, obovate, 9–11 × 4–6 mm, two internal, oblong to obelliptic,7–9 × 3–4 mm, cuculus falcate, bent around the stamens, 8–9.5 × 9–10 mm; stamens yellow, 2.5–4 mm long; ovary pale green to yellowish, pilose, 1.5–2 mm long; style yellow, 4–6 mm long. Legumes oblong, linear, green when young, mature not seen, 24–27 × 5–6.5 mm, valves chartaceous, setulose. Seeds not seen.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido Mata da pororoca, 9°48’32” S and 38°29’30” W, 584 m, 29.XII.2010, fl., C.L.S.B. Correia et al. 327 (HUNEB); Trilha sentido casa II, vindo da Pororoca, 09°49’15” S and 38°29’33” W, 670 m, 14.I.2010, C.L.S.B. Correia et al. 373 (HUNEB); Baixa do Cascavel, 09.VI.11, 09°44’08” S and 38°40’56” W, 606 m, C.L.S.B. Correia et al. 489 (HUNEB).

Chamaecrista amiciella is endemic to the Caatinga and has been recorded in southern Ceará, western Paraíba and northern Bahia, where it occurs on sandy soil at altitudes between 150-500 m (Irwin & Barneby 1978, Queiroz 2009). In the ESRC the species is rare and occurs on sandy soil at altitudes of 580-660 m. It was collected with flowers and fruits in January, December and June.

The size of the leaflets (4–11 mm) and diameter of the flowers (ca. 1 cm), distinguish it from other species occurring in the area.

3. ***Chamaecrista belemii*** (H.S.Irwin & Barneby) H.S.Irwin & Barneby var. ***belemii***, Mem. New York Bot. Gard. 35(2): 644. 1978. Figures 2c; 4a-k

Shrubs erect, profusely branched until 1.5 m tall; branches cylindrical,erect a tortuous, epidermis brown on the young branches, exfoliating and pale to greyish on the old branches. Indumentum glabrescent, constituted of trichomes glandular and tector, thin, brown to black and little orange to white, flexible, erect, sparse, c. 1.5 mm long, distributed on the branches, margin of stipules, petioles, pulvinus, rachis, pedicel, buds, sepals, ovary, legumes, rarely occurring on margin of leaflets. Stipules vinaceous to brown, lanceolate, obsolete, 0.5–2 × 0.5–1 mm, caducous. Leaves 2.2–4.8 cm long; pulvinus brown, sparsely pilose, c. 1 mm diam; petiole 7–19 mm long, c. 1.5 times longer than the rachis; without extrafloral nectaries; rachis 4–8 mm long; leaflets discolorous, 2 pairs, chartaceous, glabrous to sparsely pilose, ovate to obovate, 6–25 × 4–13 mm, apex emarginate to rounded, base asymmetrical, venation penninervous. Inflorescences racemose, terminal, sometimes grouped in panicles; 2–8 flowered, inflorescence axis 2–4.5 cm long; bracts green, lanceolate, 1–1,5 mm long; pedicel 4–20 mm long; bracteoles vinaceous, lanceolate to ovate, 0.5–1.5 × c. 0.5 mm, located along the pedicel. Buds green, rounded, 3–7 mm long. Flowers c. 3 cm diam; sepals green, ovate to obovate, abaxial surface pilose, striated, 7–10 × 3–6 mm; petals golden yellow, two external, obovate to orbicular, 19–23 × 16–18 mm, two internal, obovate, 15–17 × 11–12 mm, cuculus falcate, bent around the stamens, c. 18 × 11–13 mm; stamens yellow, 5–7 mm long; ovary green glaucous to yellowish-green; style yellow, 5–9 mm long. Legumes oblong, linear, green when young vinaceous, brown when mature, 6–38 × 3–8 mm, valves coriaceous, puberule to setulose. Seeds quadrangular to rhomboid, brown to black, glossy and pitted, 3–4 × 1–3 mm.

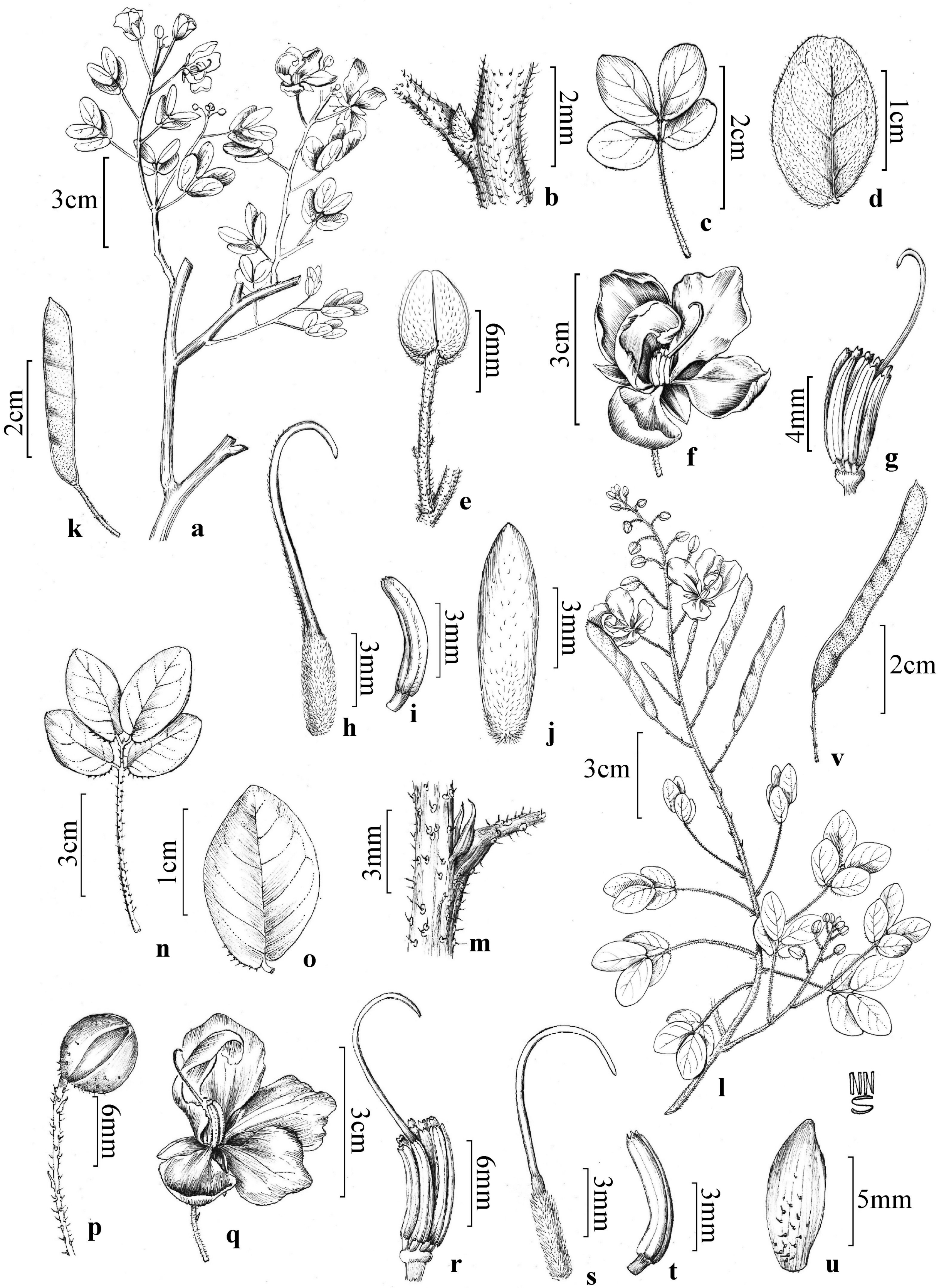

Figure 4. a-k) *Chamaecrista belemii* var. *belemii*: a) flowering branch; b) stipule; c) leaf; d) leaflet; e) bud and bracteoles; f) flower; g) androecium and gynoecium; h) pistil; i) stamen; j) sepal; k) fruit. l-v) *Chamaecrista brevicalyx* var. *brevicalyx*: l) flowering branch; m) stipule; n) leaf; o) leaflet; p) bud and bracteoles; q) flower; r) androecium and gynoecium; s) pistil; t) stamen; u) sepal; v) fruit. a-k from C.L.S.B. Correia 491; l-v from C.L.S.B. Correia 571.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Cânion Baixa da onça, 09°52'21" S and 38°37'88" W, 521 m, 24.III.2010, fl., C.L.S.B. Correia et al. 112 (HUNEB); Trilha do pau preto, sentido casa abandonada, 09°41'41" S and 38°34'56" W, 616 m, 18.V.2010, fr., A.A.S. Lopes et al. 933 (HUNEB); Cânion Baixa da onça, 09°52'21" S and 38°37'88" W, 521 m, 30.VI.2010, fl., C.L.S.B. Correia et al. 131 (HUNEB); 09.VI.2011, fl., C.L.S.B. Correia et al. 491 (HUNEB); 01.IX.2011, fl.; fr., C.L.S.B. Correia et al. 554 (HUNEB); 27.IX.2011, fr., R.R. Varjão et al. 155 (HUNEB); Trilha ao lado da casa sede, sentido curral, 09°39.84" S and 38°28'06" W, 592 m, 28.X.2010, fl., C.L.S.B. Correia et al. 323 (HUNEB); 23.XI.2010, fl., C.L.S.B. Correia et al. 267 (HUNEB); 23.XI.2010, fl., C.L.S.B. Correia et al. 293 (HUNEB); 26.VII.2011, fr., C.L.S.B. Correia et al. 511 (HUNEB); Trilha sentido Baixa da onça, 09°52'21" S and 38°37'88" W, 521 m, 30.XI.2010, fr., C.L.S.B. Correia et al. 311 (HUNEB); 30.XI.2010, fl., C.L.S.B. Correia et al. 313 (HUNEB); 09.VI.2011, fl., C.L.S.B. Correia et al. 318 (HUNEB); Trilha sentido Pedra da janela, 09°39'55" S and 38°28'02" W, 570 m, 30.XII.2010, fl., C.L.S.B. Correia et al. 357 (HUNEB).

The taxon is endemic to the Caatinga and has been recorded for the states of Bahia, Sergipe and Pernambuco on sandy soils at altitudes of 200 to 600 m (Queiroz 2009). Two varieties are recognized, both of which occur in the Caatinga, but only the *belemii* variety occurs in the study area. The taxon is very common in the ESRC, occurring in areas of shrubby to sub-shrubby Caatinga on sandy to rocky soil at ca. 616 m. It flowers from March to July and September to December, and fruits from May to September and in December.

Chamaecrista belemii var. *belemii* can be recognized in the area as a profusely branched shrub with the branches being generally tortuous and woody, epidermis pale and exfoliate on the older branches and leaves with two pairs of ovate to obovate leaflets. In the study area the taxon can be confused with *C. acosmifolia* (see comments in *C. acosmifolia*).

4. ***Chamaecrista brevicalyx*** (Benth.) H.S.Irwin & Barneby var. ***brevicalyx***, Mem. New York Bot. Gard. 35(2): 660. 1982. Figures 2d; 4l-v

Shrubs to subshrubs erect, branched until 2.5 m tall; branches cylindrical,erect, epidermis pale to whitish on the young branches, exfoliating, brown on the old branches. Indumentum hispid-papillose, constituted of trichomes glandular and tector, thin and thick, colour less to whitish or brown to black, erect, sparse, c. 0.5 mm long, distributed on the branches, pulvinus, petiole, margin of leaflets, stipules, bracts, bracteoles, pedicel, buds and sepals. Stipules green vinaceous to brown, filiform, obsolete, 1–3 × 0.5-1 mm long, caducous. Leaves 3.2–9.7 cm long; pulvinus black, glabrous, 1–1.5 mm diam.; petiole 21–56 mm long, c. 7 times longer than the rachis; without extrafloral nectaries; rachis 3–8 mm long; leaflets discolorous, 2 pairs, chartaceous to membranous, glabrous, ovate to suborbicular, 9–35 × 4–21 mm, apex acute to emarginate, base cuneate to cordate, venation penninervous. Inflorescences racemose, terminal, long, 12–26 flowered; inflorescences axis 4–21 cm long; bracts vinaceous, ovate to deltoid, 1–4 × 1–1.5 mm; pedicel 4–24 mm long; bracteoles vinaceous, ovate to deltoid, 1–3 × 0.5–1 mm, located near the receptacle. Buds vinaceous to yellowish, rounded, 2–11 mm long. Flowers c. 3 cm diam; sepals yellow to yellowish-green, lanceolate, abaxial surface sparsely pilose, striated, 9–10 × 4–6 mm; petals yellow, two external and two internal, obovate, 12–16 × 5–8 mm, cuculus spatulate to suborbicular, bent around the stamens, 18–22 × 8–11 mm; stamens yellow, 5–9 mm long; ovary green glaucous to yellowish-green, tomentose, 4–5 mm long; style yellow, 12–16 mm long. Legumes oblong, linear, when young green to vinaceous, brown when mature, 35–63 × 5–7 mm, valves chartaceous, pubescent to setulose. Seeds ellipsoids young, rhomboid to trapezoids when mature, brown to black, glossy and pitted, 5–6 × 3–4 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha da Mata da Pororoca, 09°48'72" S and 38°29'51" W, 698 m, 25.X.2010, fl., C.L.S.B. Correia et al. 236 (HUNEB); 04.IV.2011, fl., C.L.S.B. Correia et al. 427 (HUNEB); Trilha sentido Mata da Pororoca, 09°48'32" S and 38°29'30" W, 584 m, 24.XI.2010, fl.; fr., C.L.S.B. Correia et al. 296 (HUNEB); Trilha principal depois da Mata da Pororoca sentido matinha, 09°39'58" S and 38°27'61" W, 635 m, 22.XI.2010, fl., C.L.S.B. Correia et al. 255 (HUNEB); Trilha sentido casa II do ICMbio, 09°49'15" S and 38°29'33" W, 667 m, 24.XI.2010, fr., C.L.S.B. Correia et al. 276 (HUNEB); Trilha sentido Mata da Pororoca, depois da 1° encruzilhada, vindo da casa I do ICMbio, 9°47'57" S and 38°29'30" W, 584 m, 29.XII.2010, fl., C.L.S.B. Correia et al. 364 (HUNEB); Trilha sentido Mata da Pororoca, 09°48'32" S and 38°29'30" W 584 m, 24.III.2010, fl., C.L.S.B. Correia et al. 98 (HUNEB); 24.III.2010, fl., C.L.S.B. Correia et al. 100 (HUNEB); 19.V.2010, fr., M.V.V. Romão, et al. 619 (HUNEB); 29.XII.2010, fl., C.L.S.B. Correia et al. 338 (HUNEB); Trilha sentido casa II do ICMbio, 09°49'15" S and 38°29'33" W, 667 m 24.XI.2010, fl., C.L.S.B. Correia et al. 282 (HUNEB); Trilha sentido Mata da Pororoca, vindo da casa I do ICMbio, 9°45'29" S and 38°29'29" W, 584 m, 04.II.2011, fl., C.L.S.B. Correia et al. 382 (HUNEB); 04.II.2011, fl., C.L.S.B. Correia et al. 385 (HUNEB); 04.II.2011, fl., C.L.S.B. Correia et al. 386 (HUNEB); 08.IX.2011, fl., C.L.S.B. Correia et al. 569 (HUNEB); 25.X.2011, fl.; fr., C.L.S.B. Correia et al. 598 (HUNEB); Trilha sentido casa II do ICMbio, vindo da Mata da Pororoca, 09°49'14" S and 38°29'31" W, 670 m,14.I.2011, fl., C.L.S.B. Correia et al. 374 (HUNEB); 25.III.2011, fl., C.L.S.B. Correia et al. 393 (HUNEB); 25.III.2011, fl., C.L.S.B. Correia et al. 398 (HUNEB); 04.V.2011, fl., C.L.S.B. Correia et al. 458 (HUNEB); 04.V.2011, fr., C.L.S.B. Correia et al. 450 (HUNEB); Trilha sentido sul da Estação ca. 10 km da Mata da Pororoca, 09°43'18" S and 38°29'30" W, 580 m, 08.IX.2011, fl., C.L.S.B. Correia et al. 558 (HUNEB); Trilha sentido sul da Estação ca. 8 km da Mata da Pororoca, 09°42'16" S and 38°29'31" W, 579 m, 08.IX.2011, fr., C.L.S.B. Correia et al. 571 (HUNEB).

Chamaecrista brevicalyx is endemic to Brazil, being distributed in Northern Minas Gerais and Northeastern Brazil in the states of Bahia, Pernambuco, Piauí and Paraíba. The species includes two varieties: *C. brevicalyx* (Benth.) H.S.Irwin & Barneby var. *brevicalyx* and *C. brevicalyx* (Benth.) H.S.Irwin & Barneby var. *eliptica* (H.S.Irwin & Barneby) H.S.Irwin & Barneby (Irwin & Barneby 1978). The distribution of the *brevicalyx* variety coincides with the species, while the *eliptica* variety occurs in Northern Bahia (Irwin & Barneby 1978, Queiroz 2009). In the study area the species is represented only by the *brevicalyx* variety, which is very common. It was found with flowers in May to September and fruits from May to November.

In the study area the taxon can be clearly differentiated from the others by viscous branches, epidermis pale to whitish and exfoliate on the older branches and terminal, racemose inflorescences with axis 4–21 cm long.

5. ***Chamaecrista carobinha*** (H.S.Irwin & Barneby) H.S.Irwin & Barneby, Mem. New York Bot. Gard. 35: 661. 1982. Figures 2e; 5a-j

Herbs procumbent, sarmentose, little branched; branches cylindrical,erect to tortuous, epidermis green on the young branches, green-vinaceous on the old branches. Indumentum glabrescent, constituted of trichomes glandular and tector, thin, colourless to whitish and vinaceous to brown, flexible, erect and wavy, tangles, 0.5–1 mm long, distributed on the branches, pulvinus, petioles, stipules, rachis, leaflets, pedicels, bracts, bracteoles and sepals. Stipules green to green-vinaceous, filiform, obsolete, 2–4 × 0.5-1 mm long, persistent. Leaves 1.7–8.7 cm long; pulvinus green to vinaceous, pilose, 0.5–2 mm diam.; petiole 7–30 mm long, c. 1.5 times longer than the rachis; without extrafloral nectaries; rachis 4–20 mm long; leaflets discolorous, 2 pairs, chartaceous, puberulous, ovate to elliptic, 7–32 × 5–24 mm, apex rounded to emarginate, base asymmetrical to

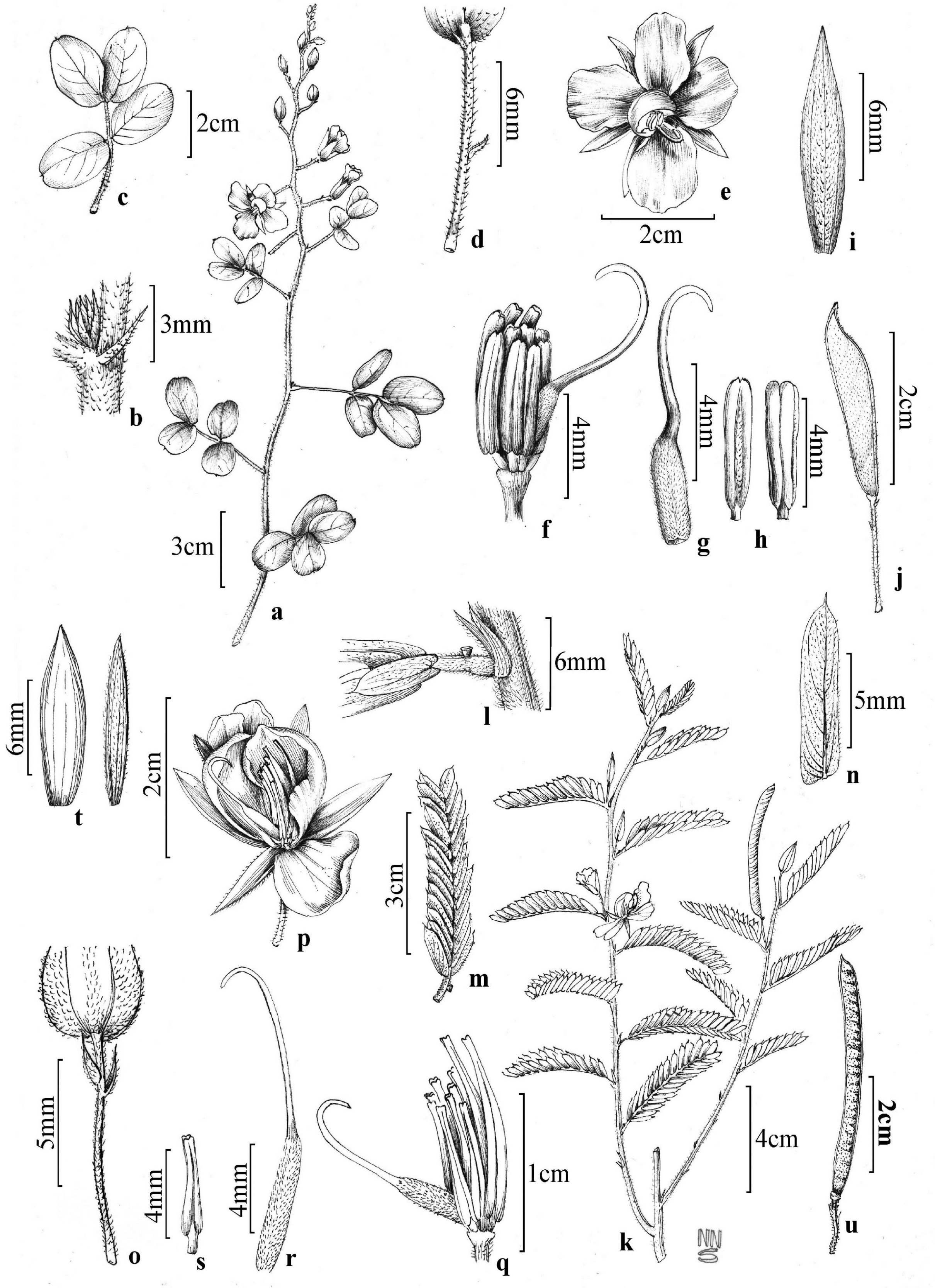

Figure 5. a-j) *Chamaecrista carobinha*: a) flowering branch; b) stipule; c) leaf; d) pedicel with bracteoles; e) flower; f) androecium and gynoecium; g) pistil; h. stamen; i) sepal; j) fruit. k-u) *Chamaecrista nictitans* var. *disadena*: k) flowering branch; l) detail of stipule and extrafloral nectary; m) leaf; n) leaflet; o) pedicel with bracteoles; p) flower; q) androecium and gynoecium; r) pistil; s) stamen; t) sepal; u) fruit. a-j from C.L.S.B. Correia 498; k-u from C.L.S.B. Correia 160.

rounded, venation penninervous. Inflorescences racemose, terminal, lax, 18–22 flowered; inflorescences axis 9–20 cm long; bracts green-vinaceous, filiform to lanceolate, 4–5 × 0.5 mm.; pedicel 4–16 mm long; bracteoles green, lanceolate to deltoid, 0.5–1 × 0.5 mm, located next to the receptacle. Buds green, acute, 2–11 mm long. Flowers c. 2.5 cm diam; sepals green to yellowish-green, lanceolate to ovate, abaxial surface pilose, striated, 5–9 × 2–3.5 mm; petals yellow, two external, oblong, 2.5–3 × 1–2.5 mm, two internal, orbicular, 2–2.5 × 1.5–2 mm, cuculus falcate, bent around the stamens, 2.5–3 × 1.5–2 mm; stamens yellow to glaucos green, 3–5 mm long; ovary glaucous green, canescent, 1.5–2 mm long; style green to yellowish, 1–1.5 mm long. Legumes oblong, slightly curved, when young green, mature not seen, 26–29 × 5–6 mm; valves chartaceous, puberulous, sparsely setulose. Seeds not seen.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido casa II vindo da Mata da Pororoca, 09°49'15" S and 38°29'33" W, 667 m, 08.VI.2011, fl., C.L.S.B. Correia et al. 484 (HUNEB); 01.VII. 2011, fl.; fr., C.L.S.B. Correia et al. 498 (HUNEB); 01.IX.2011, fl., C.L.S.B. Correia et al. 549 (HUNEB).

Chamaecrista carobinha is endemic to Caatinga and has been recorded from North Bahia and the South Piauí (Irwin & Barneby 1978, Queiroz 2009). The species is rare in the study area and was collected in well-preserved areas with sandy soil at ca. 661 m with flowers in June, July and September and fruits in September.

The species can be easily recognized in the area by its herbaceous habit procumbent to sarmentose with two pairs of ovate to elliptic leaflets (7–32 × 5–24 mm), and terminal, lax, racemose inflorescences, with axis 9–20 cm long.

6. *Chamaecrista nictitans* Moench subsp. ***disadena*** (Steud.) H.S.Irwin & Barneby var. ***disadena*** (Steud.) H.S.Irwin & Barneby, Mem. New York Bot. Gard. 35(2): 660. 1 (1982). Figures 2f; 5k-u

Herbs to subshrubs erect, little branched until 1 m tall; cylindrical branches,erect, epidermis green on the young branches, vinaceous on the old branches. Indumentum tomentose, constituted of trichomes tector, thin, blanks to colourless, flexible, erect and adpressed, slender, c. 0.5 mm long, distributed in the branches, petioles, stipules, rachis, leaflets, bracts, bracteoles, pedicels, buds and sepals. Stipules green to vinaceous, lanceolate, showy, 3–8 × 1–2 mm, persistent. Leaves 2.4–8.6 cm long; pulvinus brown, sparsely pilose, 1–2.5 mm diam.; petiole 6–8 mm long, equal or less than the rachis; 1 extrafloral nectary, vinaceous to brown, shortly stipitate, caliciform, 1–3 mm long, with head more dialated than the stipe, located next to the middle of the petiole; rachis 6–38 mm long; interfoliolar segments 1–4 mm long; leaflets discolorous, 18–21 pairs, chartaceous, pilose, oblong, 6–19 × 3–5 mm, apex acute, base cuneate, venation penninervous, midrib little excentric dividing the leaflet in a ratio of 1:1–1.5 at the base. Inflorescences fasciculate, supra-axillary, 2–5 flowered or reduced to a one flower; inflorescence axis 1.5–3.5 cm long; bracts green-vinaceous, lanceolate, 2–4 × 1–1.5 mm; pedicel 2–10.5 mm long; bracteoles green-vinaceous, lanceolate, 2–3 × c. 1 mm, located next to the middle of the pedicel. Buds green, acute, 5–15 mm long. Flowers c. 3 cm diam.; sepals green to yellowish-green, deltoid to lanceolate, the external pilose on the abaxial surface, internal glabrous, 13–16 × 2–5 mm; petals yellow to orange, two external, orbicular, 12–17 × 6–13 mm, two internal oblong, 11–12 × 3–4 mm, cuculus falcate, bent around the stamens, 12–14 × 8–9 mm; stamens yellow to yellowish-green, 6–15 mm long; ovary yellow, velutinous, 1–2 mm long; style light green to yellow, 13–15 mm long. Legumes oblong, linear, when young green to vinaceous, mature brown, 1.8–8.7 × 1–3 mm; valves chartaceous, glabrous to sparsely pilose. Seeds trapezoids, green-vinaceous, sparsely pitted, 1–1.5 × 0.3–0.5 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido Mata da Pororoca, 9°48'32" S and 38°29'30 W, 584 m, 24.III.2010, fl.; fr., C.L.S.B. Correia et al. 99 (HUNEB); Trilha sentido Pedra da janela, 09°39'55" S and 38°28'02" W, 570 m, 01.VII.2010, fl.; fr., C.L.S.B. Correia et al. 160 (HUNEB); Trilha por trás da casa sede do ICMbio, 09°39.84.2" S and 38°28'0.06" W, 592 m, 20.VIII.2010, fl.; fr., C.L.S.B. Correia et al. 218 (HUNEB); Trilha sentido Mata da Pororoca, vindo da casa I do ICMbio, 9°45'29" S and 38°29'29" W, 584 m, 29.XII.2010, fl., C.L.S.B. Correia et al. 343 (HUNEB); 29.XII.2010, fl., C.L.S.B. Correia et al. 367 (HUNEB); 14.I.2011, fl.; fr., C.L.S.B. Correia et al. 379 (HUNEB); 08.VI.2011, fl., C.L.S.B. Correia et al. 464 (HUNEB); Trilha sentido casa II, 09°49'15" S and 38°29'33" W, 667 m, 08.VI.2011, fl.; fr., C.L.S.B. Correia et al. 468 (HUNEB); 01.IX.2011, fl.; fr., C.L.S.B. Correia et al. 551 (HUNEB); Trilha principal sentido Mata da Pororoca, 9°45'29" S and 38°29'29" W, 584 m, 08.VI.2011, fl., C.L.S.B. Correia et al. 481 (HUNEB); Trilha sentido Sul da Estação, ca. 10 km da Pororoca, 09°43'18." S and 38°29'30" W, 580 m, 08.IX.2011, fl.; fr., C.L.S.B. Correia et al. 556 (HUNEB); 08.IX.2011, fl., C.L.S.B. Correia et al. 560 (HUNEB); 08.IX.2011, fl., C.L.S.B. Correia et al. 563 (HUNEB).

Chamaecrista nictitans is widely distributed in the Americas, occurring from the United States to Argentina, and includes four subspecies and 11 varieties, of which only *disadena* and *pilosa* occur in the Caatinga. In the study area, the species is represented by *Chamaecrista nictitans* var. *disadena* (Irwin & Barneby 1982, Queiroz 2009). This taxon is quite common in the ESRC, and is found in more degraded areas on sandy-clayey soil at altitudes of 570 to 696 m. It was collected with flowers and fruits from December to March and June to September.

In the study area, *Chamaecrista nictitans* subsp. *disadena* var. *disaden*a resembles *C. repens* (Vogel) H.S.Irwin & Barneby var. *multijuga* (Benth.) H.S.Irwin & Barneby, with both possessing leaves with more than 15 pairs of leaflets and supra-axillary inflorescences. Nevertheless, *Chamaecrista nictitans* subsp. *disadena* var. *disadena,* can be differentiated by stipitate extrafloral nectaries with more dialated head than the stipe and leaflets with midrib slightly excentric in a ratio of 1:1–1.5 at the base (*vs*. robust extrafloral nectaries with head less dialated than the stipe and leaflets with midrib strongly excentric in a ratio of 1:2–2.5 at the base, in *C. repens* var. *multijuga)*.

7. *Chamaecrista ramosa* (Vogel) H.S.Irwin & Barneby var. ***ramosa***, Mem. New York Bot. Gard. 35(2): 884. 1982. Figure 6a-k

Subshrubs erect to procumbent, branched until 1 m tall; branches cylindrical, erect, epidermis vinaceous to purple on the young branches, brown on the old branches. Indumentum villous, constituted to trichomes tector, thin, blanks to brown, flexible, adpressed, sparse, c. 0.5 mm long, distributed on the branches, stipules, petioles, sepals and legumes. Stipules green, deltoid to cordiform, showy, 3–6 × 2–4 mm, persistent. Leaves 7–15 cm long; pulvinus vinaceous, sparsely pilose, c. 8 mm diam.; petiole furrowed, 2–5 mm long; 1 extrafloral nectary, vinaceous to brown, stipitate, caliciform, c. 1 mm long, located on the petiole; rachis 0.5–1 mm long; leaflets discolorous, 2 pairs, chartaceous, pilose, obovate, 5–10 × 2–4 mm, apex rounded, base cuneate to truncated; venation paralelinervous. Inflorescences axillary, reduced to a single flower; bracts vinaceous, lanceolate to deltoid, 1.5–3 × 1–2.5 mm; pedicel 12–26 mm long; bracteoles brown, ovate to deltoid, 0.5–2 × 1–2 mm, located near the flower receptacle. Buds vinaceous, lanceolate, 9–14 mm long. Flowers c. 2 cm diam.; sepals green to green-vinaceous, lanceolate to elliptic or ovate to deltoid, abaxial surface pilose, multistriated, 5–11 × 2.5–5 mm; petals yellow, two external, spatulate to elliptic, 10–13.5 × 8–9 mm, two internal, obovate to orbicular, 11–15 × 7–9 mm, cuculus suborbicular to falcate, bent around the stamens, 13–14.5 × 20–23 mm; stamens yellow, 4–8.5 mm long; ovary green glaucous, panoso, 6 mm long; style green, 4–6 mm long. Legumes oblong, linear, little incurved, when young vinaceous and mature castaneous glaucous, c. 30 × c. 6 mm; valves chartaceous,

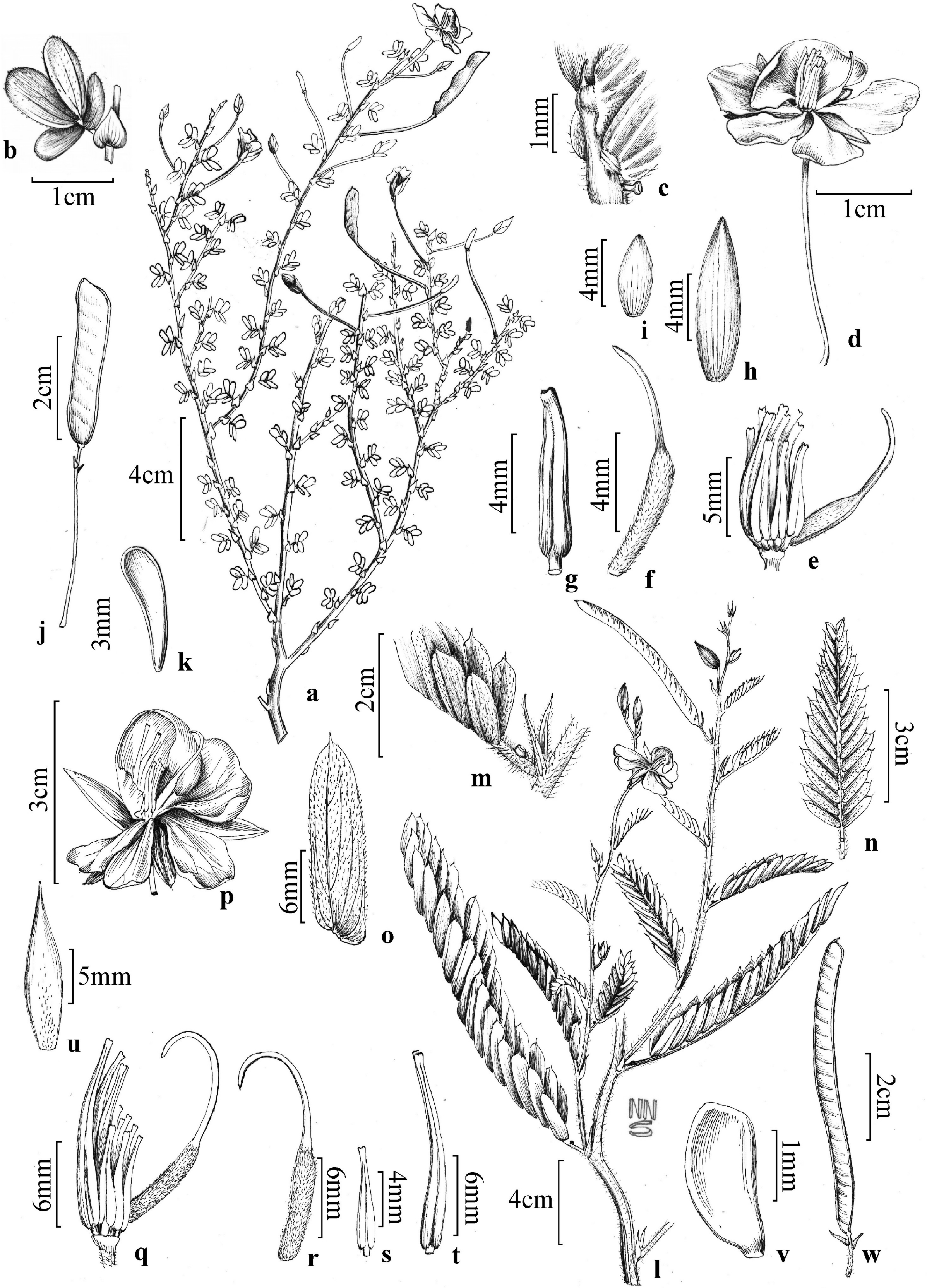

Figure 6. a-k) *Chamaecrista ramosa* var. *ramosa*: a) flowering branch; b) leaf; and stipule; c) detail of nectary; d) flower; e) androecium and gynoecium; f) pistil; g) stamen; h) external sepal; i) internal petal; j) fruit and pedicel with bracteoles; k) seed. l-w) *Chamaecrista repens* var. *multijuga*: l) flowering branch; m) detail of stipule and nectary; n) leaf; o) leaflet; p) flower; q) androecium and gynoecium; r) pistil; s) stamen; t) bigger stamen; u) sepal; v) seed; w) fruit and pedicel with bracteoles. a-k from C.L.S.B. Correia 570; l-w from C.L.S.B. Correia 494.

glabrous to puberulous. Seeds spatulated, brown, smooth and eventually pitted, 2–6 × 1.5–2 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido sul da Estação, 8 km após a Mata da Pororoca, 09°42'16" S and 38°29'31" W, 579 m, 08.IX.2011. fl.; fr., C.L.S.B. Correia et al. 561 (HUNEB); 08.IX.2011. fl.; fr., C.L.S.B. Correia et al. 570 (HUNEB).

The species occurs in South America and includes six varieties (Irwin & Barneby 1982). This species is inserted in C. sect. *Xerocalyx* that possesses taxa with limits that are considered artificial. In 2005 based on morphological data Fernandes & Nunes recognized only four varieties for the species. A biosystematic study of C. sect. *Xerocalyx* is in progress and should certainly help to more naturally delimit the taxa of this section. In the study area, the species is represented only by the *ramosa* variety, which is rare. This variety occurs from north South America to east Brazil (Irwin & Barneby 1982, Queiroz 2009).

In the study area, the taxon can be easily recognized by erect procumbent to subshrub habit with leaves having 2 pairs of leaflets with paralelinervous venation and by isolated axillary flowers.

8. *Chamaecrista repens* (Vogel) H.S.Irwin & Barneby var. *multijuga* (Benth.) H.S.Irwin & Barneby, Mem. New York Bot. Gard. 35(2): 660. 1982. Figures 2g; 6l-w

Subshrubs erect, branched until 1.5 m tall; branches cylindrical, erect, epidermis green on the young branches, vinaceous on the old branches. Indumentum vilosous, constituted to trichomes tector, thin, blanks to colourless, flexible, erect and adpressed, slender, c. 1 mm long, distributed on the branches, pulvinus, stipules, petioles, rachis, leaflets, bracts, bracteoles, buds, pedicels, sepals and young legumes. Stipules green to green-vinaceous, lanceolate, little showy, 6–8 × 0.5–1 mm, persistent. Leaves 1.6–12.1 cm long; pulvinus black, pilose, 1–2 mm diam.; petiole furrowed, 6–14 mm long; 1 extrafloral nectary, vinaceous to brown, sessile to shortly stipitate, discoid to caliciform, c. 1 mm long, head less dilated than stipe, located little above the middle of the petiole; rachis 8–113 mm long; interfoliolar segments 1–4 mm long; leaflets discolorous, 8–17 pairs, chartaceous, pilose, 6–21 × 1–7 mm, oblong, apex cuspidate, mucronate, base truncated, venation palminerveous, midrib little excentric dividing the leaflet in a ratio of 1:2–2.5 at the base. Inflorescences fasciculate, supra-axillary, 5 flowered or reduced to a single flower; pedicel 1.5–3.5 cm long; bracts green-vinaceous, lanceolate, 2–4 × 1–1.5 mm; bracteoles green-vinaceous, lanceolate, 2–3 × c. 1 mm, located next to the receptacle. Buds green, lanceolate, 5–15 mm long. Flowers c. 3 cm diam.; sepals green to yellowish-green, deltoids to elliptical, abaxial surface pilose, striated, 15–18 × 3–6 mm; petals yellow to orange, one external, orbicular, c. 22 × 21 mm, three internal, oblong, 11–17 × 6–7 mm, cuculus falcate, assimetric, bent around the stamens, 18–20 × 19–22 mm; stamens yellow to yellowish-green, 5–16 mm long; ovary yellow, tomentose, 8–9 mm long; style glaucous green to yellow, 6–8 mm long. Legumes oblong, linear, pilose, when young vinaceous, mature brown, 38–76 × 2–4 mm; valves chartaceous, sparsely puberulous. Seeds trapezoids, brown, pitted, 2–3.5 × 1–4 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido Baixa da onça, 09°52'21" S and 38º37'88" W, 521 m, 29.VI.2010. fl.; fr., C.L.S.B. Correia et al. 129 (HUNEB); Casa sede do ICMbio, 09°39.84" S and 38°28'06" W, 592 m, 06.V.2011, fl.; fr., R.R. Varjão et al. 99 (HUNEB); 01.VII.2011, fl.; fr., C.L.S.B. Correia et al. 492 (HUNEB); 01.VII.2011, fl.;fr., C.L.S.B. Correia et al. 494 (HUNEB); 01.VII.2011, fl.;fr., C.L.S.B. Correia et al. 496 (HUNEB); 27.VII.2011, fl.; fr., C.L.S.B. Correia et al. 519 (HUNEB); Trilha principal próximo a casa do Sr. Divá, 09°48'33" S and 38°29'31" W, 614 m, 27.VII.2011. fl., C.L.S.B. Correia et al. 530 (HUNEB); Trilha para cara feia próximo ao curral do dentinho, 09°47'29" S and 38°30'31" W, 588 m, 31.VIII.2011. fl., C.L.S.B. Correia et al. 537 (HUNEB).

Chamaecrista repens was reported by Irwin & Barneby (1982), as endemic to South American with two varieties: *C. repens* var. *multijuga* and *C. repens* var. *repens*. Of these two varieties, only *multijuga* occurs in the Caatinga, and is distributed in the Northeastern Region of Brazil to Minas Gerais (Irwin & Barneby 1982, Queiroz 2009). In the study area, *C. repens* var. *multijuga* is very common, occurring in anthropized areas on sandy-clayey soil at altitudes of 406 to 592 m. Flowers and fruits were recorded from May to August.

The taxon can be recognized in the study area by leaves with 8–17 pairs of leaflets, with the midrib extremely excentric in ratio of ca. 1:2–2.5 at the base and by inflorescence fasiculate, supra-axillary, with up to five flowers or reduced to a single flower. *Chamaecrista repens* var. *multijuga* can be confused with *C. nictitans* var. *disadena,* because both possess leaves with more than 8 pairs of leaflets and supra-axillary inflorescences. However, the two taxa can be differentiated by morphology of the extrafloral nectary and the position of the midrib of the leaflets, as mentioned in *C. nictitans* var. *disadena.*

9. *Chamaecrista swainsonii* (Benth.) H.S.Irwin & Barneby, Mem. New York Bot. Gard. 35(2): 701.1982. Figures 2h; 7a-k

Subshrubs erect, little branched until 0.6 m tall; branches quadrangular, fractiflex, epidermis green on the young branches, vinaceous to brown on the old branches. Indumentum vilosous, constituted by trichomes tector, thin, whitish, flexible, erect, sparse, c. 0.5 mm long, distributed on the branches, stipules, petioles, rachis, leaflets, bracts, bracteoles, pedicels, sepals and legumes. Stipules green speckled green purple on the base, past brown, acuminate to aristate, venation prominent on face adaxial, extremely showy, 4–14 × 5–17 mm, persistent. Leaves 2.4–6.2 cm long; pulvinus brown, glabrous, c. 1 mm diam; petiole furrowed, 2–13 mm long, c. 7 times greater than the rachis; 2–9 extrafloral nectary, green glaucous, stipitate, caliciform, located on petiole and on the rachis, one each interfoliolar segments, 0,4–1,0 mm long; rachis grooved, carrying a nectary, 14–42 mm long; interfoliolar segments 2–6 mm long; leaflets discolorous, 4–8 pairs, coriaceous, pubescent, 8–24 × 2.1–8.3 mm, oblong, apex cuspidate to spinescent, base rounded, venation palminerveous, prominent. Inflorescences axillary, reduced to a single flower; bracts green, purple speckled on the base, past brown, lanceolate to elliptical, 4–5 × c. 0.5 mm; pedicel 24–34 mm long; bracteoles green to green-vinaceous, elliptical to lanceolate, 1–3.5 × c. 0.5 mm, located above the middle of the pedicel. Buds green to yellowish-green, ovate to lanceolate, 9–14 mm long. Flowers c. 2 cm diam.; sepals green to yellowish-green, sparsely pilose, striated, lanceolate to deltoid, 1–14 × 3–8 mm; petals yellow glaucous to orange, two external, oblong to orbicular, 8–13.5 × 6–9.5 mm, two internal, spatulate to ovate, 10–11.5 × c. 5.5 mm, cuculus oblong to falcate, bent around the stamens, 11–13.5 × 10–11.5 mm; stamens yellow, 4–8.5 mm long; ovary green, tomentose, 4 mm long; style green, 4–5.5 mm long. Legumes oblong-linear, linear, pilose, green to green-vinaceous when young, mature brown, 12–47 × 2–6 mm; valves chartaceous, harsh. Seeds quadrangular to trapezoid, castaneous, pitted, 3–4 × 2.5–3 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina Trilha sentido Pororoca, 9°45'29" S and 38°29'29" W, 584 m, 24.III.2010, fr., C.L.S.B. et al. 102 (HUNEB); 19.V.2010, fr., M.V.V. Romão et al. 608 (HUNEB); 19.V.2010, fr., M.V.V. Romão et al. 616 (HUNEB); Trilha sentido Pedra da janela, 09°39'55" S and 38°28'02" W, 570 m 30.VI.2010, fl.; fr., R.R.O. Lima et al. 06 (HUNEB); 30.XII.2010, fl., C.L.S.B. Correia et al. 360 (HUNEB); 25.VII.2011, fl., C.L.S.B. Correia et al. 503 (HUNEB); Trilha sentido casa II, vindo da Pororoca, 09°49'15" S and 38°29'33" W, 667 m, 14.I.2011, fl., C.L.S.B. Correia et al. 370 (HUNEB); 04.II.2011, fr., C.L.S.B. Correia et al. 390

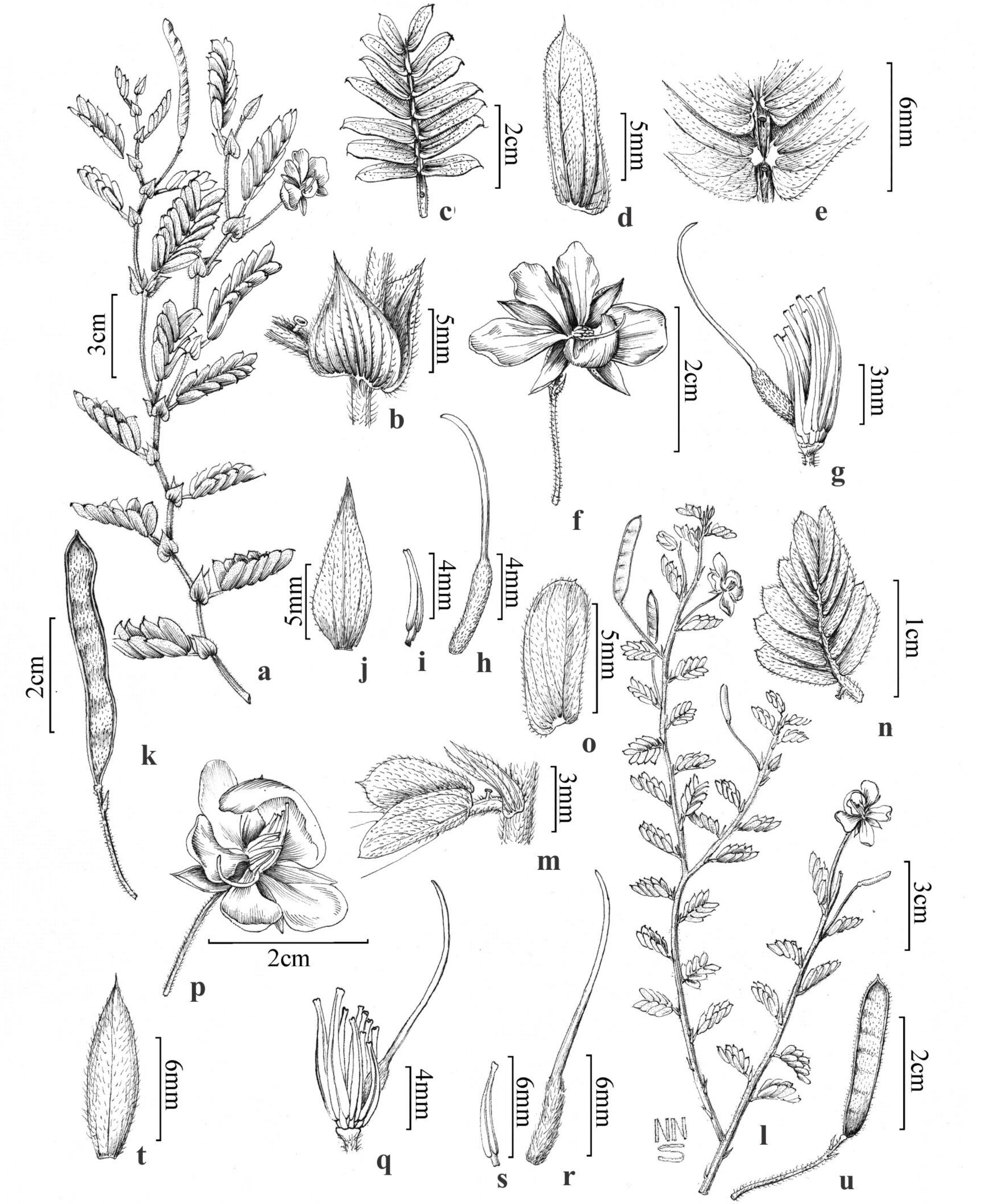

Figure 7. a-k) *Chamaecrista swainsonii*: a) flowering branch; b) stipule; c) leaf; d) leaflet; e) location of extrafloral nectary; f) flower; g) androecium and gynoecium; h) pistil; i) stamen; j) sepal; k) fruit and pedicel with bracteoles. l-u) *Chamaecrista tenuisepala:* l) flowering branch; m) detail of stipule and nectary; n) leaf; o) leaflet; p) flower; q) androecium and gynoecium; r) pistil; s) stamen; t) sepal; u) fruit and pedicel with bracteoles. a-k from C.L.S.B. Correia 390; l-u from C.L.S.B. Correia 548.

(HUNEB); 04.V.2011, fl., C.L.S.B. Correia et al. 448 (HUNEB); 08.VI.2011, fr., C.L.S.B. Correia et al. 483 (HUNEB); 01.IX.2011, fl.; fr., C.L.S.B. Correia et al. 550 (HUNEB); Trilha principal antes da Pororoca vindo da casa base, 09°39’54.8” S and 38°27’59.8” W, 618 m, 27.IX.2011, fl.; fr., C.L.S.B. Correia et al. 586 (HUNEB).

Chamaecrista swainsonii is endemic to Bahia and occurs in Caatinga, Cerrado and Restinga (Irwin & Barneby 1982, Queiroz 2009). According to Queiroz (2009), in the Caatinga the species occurs at altitudes of 680 to 1000 m. In the ESRC the species is common and was collected on sandy, sandy-clayey and rocky soils with flowers in December to June and fruiting from January to June.

It is characterized in the study area by the presence of quadrangular, fractiflex branches; acuminate to aristate stipules with prominent nervure, and leaves with 4–8 pairs of coriaceous, oblong leaflets with cuspidate to spinescent apex and palminerveous prominent venation.

10. ***Chamaecrista tenuisepala*** (Benth.) H.S.Irwin & Barneby, Mem. New York Bot. Gard. 35: 707. 1982. Figures 2i; 7l-u

Subshrub erect, little branched until 1 m tall; cylindrical branches, erect, little striated, not exfoliating, epidermis vinaceous to brown on the braches young and old. Indumentum tomentose, constituted by trichomes tector, thin, whitish, flexible, erect and wavy, tangles, c. 0.5 mm long, distributed on the branches, pulvinus, stipules, petioles, rachis, leaflets, pedicels, bracts, bracteoles, sepals, ovary and legumes. Stipules green to green-vinaceous, past brown, lanceolate to deltoid, obsolet, 1–7 × 1–2 mm, persistent. Leaves 1–3 cm long; pulvinus green, pilose, 0.5–1 mm diam.; petiole 2–4 mm long; 1 extrafloral nectary, brown to green-vinaceous, stipitate, caliciform, located on petiole, below pair of proximal leaflets, 1 –1.5 mm long; rachis 5–20 mm long; interfoliolar segments 1–3 mm long; leaflets discolorous, 5–6 pairs, chartaceous, pubescent with greater intensity on the face adaxial, 3–13 × 2–4 mm, oblong, apex acuminate to cuspidate, base asymmetrical, venation palminerveous. Inflorescences axillary, reduced to a single flower; bracts green-vinaceous, lanceolate to deltoids, 4–5 × c. 0.5 mm; pedicel 12–36 mm long; bracteoles green, lanceolate, 3–4 × c. 0.5 mm, located along the pedicel. Buds green to yellowish-green, lanceolate to ovate, 7–15 mm long. Flowers c. 1.5 cm diam.; sepals green to yellowish-green, lanceolate to deltoid, abaxial surface pilose, striated, 6–12 × 2–4 mm; petals yellow glaucous, two external, obovate, 13–18 × 8–11 mm, two internal, spatulate, 11–14 × 7–10 mm, cuculus falcate, bent around the stamens, 12–16 × 13–17 mm; stamens yellow, 3.5–11 mm long; ovary green glaucous to whitish, tomentose, 4–7 mm long; style green glaucous, 5–11 mm long. Legumes oblong, linear, green-vinaceous when young, mature brown, 9–42 × 2–6.5 mm; valves chartaceous, pilose, setulose. Seeds trapezoid, brown, pitted at base, 2–2.5 × c. 1 mm.

Material examined: BRAZIL, BAHIA: Paulo Afonso, Estação Ecológica Raso da Catarina, Trilha sentido Pedra da coruja, 09°39’55.9” S and 38°28’02.5” W 01.VII.2010. fl.; fr., C.L.S.B. Correia et al. 157 (HUNEB); Baixa da onça, 09°52’21” S and 38°37’88” W, 521 m, 24.III.2010. fl.; fr., C.L.S.B. Correia et al. 104 (HUNEB); Trilha sentido Mata da Pororoca. 9°48’32” S and 38°29’30 W, 584 m, 24.III.2010. fl.; fr., C.L.S.B. Correia et al. 101 (HUNEB); 29.XII.2010. fl.; fr., C.L.S.B. Correia et al. 340 (HUNEB); 29.XII.2010. fl.;fr., C.L.S.B. Correia et al. 342 (HUNEB); Trilha sentido Mata da Pororoca vindo da casa I, depois da 1° encruzilhada 9°47’57” S and 38°29’30” W, 584 m, 04.II.11. fl., C.L.S.B. Correia et al. 384 (HUNEB); Trilha do Transecto UFRVB, próximo a casa base do ICMbio, 9°48’50” S and 38°28’01” W, 560 m, 17.VIII.2010. fl.; fr., C.L.S.B. Correia et al. 187 (HUNEB); 29.XII.2010. fl.; fr., C.L.S.B. Correia et al. 366 (HUNEB); Trilha sentido casa II vindo da Mata da Pororoca, 09°49’15” S and 38°29’33” W, 667 m, 04.II.11. fl.; fr., C.L.S.B. Correia et al. 388 (HUNEB); 04.IV.11. fl., C.L.S.B. Correia et al. 429 (HUNEB); 08.VI.11. fl., C.L.S.B. Correia et al. 463 (HUNEB); 24.XI.11. fl.; fr., C.L.S.B. Correia et al. 280 (HUNEB).

The species is endemic to northeastern Brazil, having been recorded to southern Maranhão and Piauí to western Pernambuco and Paraíba (Irwin & Barneby 1982, Queiroz 2009). In the study area, *Chamaecrista tenuisepala* is very common and occurs on sandy, sandy-clayey and rocky soils at altitudes of 520 to 667 m. It was collected with flowers and fruits nearly every month of the year.

Chamaecrista tenuisepala can be recognized in the area by subshrub habit, lanceolated to deltate stipules, stipitate and caliciform extrafloral nectary located on the petiole below the pair of proximal leaflets, leaves with 5–6 pairs of leaflets and isolated axillary flowers of ca. 1.5 cm in diam.

Supplementary material

The following online material is available for this article:

Appendix: List of additional material examined

Acknowledgements

Thanks to the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB, PET #0023 /2007) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Proc. #552589/2011-0) for financial support. To Instituto Chico Mendes (ICMBio) for their support during field work. To anonymous reviewer by improvements. The first author thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by scholarship, the curators and technicians of the herbaria that were visited for their readiness during the consultation of the collections and Natanael Santos for the botanical illustrations.

Author Contributions

Cláudia Letícia de Souza Barros Correia: Contribution to the acquisition of data; analysis and interpretation of data; work of writing and conception and design work.

Adilva de Souza Conceição: Contribution to the acquisition of data; analysis and interpretation of data; work of writing; critical review adding intellectual content and conception and design work.

Conflicts of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Received: 27/06/2016
Revised: 07/03/2017
Accepted: 19/03/2017
Published online: 27/04/2017

Biota Neotropica 17(2): e20160282, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
inventory

Expanding the knowledge about the occurrence of anurans in the highest amphibian diversity area of Atlantic Forest: Parque Estadual da Serra do Mar, São Paulo, Brazil

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DA SILVA, F.R., LYRA, M.L., HADDAD, C.F.B., ROSSA-FERES, D.C. **Expanding the knowledge about the occurrence of anurans in the highest amphibian diversity area of Atlantic Forest: Parque Estadual da Serra do Mar, São Paulo, Brazil**. Biota Neotropica. 17(2): e20160282. http://dx.doi.org/10.1590/1676-0611-BN-2016-0282

Abstract**:** The vegetation cover in the Atlantic Forest has been converted to human use or degraded by human activities, which declined the vegetation to 16% of its original extent. Although several protected areas have been created in this Neotropical biome over the past decades, our knowledge of the amphibian species within these areas is still far from adequate. Here, we present lists of anuran species from three different areas of Parque Estadual da Serra do Mar: núcleos Curucutu, Santa Virgínia and São Sebastião. To survey anuran species, we visited six sampling units (two ponds, two streams and two trails inside forest fragments) in each locality. Samplings were accomplished from December 2014 to February 2015, December 2015 to February 2016 and December 2016 to February 2017, totalizing 27 days of field samplings in each sampling unit. We recorded 34 anuran species in the Núcleo Curucutu, 44 species in the Núcleo São Sebastião and 42 species in the Núcleo Santa Virgínia, totalizing 65 species belonging to 12 families (number of species in parentheses): Bufonidae (5), Brachycephalidae (6), Centrolenidae (1), Craugastoridae (1), Cycloramphidae (2), Hemiphractidae (1), Hylidae (29), Hylodidae (3), Leptodactylidae (12), Microhylidae (2), Odontophrynidae (2), and Phyllomedusidae (1). We hope that these lists can be useful for future studies as well as helping in the management and conservation planning of these protected areas.

Keywords: *Amphibians, Biodiversity, Conservation, Hotspots, Species list.*

Expandindo o conhecimento sobre a ocorrência de anuros na área com maior diversidade de anfíbios da Floresta Atlântica: Parque Estadual da Serra do Mar, São Paulo, Brasil

Resumo**:** A cobertura vegetal da Floresta Atlântica foi convertida para uso humano ou degradada pela atividade humana permanecendo atualmente 16% da sua extensão original. Embora muitas áreas protegidas tenham sido criadas neste bioma neotropical nas últimas décadas, nosso conhecimento sobre as espécies de anfíbios nestas áreas ainda está longe do adequado. Aqui, nós apresentamos listas das espécies de anuros para três diferentes áreas do Parque Estadual da Serra do Mar: núcleos Curucutu, Santa Virgínia e São Sebastião. Para amostrar as espécies de anuros, visitamos seis locais de amostragem (duas poças, dois riachos e duas trilhas) em cada localidade. As amostragens foram realizadas de dezembro 2014 a fevereiro de 2015, dezembro de 2015 a fevereiro de 2016 e de dezembro de 2016 a fevereiro de 2017, totalizando 27 dias de amostragens de campo em cada ponto de amostragem. Registramos 34 espécies de anuros no Núcleo Curucutu, 44 espécies no Núcleo São Sebastião, e 42 espécies no Núcleo Santa Virgínia, totalizando 65 espécies pertecentes a 12 famílias (número de espécies entre parênteses): Bufonidae (5), Brachycephalidae (6), Centrolenidae (1), Craugastoridae (1), Cycloramphidae (2), Hemiphractidae (1), Hylidae (29), Hylodidae (3), Leptodactylidae (12), Microhylidae (2), Odontophrynidae (2), e Phyllomedusidae (1). Esperamos que estas listas possam ser úteis para futuros estudos assim como auxiliar os planos de manejo e conservação destas áreas protegidas.

Palavras chaves: *Anfíbios, Biodiversidade, Conservação, Hotspots, Lista de espécies.*

Introduction

The Atlantic Forest, a global hotspot for biodiversity conservation (Mittermeier et al. 2004), originally covered 1.3 million km^2, of which between 84% and 89% have been converted to human use or degraded by human activities (Ribeiro et al. 2009). This biome harbors one of the highest diversity of amphibians in the world, hosting approximately 600 species of amphibians (Haddad et al. 2013). Although several protected areas have been created in this Neotropical biome over the past three decades, our knowledge of the amphibian species within these areas is still far from adequate. For example, the Parque Estadual da Serra do Mar (PESM) in the state of São Paulo, Brazil, is the largest protected area of the entire Atlantic Forest biome but some administrative areas such as Núcleo São Sebastião still do not have an amphibian survey published. This is worrisome because PESM harbors the highest amphibian diversity of the Atlantic Forest biome (Loyola et al. 2014). Here, we surveyed amphibian species of Núcleo Curucutu, Núcleo Santa Virgínia and Núcleo São Sebastião and present the first list of amphibian species for Núcleo São Sebastião. Natural history knowledge and species composition are the basic dataset for ecology, systematics, biogeography, and conservation biology (e.g., Collen et al. 2008, Da Silva et al. 2012, 2014); therefore, we hope that our lists can be useful for future studies as well as helping in the management and conservation planning of these protected areas.

Material and Methods

1. Study area

The PESM (23°13’8.4”S and 24°5’42.0”S, 44°43’12.0”W and 46°33’50.4”W) is the largest protected area in the Atlantic Forest biome with 315,390 ha in the state of São Paulo, southeastern Brazil (Figure 1). Due to its large extension the PESM is managed by 11 administrative divisions named: Núcleo Bertioga, Núcleo Caminhos do Mar, Núcleo Caraguatatuba, Núcleo Cunha, Núcleo Curucutu, Núcleo Itariru, Núcleo Itutinga Pilões, Núcleo Padre Dória, Núcleo Picinguaba, Núcleo Santa Virgínia and Núcleo São Sebastião. In this study, we carried out species inventories in three of these localities (Figure 1): (i) Núcleo Curucutu (23°59’6.0”S and 46°44’34.8”W), with 12,029 ha is encompassed by four municipalities (Itanhaém, Juquitiba, Mongaguá and São Paulo) and altitudes varying from 15 to 870 m asl; (ii) Núcleo Santa Virgínia (23°21’3.6”S and 45°8’6.0”W), with 17,500 ha is encompassed by five municipalities (São Luiz do Paraitinga, Cunha, Caraguatatuba, Ubatuba and Natividade da Serra), and altitudes varying from 740 to 1620 m asl; and (iii) Núcleo São Sebastião (23°43’1.2”S and 45°42’10.8”W), with 26,268 ha is encompassed by the municipality of São Sebastião and altitudes varying from sea level to 1200 m asl.

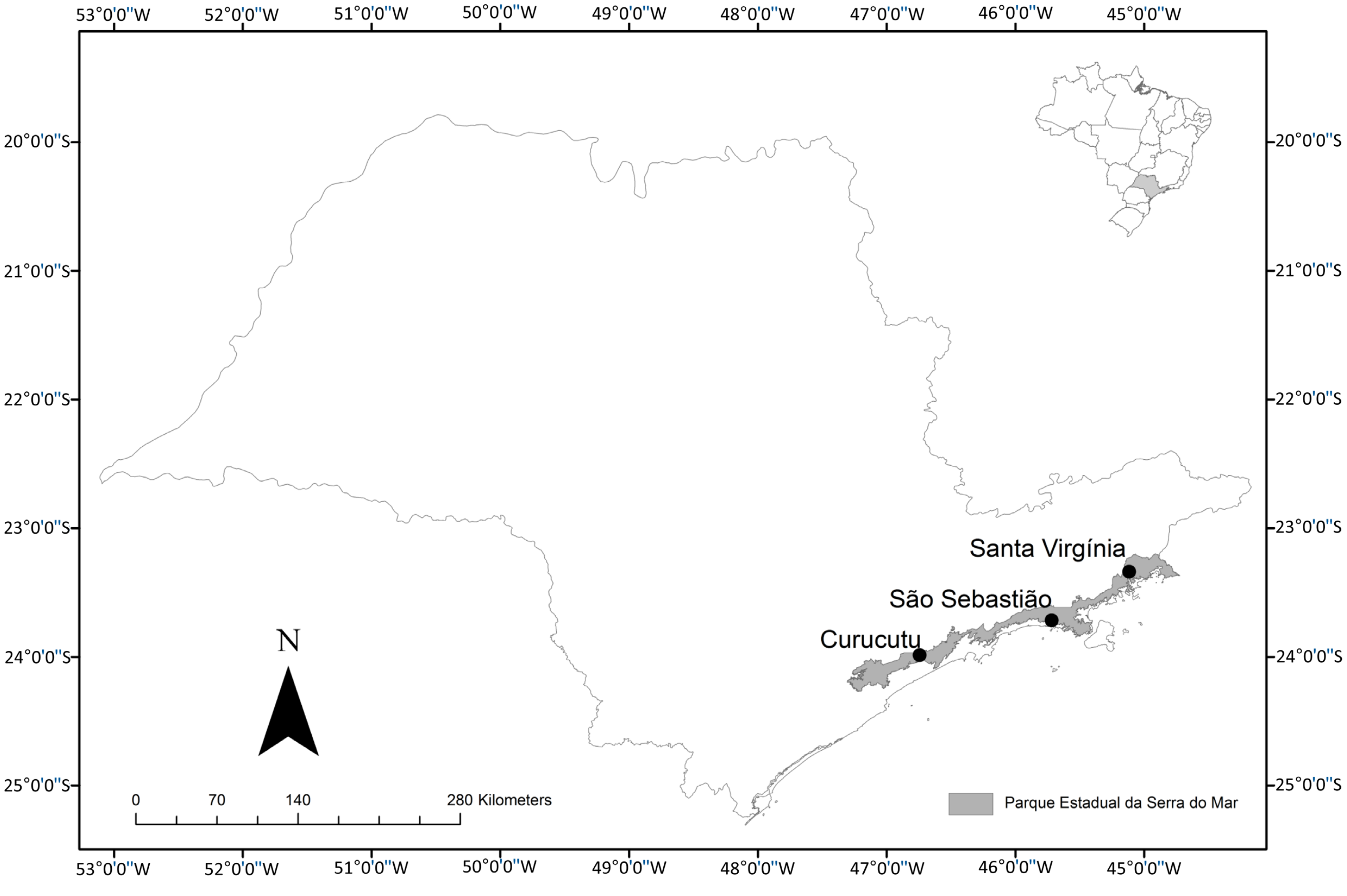

Figure 1. Map of the state of São Paulo, Brazil, highlighting the area of the Parque Estadual da Serra do Mar and the location of Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virginia.

2. Survey techniques

In each of the three localities we sampled amphibian species in two ponds, two streams and two trails inside forest fragments (Table 1, Figure 2) from December 2014 to February 2015, December 2015 to February 2016 and December 2016 to February 2017 totalizing 27 days of field samplings in each sampling unit. We selected this period because is the time of the year when most of annual rainfall occurs and most anuran species are active. We sought to choose sampling units with similar size area and physiognomic characteristics in the three núcleos (Figure 2). We carried out the surveys on streams and trails inside forest fragments considering always the same extension of 100 m in all núcleos. Furthermore, we selected trails in which there were no water bodies along the extension sampled. We used three methods to record the presence of anurans in each locality: (i) surveys at breeding sites (Scott & Woodward 1994) - we recorded calling males from 19.00 hours to 24.00 hours; (ii) survey of larvae with dipnetting - we used a long and wire hand net (3 mm^2 mesh size) along the margins of ponds and streams, sampling the available microhabitats from 12.00 hours to 18.00 hours; and (iii) visual encounter (Crump & Scott Jr. 1994) - we walked slowly for 30 minutes in trails inside the forest fragment, streams and around ponds looking at microhabitats for individuals hidden under trunks, bromeliads, stones, branches, and leaf litter. All collected specimens (Table 2) were anesthetized and killed, fixed in 10% formaldehyde and stored in 70% ethanol (adults) or 10% formaldehyde (tadpoles). We also collected tissue samples that were fixed in 100% ethanol and maintained in -20ºC in freezers (Table 2). All adult specimens and tissues samples are housed in the Coleção de Anfíbios Célio F. B. Haddad (CFBH), Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro and Coleção de Anfíbios do Departamento de Zoologia e Botânica (DZSJRP), Universidade Estadual Paulista, São José do Rio Preto (Appendix 1). All collected tadpoles are housed in the Laboratório de Ecologia Teórica: Integrando Biologia, Espaço e Tempo (LET.IT.BE), Departamento de Ciências Ambientais, Universidade Federal de São Carlos, campus Sorocaba (Appendix 1).

Table 1. Geographical coordinates of sample units used in the survey of anurans in Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virgínia of the Parque Estadual da Serra do Mar, state of São Paulo, southeastern Brazil. Geographical coordinates in degrees, minutes and seconds, based on the WGS84 datum.

SITES	LATITUDE (S)	LONGITUDE (W)	ALTITUDE (m)
NÚCLEO CURUCUTU			
POND 1	23°58’50.69”	46°44’08.32”	795
POND 2	23°59’13.90”	46°44’34.43”	794
STREAM 1	23°59’20.82”	46°44’44.09”	801
STREAM 2	23°59’17.76”	46°44’56.64”	793
TRAIL 1	23°59’06.78”	46°44’35.52”	788
TRAIL 2	23°59’17.04”	46°44’36.89”	796
NÚCLEO SÃO SEBASTIÃO			
POND 1	23°44’01.13”	45°45’29.31”	21
POND 2	23°43’08.15”	45°43’09.73”	72
STREAM 1	23°43’03.78”	45°43’05.81”	71
STREAM 2	23°43’01.70”	45°42’11.29”	335
TRAIL 1	23°42’48.37”	45°43’34.46”	205
TRAIL 2	23°43’01.27”	45°42’09.47”	335
NÚCLEO SANTA VIRGÍNIA			
POND 1	23°19’55.80”	45°5’49.49”	979
POND 2	23°21’03.93”	45°8’07.09”	964
STREAM 1	23°19’52.14”	45°5’51.00”	981
STREAM 2	23°20’36.96”	45°7’45.12”	938
TRAIL 1	23°20’14.39”	45°8’07.14”	1000
TRAIL 2	23°20’40.44”	45°7’58.44”	951

3. Data Analysis

To assess the sampling efficiency of surveys separately for each locality, we used species accumulation curve (Gotelli & Colwell 2001) generated from the data of incidence of anurans during the inventory period. The total number of species recorded each day was considered as a sample, totaling 27 samples. Considering the diverse richness estimators available, we chose to use the Jackknife algorithm first order based on its performance when compared to other estimators (Magurran 2004, Walther & Moore 2005, Hortal et al. 2006). All analyzes were performed in R v 3.2.2 (R Core Team 2015) with 1,000 randomizations using *vegan* (Oksanen et al. 2016) and *BiodiversityR* (Kindt & Coe 2005) packages. Taxonomic nomenclature follows Frost (2017). The species conservation status was obtained from The International Union for Conservation of Nature Red List of Threatened Species (IUCN 2016), Brazil Red Book of Threatened Species of Fauna (ICMBio 2015) and Faunal Species of São Paulo state Threatened with Extinction (Garcia et al. 2009).

Figure 2. Photographies of ponds (a-b), streams (c-d) and trails inside forest fragments (e-f) used as sample units in the survey of anurans in the núcleos Curucutu, São Sebastião and Santa Virgínia of the Parque Estadual da Serra do Mar, state of São Paulo, southeastern Brazil.

Table 2. Anuran species composition and abundance from Núcleo Curucutu (PECU), Núcleo São Sebastião (PESB) and Núcleo Santa Virgínia (PESV) of the Parque Estadual da Serra do Mar, state of São Paulo, southeastern Brazil. STATUS IUCN = threatened species categories used in the IUCN Red List Data (IUCN 2016); STATUS BRAZIL = categories of threatened species used in the Brazil Red Book of Threatened Species of Fauna (ICMBio 2015); STATUS SÃO PAULO = categories of threatened species used in the São Paulo Red Book of Threatened Species of Fauna (Garcia et al. 2009): DD (Data Deficient), LC (Least Concern) and CE (Critically Endangered). POP. TREND = current population trends used in IUCN Red List Data: increasing, decreasing, stable or unknown. * = species not evaluated or not cited. # Cryptic species: in this case, abundance was defined as only one species in the fieldwork.

Family/Species	PECU	PESB	PESV	STATUS IUCN	STATUS BRAZIL	STATUS SÃO PAULO	POP. TREND
BUFONIDADE							
Dendrophryniscus brevipollicatus Jiménez de la Espada, 1870	8	1	1	LC	LC	LC	Stable
Dendrophryniscus sp.	1			*	*	*	*
Rhinella hoogmoedi Caramaschi & Pombal, 2006		1		LC	LC	LC	Unknown
Rhinella icterica (Spix, 1824)	16	2	27	LC	LC	LC	Stable
Rhinella ornata (Spix, 1824)	11	14	5	LC	LC	LC	Stable
BRACHYCEPHALIDAE							
Brachycephalus pitanga Alves, Reis & Haddad, 2009			18	*	*	*	*
Ischnocnema gr. *guentheri* (CS1 lineage, Gehara et al. 2013)			16[#]	*	*	*	*
Ischnocnema gr. *guentheri* (CS3 lineage, Gehara et al. 2013)		12		*	*	*	*
Ischnocnema gr. *guentheri* (CS4 lineage, Gehara et al. 2013)			16[#]	*	*	*	*
Ischnocnema henselii (Peters, 1870)	24			LC	LC	*	Unknown
Ischnocnema parva (Girard, 1853)	5	2	43	LC	LC	LC	Decreasing
CENTROLENIDAE							
Vitreorana uranoscopa (Müller, 1924)	20	12	24	LC	LC	LC	Decreasing
CRAUGASTORIDAE							
Haddadus binotatus (Spix, 1824)	7	4	3	LC	LC	LC	Stable
CYCLORAMPHIDAE							
Cycloramphus boraceiensis Heyer, 1983		25		LC	LC	DD	Decreasing
Thoropa taophora (Miranda-Ribeiro, 1923)		1		*	LC	LC	*
HEMIPHRACTIDAE							
Fritziana sp. (aff. *fissilis*)	22	10	9	*	*	*	*
HYLIDAE							
Aplastodiscus albofrenatus (Lutz, 1924)		1	4	LC	LC	*	Stable
Aplastodiscus leucopygius (Cruz & Peixoto, 1985)	1	2	10	LC	LC	LC	Stable
Bokermannohyla izecksohni (Jim & Caramaschi, 1979)	1			CE	DD	LC	Decreasing
Bokermannohyla circumdata (Cope, 1871)	4	1	6	LC	LC	LC	Decreasing
Bokermannohyla hylax (Heyer, 1985)	4	9	4	LC	LC	LC	Decreasing
Dendropsophus berthalutzae (Bokermann, 1962)		23	1	LC	LC	LC	Stable
Dendropsophus elegans (Wied-Neuwied, 1824)	2	3	1	LC	LC	LC	Stable
Dendropsophus microps (Peters, 1872)		2	37	LC	LC	LC	Stable
Dendropsophus minutus (Peters, 1872)	7	6	26	LC	LC	LC	Stable
Dendropsophus seniculus (Cope, 1868)		1	15	LC	LC	LC	Stable
Boana albomarginata (Spix, 1824)	8	15	5	LC	LC	LC	Stable
Boana albopunctata (Spix, 1824)	40		3	LC	LC	LC	Stable
Boana bandeirantes (Caramaschi & Cruz, 2013)	2		7	*	LC	*	*
Boana bischoffi (Boulenger, 1887)	11		2	LC	LC	LC	Stable
Boana faber (Wied-Neuwied, 1821)	40	5	26	LC	LC	LC	Stable
Boana pardalis (Spix, 1824)			5	LC	LC	LC	Stable
Boana semilineata (Spix, 1824)	4	12		LC	LC	LC	Stable
Ololygon argyreornata (Miranda-Ribeiro, 1926)		1		LC	LC	LC	Stable
Ololygon littoralis (Pombal & Gordo, 1991)		28		LC	LC	LC	Decreasing
Ololygon cf. *obtriangulata* (Lutz, 1973)			1	LC	LC	LC	Decreasing
Ololygon perpusilla (Lutz & Lutz, 1939)	52	5	20	LC	LC	LC	Stable
Ololygon rizibilis (Bokermann, 1964)	1	4	4	LC	LC	LC	Decreasing

http://dx.doi.org/10.1590/1676-0611-BN-2016-0282

Table 2. Continued...

Family/Species	PECU	PESB	PESV	STATUS IUCN	STATUS BRAZIL	STATUS SÃO PAULO	POP. TREND
Scinax crospedospilus (Lutz, 1925)	1	5	5	LC	LC	LC	Stable
Scinax fuscovarius (Lutz, 1925)	1		1	LC	LC	LC	Stable
Scinax hayii (Barbour, 1909)		10	26	LC	LC	LC	Stable
Scinax perereca Pombal, Haddad & Kasahara, 1995	8			LC	LC	LC	Unknown
Scinax tymbamirim Nunes, Kwet & Pombal, 2012	36	22	5	*	*	*	*
Trachycephalus mesophaeus (Hensel, 1867)		1		LC	LC	LC	Decreasing
Trachycephalus imitatrix (Miranda-Ribeiro, 1926)			2	LC	LC	DD	Decreasing
HYLODIDAE							
Hylodes asper (Müller, 1924)		15		LC	LC	LC	Stable
Hylodes phyllodes Heyer & Crocoft, 1986		15	10	LC	LC	LC	Decreasing
Megaelosia cf. *bocainensis* Giaretta, Bokermann & Haddad, 1993			1	DD	DD	DD	Unknown
LEPTODACTYLIDAE							
Adenomera ajurauna (Berneck, Costa & Garcia, 2008)	10			DD	*	LC	Stable
Adenomera marmorata Steindachner, 1867	20	70	32	LC	LC	LC	Stable
Adenomera sp.		15		*	*	*	*
Leptodactylus furnarius Sazima & Bokermann, 1978	24			LC	LC	LC	Stable
Leptodactylus fuscus (Schneider, 1799)			3	LC	LC	LC	Stable
Leptodactylus plaumanni Ahl, 1936	3			LC	LC	*	Stable
Leptodactylus latrans (Steffen, 1815)	7	1	1	LC	LC	LC	Stable
Paratelmatobius cardosoi Pombal & Haddad, 1999	13			DD	LC	LC	Unknown
Physalaemus atlanticus Haddad & Sazima, 2004		10		VU	LC	LC	Unknown
Physalaemus bokermanni Cardoso & Haddad, 1985		20		DD	LC	LC	Decreasing
Physalaemus cuvieri Fitzinger, 1826	25		7	LC	LC	LC	Stable
Physalaemus olfersii (Lichtenstein & Martens, 1856)		3	14	LC	LC	LC	Stable
MICROHYLIDAE							
Chiasmocleis leucosticta (Boulenger, 1888)		2		LC	LC	LC	Stable
Myersiella microps (Duméril & Bibron, 1841)		4		LC	LC	LC	Stable
ODONTOPHRYNIDAE							
Proceratophrys boiei (Wied-Neuwied, 1824)		2	2	LC	LC	LC	Stable
Proceratophrys melanopogon (Miranda-Ribeiro, 1926)		4		LC	LC	LC	Decreasing
PHYLLOMEDUSIDAE							
Phrynomedusa dryade Baêta, Giasson, Pombal & Haddad, 2016			10	*	*	*	*
Number of Species	34	44	42				

Results and Discussion

We recorded 34 species in Núcleo Curucutu, 44 species in Núcleo São Sebastião and 42 species in Núcleo Santa Virgínia (Tabela 2, Figures 3-6), totalizing 65 anuran species belonging to 12 families (number in parentheses): Bufonidae (5), Brachycephalidae (6), Centrolenidae (1), Craugastoridae (1), Cycloramphidae (2), Hemiphractidae (1), Hylidae (29), Hylodidae (3), Leptodactylidae (12), Microhylidae (2), Odontophrynidae (2) and Phyllomedusidae (1). From the 65 species recorded, 20 species (30.7%) occurred in the three núcleos, 14 species (21.5%) occurred in two núcleos, eight species (12.3%) occurred only at Núcleo Curucutu, nine species (13.8%) occurred only at Núcleo Santa Virgínia and 14 species (21.5%) occurred only at Núcleo São Sebastião (Table 2). The observed species richness was smaller than the estimated species richness (Figure 7), indicating that 4.8 more species could be recorded at núcleos Curucutu, 6.7 more species at Núcleo Santa Virgínia and 5.7 more species at the Núcleo São Sebastião if we increase the sampling effort or utilize other sampling methods (e.g., pitfall traps). For example, Malagoli (2008, 2013) using data gathered from fieldwork, scientific collection and grey literature recorded 66 anuran species to Núcleo Curucutu. Giasson (2008) carried out 25 excursions to Núcleo Santa Virgínia from January 2003 to December 2006 and recorded 50 anuran species. However, we are aware that these studies used different sampling efforts and their results are not comparable to species richness observed in our study.

According to the threatened species categories used in IUCN (2016), *Bokermannohyla izecksohni* is classified as critically endangered, *Physalaemus atlanticus* is classified as vulnerable, *Adenomera ajurauna*, *Megaelosia* cf. *bocainensis*, *Paratelmatobius cardosoi* and *P. bokermanni* are classified as data deficient and other anuran species are classified as least concern (Table 2).On the other hand, considering threatened species categories used in the Brazilian (ICMBio 2015) and state of São Paulo (Garcia et al. 2009) lists, *B. izecksohni*, *Cycloramphus boraceiensis*, *Trachycephalus imitatrix*

Figure 3. Anuran species recorded at the Parque Estadual da Serra do Mar, Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virgínia, state of São Paulo, southeastern Brazil. a = *Rhinella icterica*, b = *R. hoogmoedi*, c = *R. ornata*, d = *Dendrophryniscus brevipollicatus*, e = *Brachycephalus pitanga*, f = *Ischnocnema* gr. *guentheri* (CS1 lineage Gehara et al. 2013), g = *Ischnocnema* gr. *guentheri* (CS4 lineage Gehara et al. 2013), h = *I. henselii*, i = *I. parva*, j = *Haddadus binotatus*, k = *Proceratophrys melanopogon*, l = *Cycloramphus boraceiensis*, m = *Hylodes asper*, n = *H. phyllodes* and o = *Thoropa taophora*.

Figure 4. Anuran species recorded at the Parque Estadual da Serra do Mar, Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virgínia, state of São Paulo, southeastern Brazil. p = *Megaelosia* cf. *bocainensis*, q = *Adenomera marmorata*, r = *Adenomera* sp., s = *Leptodactylus furnarius*, t = *L. fuscus*, u = *L. latrans*, v = *Physalaemus atlanticus*, w = *P. bokermanni*, x = *P. cuvieri*, y = *P. olfersii*, z = *Paratelmatobius cardosoi*, a1 = *Chiasmocleis leucosticta*, b1 = *Myersiela microps*, c1 = *Fritziana* sp. (aff. *fissilis*) and d1 = *Phrynomedusa dryade*.

Figure 5. Anuran species recorded at the Parque Estadual da Serra do Mar, Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virgínia, state of São Paulo, southeastern Brazil. e1 = *Aplastodiscus leucopygius*, f1 = *Bokermannohyla circumdata*, g1 = *B. hylax*, h1 = *B. izecksohni*, i1 = *Dendropsophus berthalutzae*, j1 = *D. elegans*, k1 = *D. microps*, l1 = *D. minutus*, m1 = *D. seniculus*, n1 = *Boana albomarginata*, o1 = *B. albopunctata*, p1 = *B. bandeirantes*, q1 = *B. bischoffi*, r1 = *B. faber* and s1 = *B. pardalis*.

Figure 6. Anuran species recorded at the Parque Estadual da Serra do Mar, Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virgínia, state of São Paulo, southeastern Brazil. t1 = *B. semilineata*, u1 = *Ololygon argyreornata*, v1 = *O. littoralis*, w1 = *O. perpusilla*, x1 = *O. rizibilis*, y1 = *Scinax crospedospilus*, z1 = *S. fuscovarius*, a2 = *S. hayii*, b2 = *S. perereca*, c2 = *S. tymbamirim*, d2 = *Trachycephalus imitatrix*, e2 = *T. mesophaeus* and f2 = *Vitreorana uranoscopa*.

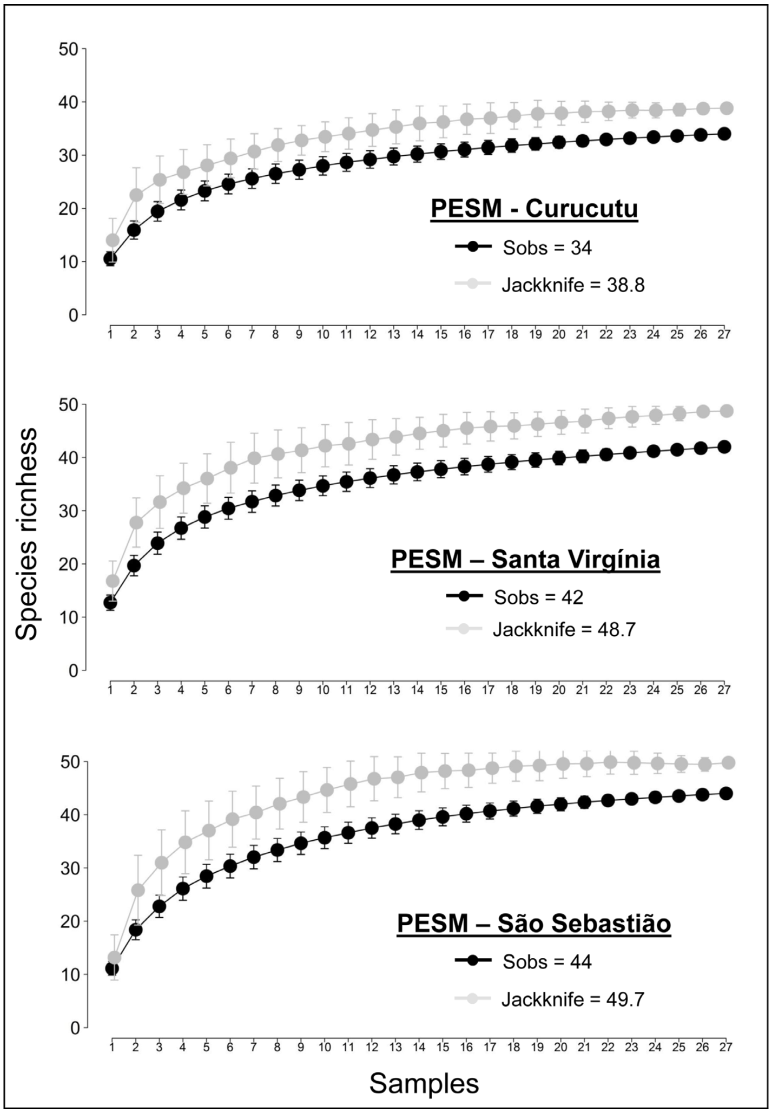

Figure 7. Species accumulation curve (black line) and Jackknife algorithm first order (gray line) representing the anuran species richness observed and estimated, respectively, based on 18 samples from December 2014 to February 2017 at the Parque Estadual da Serra do Mar, Núcleo Curucutu, Núcleo São Sebastião and Núcleo Santa Virgínia, state of São Paulo, southeastern Brazil. The dots show the mean generated by 1000 randomizations and the vertical bars indicate the standard deviation.

and *Megaelosia* cf. *bocainensis* are classified as data deficient and other anuran species are classified as least concern (Table 2). Considering the trend populations of IUCN (2016), 14 species have declining populations (*Ischnocnema parva*, *Vitreorana uranoscopa*, *C. boraceiensis*, *B. izecksohni*, *B. circumdata*, *B. hylax*, *Ololygon littoralis*, *Ololygon* cf. *obtriangulata*, *O. rizibilis*, *T. imitatrix*, *T. mesophaeus*, *Hylodes phyllodes*, *P. bokermanni* and *Proceratophrys melanopogon*), and other six species have unknown population trends (*I. henselli*, *Scinax perereca*, *Megaelosia* cf. *bocainensis*, *P. cardosoi*, *P. atlanticus* and *Rhinella hoogmoedi*) (Table 2). It is important to recognize that IUCN trend populations it is not consensual among researchers and it may contain imprecisions. For example, the populations of widespread species with recognized tolerance to anthropogenic habitats such as *T. mesophaeus* probably may not be declining (Carvalho-e-Silva & Garcia 2004).

We registered 1282 individuals being that *Ololygon perpusillus* and *Boana albopunctata* were the most abundant species at Núcleo Curucutu (Figure 8B), *Ischnocnema parva* and *Dendropsophus microps* were the most abundant species at Núcleo Santa Virgínia (Figure 8C) and *Adenomera marmorata* and *Ololygon littoralis* were the most abundant species at Núcleo São Sebastião (Figure 8D). Most anuran species were recorded more than once during the sampling period; however, *Thoropa taophora*, *B. izecksohni*, *O. argyreornata*, *Ololygon* cf. *obtriangulata*, *T. mesophaeus*, *Megaelosia* cf. *bocainensis* and *R. hoogmoedi* were recorded on only one occasion based on a single individual (Figure 8A). Except for *Megaelosia* cf. *bocainensis* whose natural history and population trend are unknown, *Thoropa taophora*, *B. izecksohni*, *O. argyreornata*, *Ololygon* cf. *obtriangulata*, *T. mesophaeus* and *R. hoogmoedi* are common species that reproduce during one or few days along the year (i.e. explosive breeding). Therefore, the low abundance recorded for these species probably is due to sampling effort. Information about species richness and composition are the basic dataset for ecology, systematics, biogeography and conservation biology (e.g., Collen et al. 2008, Da Silva et al. 2012, 2014) as well as they are relevant to subsidize management plans and conservation strategies. Therefore, we hope that these lists can be useful for future studies as well as helping in the management and conservation planning of these protected areas.

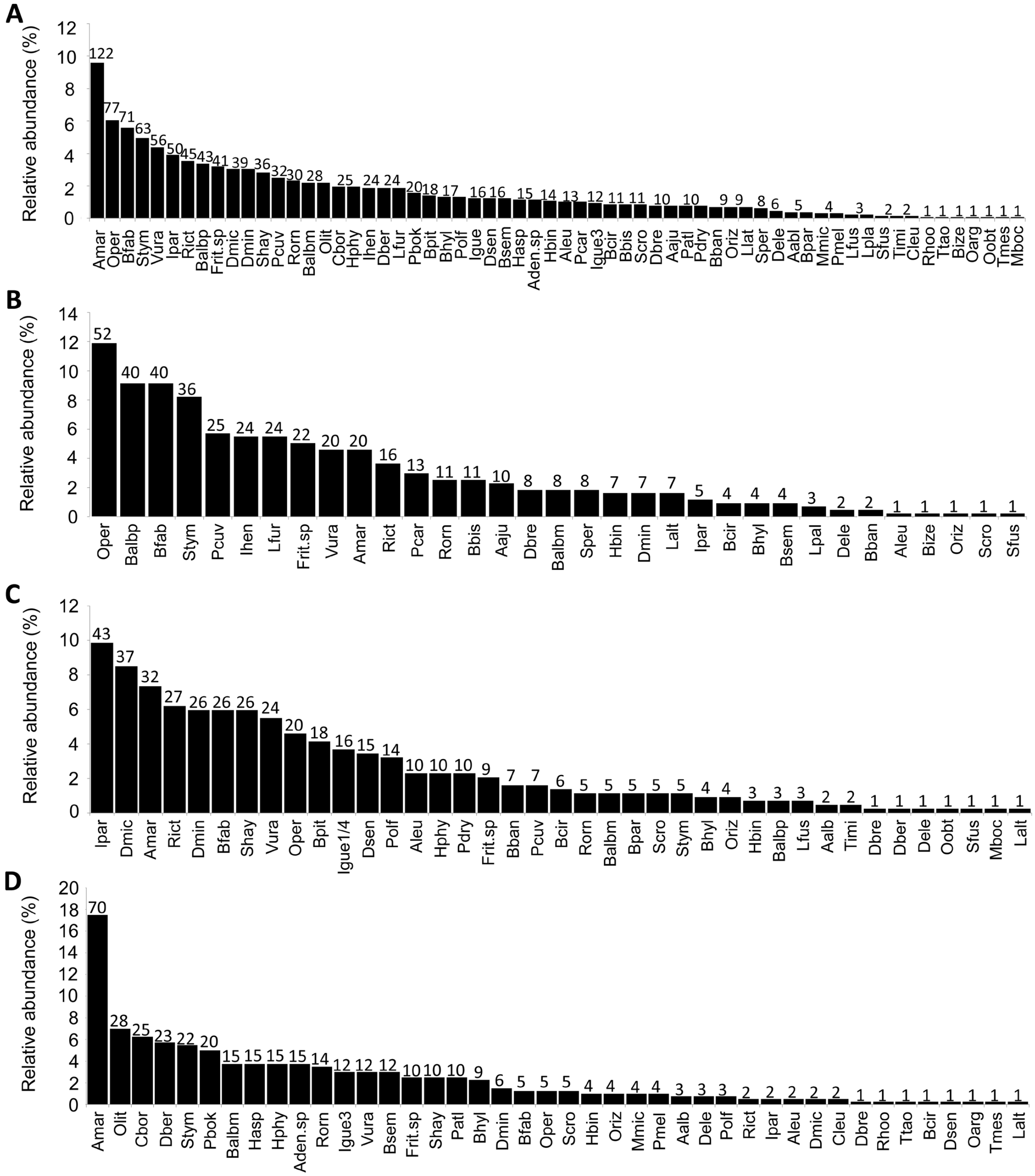

Figure 8. Relative abundance of anuran species (%) observed at the Parque Estadual da Serra do Mar, state of São Paulo, southeastern Brazil. (A) sum of all individuals observed at the Núcleo Curucutu, Núcleo Curucutu and Núcleo Santa Virgínia; (B) individuals observed only at the Núcleo Santa Virgínia; (C) individuals observed only at the Núcleo São Sebastião; (D) individuals observed only at the Núcleo São Sebastião. The total number of recorded individuals per species is indicated above each column. Anuran species: Rict, *Rhinella icterica*; Rhoog, *R. hoogmoedi*; Rorn, *R. ornata*; Dbre, *Dendrophryniscus brevipollicatus*; Bpit, *Brachycephalus pitanga*; Igue1, *Ischnocnema* gr. *guentheri* (CS1 lineage Gehara et al. 2013); Igue3, *I.* gr. *guentheri* (CS3 lineage Gehara et al. 2013); Igue4, *I.* gr. *guentheri* (CS4 lineage Gehara et al. 2013); Ihen, *I. henselii*; Ipar, *I. parva*, Hbin, *Haddadus binotatus*; Pmel, *Proceratophrys melanopogon*; Cbor, *Cycloramphus boraceiensis*; Hasp, *Hylodes asper*; Hphy, *H. phyllodes*; Ttao, *Thoropa taophora*; Mboc, *Megaelosia* cf. *bocainensis*; Amar, *Adenomera marmorata*; Aden.sp, *Adenomera* sp.; Lfur, *Leptodactylus furnarius*; Lfus, *L. fuscus*; Llat, *L. latrans*; Patl, *Physalaemus atlanticus*; Pbok, *P. bokermanni*; Pcuv, *P. cuvieri*; Polf, *P. olfersii*; Pcar, *Paratelmatobius cardosoi*; Cleu, *Chiasmocleis leucosticta*; Mmic, *Myersiela microps*; Frit.sp, *Fritziana* sp. (aff. *fissilis*); Pdry, *Phrynomedusa dryade*; Aleu, *Aplastodiscus leucopygius*; Bcir, *Bokermannohyla circumdata*; Bhyl, *B. hylax*; Bize, *B. izecksohni*; Dber, *Dendropsophus berthalutzae*; Dele, *D. elegans*; Dmic, *D. microps*; Dmin, *D. minutus*; Dsen, *D. seniculus*; Balbm, *Boana albomarginata*; Balbp, *B. albopunctata*; Bban, *B. bandeirantes*; Bbis, *B. bischoffi*; Bfab, *B. faber*; Bpar, *B. pardalis*; Bsem, *B. semilineata*; Oarg, *Ololygon argyreornata*; Olit, *O. littoralis*; Oper, *O. perpusilla*; Oriz, *O. rizibilis*; Scro, *Scinax crospedospilus*; Sfus, *S. fuscovarius*; Shay, *S. hayii*; Sper, *S. perereca*; Stym, *S. tymbamirim*; Timi, *Trachycephalus imitatrix*; Tmes, *T. mesophaeus*; Vura, *Vitreorana uranoscopa*.

Supplementary material

The following online material is available for this article:

Appendix 1: Vouchers of anurans collected at núcleos Curucutu, São Sebastião and Santa Virgínia of the Parque Estadual da Serra do Mar state of São Paulo, southeastern Brazil. VOUCHER AND TISSUES = register number of the Coleção de Anfíbios Célio F. B. Haddad, Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro (CFBH); Coleção de Anfíbios do Departamento de Zoologia e Botânica, Universidade Estadual Paulista, São José do Rio Preto (DZSJRP); Laboratório de Ecologia Teórica:Integrando Biologia, Espaço e Tempo (LET.IT.BE), Departamento de Ciências Ambientais, Universidade Federal de São Carlos, campus Sorocaba (FRS). * Species recorded by vocalization in the fieldwork.

Acknowledgements

We thank the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for providing collection permits (SISBIO/41282-1) and the Instituto Florestal de São Paulo for the permission to perform the study in the Parque Estadual da Serra do Mar, núcleos Curucutu, São Sebastião and Santa Virgínia (Proc. 260108-004.608/2014). We thank all the staff of the núcleos Curucutu, São Sebastião and Santa Virgínia for helping us during fieldworks. FRS thanks São Paulo Research Foundation (FAPESP - grant #2013/50714-0) for financial support. MLL thanks FAPESP and Coordenação de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) for postdoctoral fellowships (grants #2010/50124-0; 88881.062205/2014-01). CFBH thanks Fundação Grupo Boticário de Proteção à Natureza and FAPESP (grants #2013/50741-7 and #2014/50342-8) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Proc. 302518/2013-4) for a research fellowship. DCRF thanks CNPq (Proc. 303522/2013-5).

Author Contributions

Fernando Rodrigues da Silva: contributed in the acquisition, analysis, data interpretation and writing of the work. Mariana Lúcio Lyra: contributed in the molecular analysis, data interpretation and writing of the work. Célio Fernando Baptista Haddad: contributed in the identification of species, data interpretation and writing of the work. Denise de Cerqueira Rossa-Feres: contributed in the data interpretation and writing of the work.

Conflicts of interest

The author(s) declare(s) that they have no conflict of interest related to the publication of this manuscript.

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Received: 19/10/2016
Revised: 18/05/2017
Accepted: 17/06/2017

Biota Neotropica 17(2): e20170351, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
Inventory

Bats (Mammalia, Chiroptera) from the Nísia Floresta National Forest, with new records for the state of Rio Grande do Norte, northeastern Brazil

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BARROS, M. A. S., MORAIS, C. M. G., FIGUEIREDO, B. M. B., de MOURA JÚNIOR, G. B., RIBEIRO, F. F. dos S., PESSOA, D. M. A., ITO, F., BERNARD, E. **Bats (Mammalia, Chiroptera) from the Nísia Floresta National Forest, with new records for the state of Rio Grande do Norte, northeastern Brazil.** Biota Neotropica. 17(2): e20170351. http://dx.doi.org/10.1590/1676-0611-BN-2017-0351

Abstract: The state of Rio Grande do Norte is considered a data gap for bat species records in Brazil. The state is also currently target of large economic projects with potential impacts on bats, especially wind farms and mining enterprises. In addition, Rio Grande do Norte has few conservation units in which there is no systematic study on bat fauna. The Nísia Floresta National Forest (NFNF), a federally protected area of 174 hectares, is located in the eastern coast of Rio Grande do Norte and corresponds to one of the last remnants of Atlantic Forest in the state, in its northernmost limits. A bat inventory was conducted in NFNF using mist nets set at ground level, from sunset to sunrise, from December 2011 to December 2012, totaling 25 sampling nights. We captured 1,379 bats belonging to four families and 16 species. *Artibeus planirostris* (Phyllostomidae) was the most frequently captured species (n = 685; 50%), followed by *Myotis lavali* (Vespertilionidae) (n = 248; 18%) and *Phyllostomus discolor* (Phyllostomidae) (n = 147; 11%). *Peropteryx leucoptera*, *Phyllostomus discolor*, *Phyllostomus hastatus*, *Lophostoma brasiliense*, *Lasiurus blossevillii*, *Myotis lavali*, and *Promops nasutus* are new records for Rio Grande do Norte, increasing the current number of bat species from 25 to 32 in this state. Further inventories, especially using acoustic surveys with bat detectors, might add more species to the NFNF bat list.

Key-words: *Atlantic Rainforest, chiropteran inventory, conservation units, Neotropical biodiversity, protected areas.*

Morcegos (Mammalia, Chiroptera) da Floresta Nacional de Nísia Floresta, com novos registros para o estado do Rio Grande do Norte, nordeste do Brasil

Resumo: O estado do Rio Grande do Norte é considerado uma lacuna de informações sobre ocorrência de morcegos no Brasil. O estado também é atualmente alvo de grandes empreendimentos com potencial impacto sobre a quiropterofauna, especialmente no setor de energia eólica e mineração. Além disso, apresenta poucas unidades de conservação, e estas não possuem sua quiropterofauna estudada de maneira sistematizada. A Floresta Nacional de Nísia Floresta (FNNF), uma unidade de conservação federal de 174 hectares, localiza-se na costa leste do Rio Grande do Norte e corresponde a um dos últimos remanescentes de Mata Atlântica no estado e no limite norte do bioma. Foi realizado um inventário de morcegos na FNNF com a utilização de redes de neblina armadas no nível do solo, do por do sol ao amanhecer, de dezembro de 2011 a dezembro de 2012, totalizando 25 noites de amostragem. Nós capturamos 1379 morcegos pertencentes a quatro famílias e 16 espécies. *Artibeus planirostris* (Phyllostomidae) foi a espécie mais frequentemente capturada (n = 685; 50%), seguida por *Myotis lavali* (Vespertilionidae) (n = 248; 18%) e *Phyllostomus discolor* (Phyllostomidae) (n = 147; 11%). *Peropteryx leucoptera*, *Phyllostomus discolor*, *Phyllostomus hastatus*, *Lophostoma brasiliense*, *Lasiurus blossevillii*, *Myotis lavali* e *Promops nasutus* são novos registros para o Rio Grande do Norte, aumentando o número atual de espécies de morcegos no estado de 25 para 32. Inventários adicionais, especialmente utilizando amostragens acústicas com detectores de morcegos, tendem a acrescentar novas espécies à lista de morcegos da FNNF.

Palavras-chave: *Mata Atlântica, inventário de quirópteros, unidade de conservação, biodiversidade Neotropical, áreas protegidas.*

Introduction

Brazil is a mega-biodiversity country (Mittermeier et al. 1997) with one of the highest species richness of bats in the world. Currently, nine families, comprising 178 species, are known in the Brazilian territory (Nogueira et al. 2014). Nevertheless, information on distribution and occurrence of bat species in Brazil is highly fragmented. There are no records of bat species for nearly 60% of the Brazilian area, and 8% of the country can be considered minimally surveyed in terms of bats (Bernard et al. 2011). One of the most significant data gaps about bat occurrence in Brazil corresponds to the state of Rio Grande do Norte (Bernard et al. 2011), located in the northeast part of the country. Currently, Rio Grande do Norte is the state with the lowest number of localities surveyed for bats (= 12) and the lowest bat richness (= 24 species) in northeastern Brazil (Garcia et al. 2014). Concomitantly, this state has been attracting investments in large economic projects with potential impact on bats, such as wind farms and mining development.

The east coast of Rio Grande do Norte corresponds to the northern limit of the Atlantic Forest biome (IBGE 2004), a hotspot for biodiversity conservation due to high rates of endemism and habitat loss (Myers et al. 2000). Five of the 10 bat species currently considered endemic to Brazil occur exclusively in the Atlantic Forest (Nogueira et al. 2014). The region originally covered an area of 150 million hectares, but currently, only 12% of the original vegetation remains, mostly distributed in small forest fragments below a size of 50 hectares (Ribeiro et al. 2009). Similarly, the current area of the Atlantic Forest corresponds to 12% of its original domain in the state of Rio Grande do Norte, with about 27,000 hectares of natural non-forest vegetation (including mangroves and salt marshes) and 16,000 hectares of forest remnants (Fundação SOS Mata Atlântica & INPE 2014). One of these remnants is the Nísia Floresta National Forest (Floresta Nacional de Nísia Floresta), a federally protected area of 174 hectares created in 2001 (MMA 2012).

Although the coastline of Rio Grande do Norte is considered an area of high biological importance and a priority area for biodiversity conservation of the Atlantic Forest (Conservation International do Brasil et al. 2000), this region is poorly surveyed in terms of bats. The scarce information about bat occurrence along the coastal region of Rio Grande do Norte is dispersed in a few research papers and non-published dissertations and theses (reviewed by Garcia et al. 2014). Bats are diverse and abundant (Altringham 1996), indicators of habitat disturbance (Jones et al. 2009), and play important ecological roles as insect controllers (Boyles et al. 2011), pollinators (Fleming et al. 2009), and seed dispersers (Lobova et al. 2009) in the Neotropics. Considering the lack of information about the bat fauna in Rio Grande do Norte and the biological relevance of the remaining Atlantic Forest fragments in this state, the objective of this study was to carry out the first bat inventory at the Nísia Floresta National Forest, northeastern Brazil.

Material and Methods

1. Study Area

Our study was carried out at the Nísia Floresta National Forest (hereafter termed NFNF), a federal protected area located in the municipality of Nísia Floresta, state of Rio Grande do Norte, northeastern Brazil (latitude: 06º05’12,4” S; longitude: 035º11’04,0” W). The NFNF covers an area of 174.95 ha, with a maximum altitude of 100 m asl (MMA 2012). The climate in this region is classified as tropical savannah (Aw) (Peel et al. 2007) with an average temperature of 27°C (maximum: 30°C; minimum: 21°C) (IDEMA 2013). Normal annual rainfall is 1,522 mm, with a distinctive rainy season from March to August and a dry season from September to February (IDEMA 2013).

The study area is located in the Atlantic Forest biome (IBGE 2004), in a region originally covered by rainforest that has been historically exploited for sugarcane production. The NFNF area has been used as a forestry experiment station from the 1960s to the early 1980s and consists of both secondary native forest (≈ 60% of the total area) and experimental forestry sites (≈ 40% of the total area) (MMA 2012). Within the native forest, the predominant vegetation is seasonal semideciduous forest (“floresta estacional semidecidual”) (≈ 45% of the NFNF total area) in the west-central part of the NFNF, followed by coastal tableland (“tabuleiro litorâneo”) (≈ 15% of the NFNF total area) in the northern part of the NFNF (MMA 2012).

Bat surveys took place at the experimental forestry area in the southeastern part of the NFNF. This area exhibits both regenerating seasonal semideciduous forest and introduced plants, including native and exotic species planted during forestry experiments. The dominant tree species are *Tapirira guianensis* (family Anacardeaceae), *Byrsonima crassifolia* (family Malpighiaceae), *Mischocarpus sundaicus* (family Sapindaceae), *Cordia nodosa* (family Boraginaceae), *Plathymenia reticulata* (family Leguminosae), *Caesalpinia ferrea* (family Leguminosae), and exotic species of *Eucalyptus* (family Myrtaceae) (MMA 2012). *Tapirira guianensis* (locally called “cupiúba”) is common throughout the entire area, while the density of the other species varies among different sites within the NFNF experimental forest zone (MMA 2012).

2. Sampling

Bat surveys were conducted using mist nets (12 × 3 m, mesh 19 mm, five shelves, Ecotone®) from December 2011 to December 2012. Sampling was performed during a total of 25 nights, one in December 2011 and two consecutive nights per month from January to December 2012. At each night, eight mist nets were set at ground level along forest trails and edges. The mist nets were set at different sites within the NFNF experimental forestry area in December 2011 and at the same spots from January to December 2012 (six along the first 300 m of the NFNF main trail and two at forest edges near the trailhead). Nets were opened at sunset and remained open for 11 h. The total sampling effort was 79,200 h.m^2 (3,168 h.m^2 per night), calculated according to Straube & Bianconi (2002) by multiplying the following factors: mist net area (m^2), hours of exposure (h), number of nets, and number of sampling nights.

Captured bats were removed from nets, kept in individual cotton bags, and checked for sex and reproductive status (according to Racey 2009), age (according to Brunet-Rossinni & Wilkinson 2009), forearm length (using a digital caliper), and body mass (with a spring scale). Bats were then immediately released at the capture site, except a number between 1–10 individuals per species which were killed by ether inhalation and collected as voucher specimens (permit SISBIO/ICMBio #30730-2), as well as individuals who accidentally died during capturing or handling processes. These specimens were fixed in formaldehyde (10% solution), preserved in ethanol (70% solution), and deposited in the Mammal Collection of the Federal University of Pernambuco (UFPE).

Voucher specimens were taxonomically identified according to the keys and descriptions of Araújo & Langguth (2010), Cloutier & Thomas (1992), Fonseca & Pinto (2004), Greenhall et al. (1983), Gregorin & Taddei (2002), Jones & Hood (1993), Lim et al. (2010), Moratelli et al. (2011), Reis et al. (2007), Simmons & Voss (1998), Velazco (2005), and Webster (1993). In specimens for which morphological identification was not completely conclusive, tissue samples (liver pieces) were used to molecular identification. DNA extraction was conducted using the Biopur Mini Spin Plus kit according to the manufacturer’s instructions. A conserved region of 360 bp of the cytochrome subunit b gene was amplified by PCR with the universal primers L14841 (5′-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) and H15149 (5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′)

(Kocher et al. 1989). Amplifications were performed in a final volume of 25 µl containing 12.5 µl of 2X Taq Master Mix (Vivantis Technologies), 0.5 µl of MgCl2 (50 mM), 1.0 µl of each primer (2.0 mM), and 10 ng of extracted DNA, according to Bobrowiec et al. (2015). The PCR products were purified and sequenced with the sequencing kit Big Dye® Terminator v3.1 (Life Technologies), according to the protocol provided, in both forward and reverse directions, in ABI 3500 automatic sequencer. To obtain a consensus sequence, we aligned and edited the sequences obtained in the program BioEdit v. 7.2.5 (Hall 1999). Then, we compared the sequences to homologous sequences of Cytb deposited in GenBank. In addition, we aligned the consensus sequences with sequences of the possible species, based on information on distribution and occurrence of bat species in northeastern Brazil.

Results

We captured a total of 1,379 bats of 16 species and four families (Table 1, Figure 1). The richest family was Phyllostomidae (12 species); the families Vespertilionidae, Emballonuridae, and Molossidae were represented by one to two species each. *Artibeus planirostris* (Phyllostomidae) was the most frequently captured species (50%), followed by *Myotis lavali* (Vespertilionidae) (18%) and *Phyllostomus discolor* (Phyllostomidae) (11%). The capture frequencies of the other species were less than 10%.

All captured species are vouchered by specimens in the Mammal Collection of the Federal University of Pernambuco (Appendix 1). Molecular taxonomic identification was carried out in three specimens (UFPE 3297, UFPE 3191, and UFPE 3198), in order to confirm the identification of the species from the genera *Carollia* and *Myotis*. The taxonomic identification of the specimens was confirmed as *Carollia perspicillata* and *Myotis lavali* (similarity was 100% in all cases; see gene sequences in the online Supplementary Material 1 and 2).

Discussion

Although our sampling was limited to one habitat type (experimental forestry area) within the NFNF, this is the first systematic inventory of bats in a protected area in the state of Rio Grande do Norte. Our results are regionally relevant and provide new information on bat composition in this state with a poorly known and described bat fauna. Approximately 44% of all captured species are new state records; we report for the first time the occurrence of *Myotis lavali*, *Phyllostomus discolor*, *Phyllostomus hastatus*, *Lophostoma brasiliense*, *Lasiurus blossevillii*, *Peropteryx leucoptera*, and *Promops nasutus* in Rio Grande do Norte. In addition, we confirm the occurrence of seven species (*Artibeus lituratus*, *Artibeus planirostris*, *Carollia perspicillata*, *Desmodus rotundus*, *Platyrrhinus lineatus*, *Sturnira lilium*, and *Trachops cirrhosus*) by providing voucher specimens, which were lacking in the previous records in grey and peer-reviewed literature (reviewed by Garcia et al. 2014). Only the previous records of *Dermanura cinerea* and *Glossophaga soricina* are vouchered by specimens in collections (Handley Jr. 1987, Webster 1993). The seven new species records increase the current number of bat species from 25 to 32 in Rio Grande do Norte (Barros 2014, Garcia et al. 2014).

Artibeus planirostris is a primarily frugivorous bat (Hollis 2005) and accounted for about 50% of total bat captures. This high frequency is probably associated with the availability of food resources in our study area, in which several fruit trees could be identified near the main forest trail. These bats were often captured carrying fruits of *Ficus* spp. (family Moraceae) and *Cecropia* spp. (family Urticaceae), which are largely consumed by *Artibeus* species in the Neotropics (Lobova et al. 2009). *Artibeus planirostris* is also often captured by mist-netting in the urban area of Natal city; this species accounted for 96% (N = 260) of bat captures in green areas within the campus of the Federal University of Rio Grande do Norte (UFRN) (M.A.S. Barros, unpublished data). This suggests that *A. planirostris* is an abundant bat species both in natural and anthropic areas in the Atlantic Forest biome in this state. Apart from the present

Table 1. Numbers of bat captures by mist-netting in forest trails and edges at the Nísia Floresta National Forest (Floresta Nacional de Nísia Floresta), state of Rio Grande do Norte, northeastern Brazil, from December 2011 to December 2012.

Taxon	Number of females	Number of males	Total number	Frequency
Family Emballonuridae				
Peropteryx leucoptera Peters, 1867	–	1	1	0.1%
Family Phyllostomidae				
Artibeus lituratus (Olfers, 1818)	7	4	11	0.8%
Artibeus planirostris (Spix, 1823)	342	343	685	49.7%
Carollia perspicillata (Linnaeus, 1758)	22	18	40	2.9%
Dermanura cinerea Gervais, 1856	16	13	29	2.1%
Desmodus rotundus (É. Geoffroy, 1810)	–	1	1	0.1%
Glossophaga soricina (Pallas, 1766)	63	57	120	8.7%
Lophostoma brasiliense Peters, 1866	3	5	8	0.6%
Phyllostomus discolor (Wagner, 1843)	71	76	147	10.7%
Phyllostomus hastatus (Pallas, 1767)	6	5	11	0.8%
Platyrrhinus lineatus (É. Geoffroy, 1810)	24	17	41	3.0%
Sturnira lilium (É. Geoffroy, 1810)	8	26	34	2.5%
Trachops cirrhosus (Spix, 1823)	1	–	1	0.1%
Family Molossidae				
Promops nasutus (Spix, 1823)	–	1	1	0.1%
Family Vespertilionidae				
Lasiurus blossevillii ([Lesson, 1826])*	–	1	1	0.1%
Myotis lavali Moratelli, Peracchi, Dias & Oliveira, 2011	5	243	248	18.0%
Total			1379	100.0%

*According to Nogueira et al. (2014).

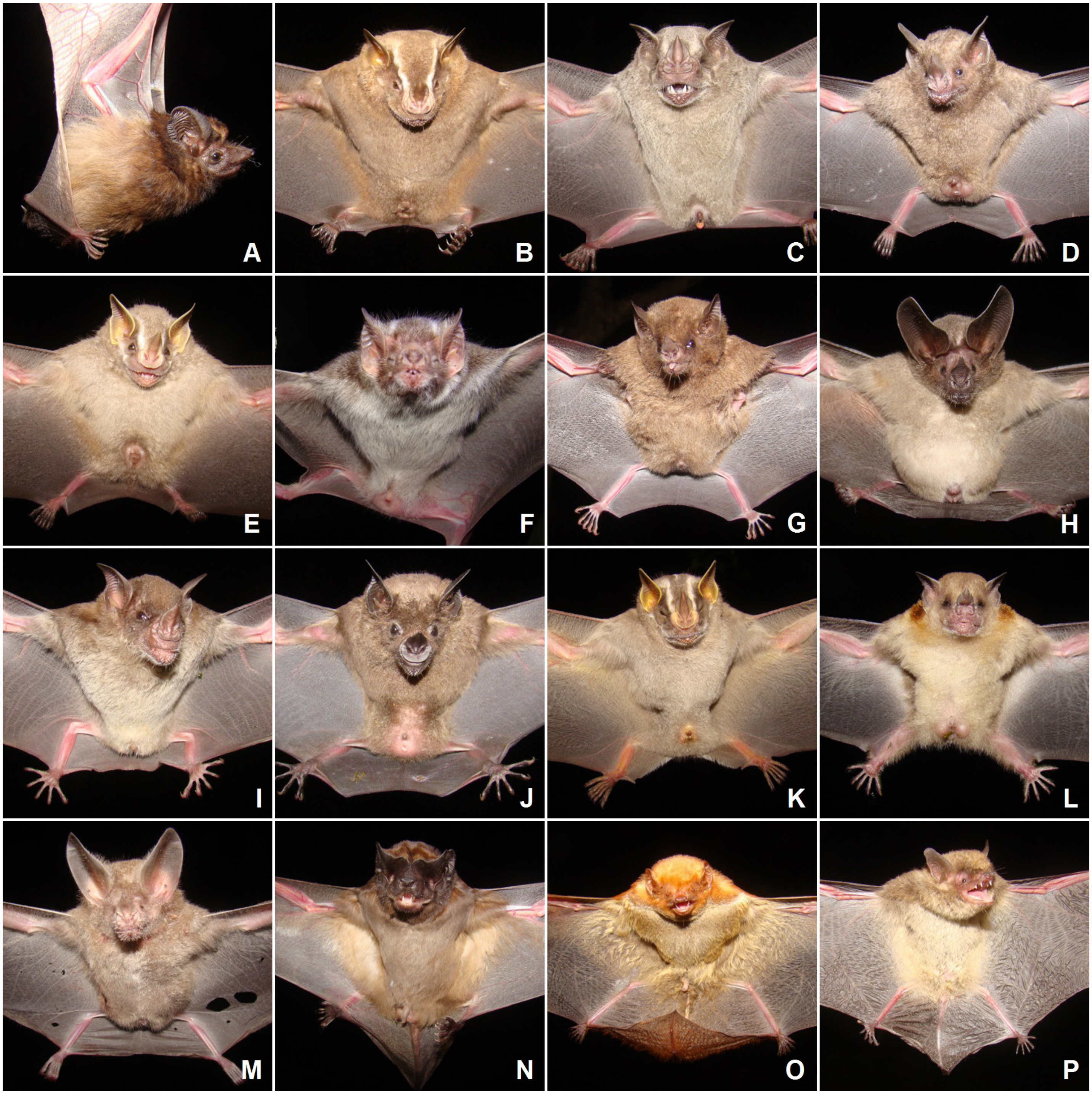

Figure 1. Bat species (Mammalia, Chiroptera) captured by mist-netting in forest trails and edges at the Nísia Floresta National Forest (Floresta Nacional de Nísia Floresta), state of Rio Grande do Norte, northeastern Brazil, from December 2011 to December 2012. (a) *Peropteryx leucoptera*; (b) *Artibeus lituratus*; (c) *Artibeus planirostris*; (d) *Carollia perspicillata*; (e) *Dermanura cinerea*; (f) *Desmodus rotundus*; (g) *Glossophaga soricina*; (h) *Lophostoma brasiliense*; (i) *Phyllostomus discolor*; (j) *Phyllostomus hastatus*; (k) *Platyrrhinus lineatus*; (l) *Sturnira lilium*; (m) *Trachops cirrhosus*; (n) *Promops nasutus*; (o) *Lasiurus blossevillii*; e (p) *Myotis lavali*.

study, there are currently six additional records of *A. planirostris* in Rio Grande do Norte: in Ponta Negra, Natal (Varela-Freire 1997), and in five caves, one (Gruta da Carrapateira) in Felipe Guerra (Ferreira et al. 2010, Cordero-Schmidt et al. 2016), one (Caverna do Serrote Preto) in Lajes, one (Gruta Casa de Homens) in Caraúbas, and two (Caverna Lajedo Grande and Caverna da Pedrada) in Governador Dix-Sept Rosado (Cordero-Schmidt et al. 2016).

Myotis lavali, the second most frequently captured species (18%), is a recently described species (Moratelli et al. 2011) whose distribution limits and natural history are poorly known. Our record is a new marginal point of occurrence and extends the distribution of the species by about 470 km southeast (from Russo–CE) and about 210 km north (from São Lourenço da Mata–PE) (Moratelli & Wilson 2013). In northeastern Brazil, this species occurs mainly in xeric forests and shrublands within the Caatinga biome

(Novaes & Laurindo 2014, Novaes et al. 2015, Silva et al. 2015); this is the second peripheral record of *M. lavali* in the adjacent Atlantic Forest, besides a specimen captured in a seasonal lowland forest area near the coast of Pernambuco (Moratelli & Wilson 2013). The high number of captures in the NFNF suggests these bats often forage at low heights near to the ground and forest edges. In addition, males were highly predominant in our study area (98% of *M. lavali* captures), in contrast to Caatinga sites in the states of Ceará and Pernambuco where sexes occurred in similar proportions (Willig 1983, species originally identified as *M. nigricans*, but reclassified as *M. lavali*; see Moratelli & Wilson 2013). This extremely male-biased sex ratio suggests the occurrence of sex-specific roosting and/or foraging areas at least in a part of the *M. lavali* distribution. Spatial sexual segregation has been reported for other *Myotis* species in temperate regions, whose patterns are related mainly to differences in energetic demands between sexes throughout the year (Barclay 1991, Cryan et al. 2000, Russo 2002, Dietz et al. 2006, Encarnação 2012, Angell et al. 2013). These studies observed that males tend to occupy poor quality habitats in terms of food availability and weather conditions (frequently located at higher altitudes) in comparison to reproductive females. We captured only five *M. lavali* females at the NFNF experimental forestry area: two non-reproductive (in August and December 2012), two lactating (in October and November 2012) and one both pregnant and lactating female (in December 2012). It is possible that females (reproductive or not) occur in higher proportions in more suitable habitats in the northern portion of the NFNF not sampled in our study (e.g. near ponds or in secondary native forest areas), or that sexual segregation occurs in a larger spatial scale. Both hypotheses require investigation; although sexual segregation was observed inside roosts in *M. nigricans* (Wilson 1971) and suggested by sperm storage in *M. albescens* and *M. simus* (Wilson & Findley 1971), there is no information on possible sex differences in landscape use by *Myotis* species in the Neotropics.

Phyllostomus discolor was the third most common species (11%) at the NFNF experimental forestry area. This is an omnivorous bat that feeds mainly on nectar and pollen, but also on fruits and insects (Kwiecinski 2006). We believe its generalist diet enables this species to occupy a wide variety of habitats and be one of the most common bats in our study area. Moreover, the availability of flower resources along the NFNF forest trail attract *P. discolor* bats, which were frequently captured in mist nets near flowering trees of *Parkia* spp. (family Leguminosae). Flowers of several species of *Parkia* are often visited and pollinated by *P. discolor* in northern and northeastern Brazil (Carvalho 1961, Hopkins 1984, Piechowski et al. 2010); *P. discolor* possibly plays an important role as pollinator of *Parkia* species at the FNNF. The record of *P. discolor* in the state of Rio Grande do Norte was expected, since it occurs in the neighboring states of Ceará (Fabián 2008) and Paraíba (Feijó & Langguth 2011). In northeastern Brazil, this species occurs in moist and dry forests in Caatinga (Souza et al. 2004, Novaes & Laurindo 2014) and Atlantic Forest biomes (Mikalauskas 2005, Faria et al. 2006).

As well as *Phyllostomus discolor*, the species *Phyllostomus hastatus*, *Lophostoma brasiliense*, and *Lasiurus blossevillii* occur both in the states of Ceará and Paraíba (Alencar et al. 1976, Mares et al. 1981, Fabián 2008, Feijó & Langguth 2011, Peracchi et al. 2011) and therefore, Rio Grande do Norte is part of their expected distributions. *Promops nasutus*, however, was recorded only in four localities in the northeastern region of Brazil: at its type locality in Rio São Francisco (Spix 1823, Thomas 1915), Lamarão (Goodwin & Greenhall 1962), and Rio Preto (Gregorin & Chiquito 2010) in the state of Bahia, and at a not informed site in the south of the state of Piauí (Tavares et al. 2008). The present record extends the geographic distribution of this species to 750 km north (from Lamarão–BA) and at least 700 km east (from the southeastern border of Piauí). It suggests that *Promops nasutus* is widely distributed throughout northeastern Brazil, probably occurring in the states of Ceará, Paraíba, Pernambuco, Alagoas, and Sergipe. The low number of records is possibly due to the foraging behavior of the genus *Promops*, whose species hunt insects in the open space high above the ground or canopy (Schnitzler & Kalko 2001), and are therefore rarely captured by mist-netting at the ground level. The species *Peropteryx leucoptera* is also poorly recorded in northeastern Brazil; it was observed in Sapé in the state of Paraíba (Feijó & Langguth 2011), in Itamaracá (Cruz et al. 2002) and Formoso (Guerra 1980) in the state of Pernambuco, and in Capela in the state of Sergipe (Mikalauskas et al. 2014). This species apparently has a disjunct geographic distribution, occurring throughout northern South America and separately in the coastal region of extreme northeastern Brazil (Mikalauskas et al. 2014). Our record extends the distribution of *Peropteryx leucoptera* to 110 km north (from Sapé–PB) in the northeastern region of Brazil, corresponding to the northern limit the of distribution of this species in the Atlantic Forest biome.

Among the bat species previously recorded in Rio Grande do Norte, *Desmodus rotundus* and *Trachops cirrhosus* (cited as *Trachops* sp.) were observed inside caves in the western region of the state in the Caatinga biome (Ferreira et al. 2010). Therefore, we recorded these bat species for the first time in an Atlantic Forest area in the state of Rio Grande do Norte. The species *Artibeus lituratus*, *Carollia perspicillata*, *Sturnira lilium*, *Dermanura cinerea*, *Glossophaga soricina*, and *Platyrrhinus lineatus* were recorded in Atlantic Forest areas near NFNF (≈ 18–22 km). *Artibeus lituratus*, *Carollia perspicillata*, and *Sturnira lilium* occur at the Parque Estadual do Jiquí in the municipality of Parnamirim, a 398 hectares protected area that exhibits forest remnants and hydric resources (Farias 2009); *Dermanura cinerea*, *Glossophaga soricina*, and *Platyrrhinus lineatus* were recorded in the Parque Estadual do Jiquí and also in the municipality of Natal (Handley Jr. 1987, Webster 1993, Varela-Freire 1997, Farias 2009). *Artibeus planirostris* occurs both in the Atlantic Forest and Caatinga biomes in the state of Rio Grande do Norte (Ferreira et al. 2010, Varela-Freire 1997, Cordero-Schmidt et al. 2016, Present study).

Although the NFNF is a relatively small protected area in regeneration surrounded by farms and croplands, it apparently is an important habitat for bats. The bats from NFNF are morphologically and ecologically diverse, including species that feed primarily on fruits (*Artibeus planirostris*, *Artibeus lituratus*, *Dermanura cinerea*, *Platyrrhinus lineatus*, *Sturnira lilium*, and *Carollia perspicillata*), pollen/nectar (*Glossophaga soricina* and *Phyllostomus discolor*), arthropods and small vertebrates (*Trachops cirrhosis* and *Phyllostomus hastatus*), insects (*Peropteryx leucoptera*, *Myotis lavali*, *Lasiurus blossevillii*, *Promops nasutus*, and *Lophostoma brasiliense*), and blood (*Desmodus rotundus*). Thus, the bats recorded in the present study provide ecosystem services such as seed dispersal, pollination, and control of insect populations in the region of the NFNF. Bats from the family Phyllostomidae corresponded to the largest number of captures, which is in accordance with most inventories in the Neotropics (e.g. Bernard et al. 2001, Esbérard et al. 2006, Novaes & Laurindo 2014). This pattern is related both to the high abundance of phyllostomid bats in Brazilian tropical areas and to the selectivity of the mist-netting method, which tends to capture bats that forage near the ground and vegetation (such as fruit-eating and nectar-feeding bats). Further inventories, especially using acoustic surveys and sampling areas further north of the NFNF, might add more species to the present bat list.

Supplementary material

The following online material is available for this article:

Supplementary Material 1: Consensus sequences of a specimen of Carollia perspicillata (Chiroptera, Phyllostomidae), collected as voucher (Mammal Collection of the Federal University of Pernambuco; collection number UFPE 3297) in 2012 at the Nísia Floresta National Forest, Rio Grande do Norte, northeastern Brazil.

Supplementary Material 2: Alignment of consensus sequences of two specimens of Myotis lavali (Chiroptera, Vespertilionidae), collected as vouchers (Mammal Collection of the Federal University of Pernambuco; collection numbers UFPE 3191 and 3198) in 2012 at the Nísia Floresta National Forest, Rio Grande do Norte, northeastern Brazil, and sequences from Myotis nigricans, Myotis levis and Myotis lavali.

Appendix 1: List of bat specimens collected as vouchers at the Nísia Floresta National Forest (Floresta Nacional de Nísia Floresta), state of Rio Grande do Norte, northeastern Brazil, from December 2011 to December 2012. This material is deposited in the Mammal Collection of the Federal University of Pernambuco (Universidade Federal de Pernambuco).

Acknowledgments

We thank Patrícia Pereira da Silva Macêdo, Cláudio José Barbosa Pinto (Instituto Chico Mendes de Conservação da Biodiversidade), and Paulo Roberto Fernandes de Medeiros (Floresta Nacional de Nísia Floresta) for logistical support in the study area.

Author Contributions

Marília A. S. Barros: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation.

Camila Martins Gomes Morais: Contribution to data collection; Contribution to manuscript preparation.

Bruna Maria Braga Figueiredo: Contribution to data collection; Contribution to manuscript preparation.

Gilberto Benigno de Moura Júnior: Contribution to data collection; Contribution to manuscript preparation.

François Fernandes dos Santos Ribeiro: Contribution to data collection; Contribution to manuscript preparation.

Daniel Marques de Almeida Pessoa: Contribution to critical revision, adding intellectual content.

Fernanda Ito dos Santos: Contribution to data analysis and interpretation; Contribution to manuscript preparation.

Enrico Bernard: Contribution to critical revision, adding intellectual content.

Conflicts of interest

The authors declare no conflict of interest.

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Received: 04/04/2017
Accepted: 14/05/2017

Biota Neotropica 17(2): e20160299, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
article

Establishment of a cryopreserved biobank for the Culture Collection of Freshwater Microalgae (CCMA-UFSCar), São Paulo, Brazil

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TESSAROLLI, L.P., DAY, J.G., VIEIRA, A.A.H. **Establishment of a cryopreserved biobank for the Culture Collection of Freshwater Microalgae (CCMA-UFSCar), São Paulo, Brazil**. Biota Neotropica. 17(2): e20160299, http://dx.doi.org/10.1590/1676-0611-BN-2016-0299

Abstract: The Culture Collection of Freshwater Microalgae (CCMA-UFSCar, Coleção de Culturas de Microalgas de Água-Doce), based at the Universidade Federal de São Carlos, plays an important role in underpinning Brazilian microalgal research, providing biological materials, substrates and training personnel for a large proportion of the past and current projects in this area. However, recent efforts to expand the culture catalogue/holdings have reached a ceiling because of the logistical practicality of maintaining actively growing cultures. In order to reduce the costs associated with the maintenance regime of cultures, efforts were initiated on the establishment of a cryopreserved biobank for long term maintenance of cultures, thus minimizing the efforts associated with handling of material, as successfully frozen cultures, in theory, could be maintained effectively indefinitely, with the advantage of the stability of cells characteristics. Initial tests were performed on 93 strains including exemplar taxa across the different taxonomic groups in the collection catalogue. The highest levels of success were achieved for the smaller taxa, such as the small green algae; while for the larger and more complex organisms limited to no success was obtained. For the strains tested, over 70% had positive good levels of post-thaw viability and regenerated phenotypically normal cultures.

Keywords: *Cryopreservation; freshwater microalgae; biobank*

Estabelecimento de um biobanco criopreservado para a Coleção de Culturas de Microalgas de Água-Doce (CCMA-UFSCar), São Paulo, Brasil

Resumo: A Coleção de Culturas de Microalgas de Água-Doce (CCMA-UFSCar), localizada na Universidade Federal de São Carlos tem um importante papel na sustentação da pesquisa brasileira em microalgas, provendo material biológico, substratos e treinamento de pessoal para uma grande fração dos projetos atuais nessa área. Entretanto, os recentes esforços para expandir o catálogo de culturas atingiram um limite máximo em praticidade logística para a manutenção de culturas metabolicamente ativas. Buscando reduzir os custos do regime de manutenção das culturas, foi-se iniciado o estabelecimento de um banco criopreservado para a manutenção em longo prazo desses organismos, minimizando os gastos com manuseio do material, visto que culturas congeladas com sucesso podem, em teoria, serem mantidas por prazos indeterminados, ainda com a vantagem da estabilidade das características nas culturas. Testes iniciais foram realizados com 93 linhagens, incluindo organismos de diferentes grupos taxonômicos mantidos na coleção de culturas. As maiores taxas de sucesso foram observadas nos organismos menores, como as pequenas algas verdes, enquanto os organismos maiores e mais complexos obtiveram sucesso limitado. Em geral, para as linhagens testadas, acima de 70% dos organismos apresentaram resposta positiva ao processo.

Palavras-chave: *criopreservação, microalgas de água-doce, biobancos.*

Introduction

The Culture Collection of Freshwater Microalgae (Coleção de Culturas de Microalgas de Água-doce – CCMA-UFSCar), based at Universidade Federal de São Carlos was the first of its genre to be established in Brazil, in 1977, by Dr. Armando Vieira (Lourenço & Vieira 2004). It is currently the largest microalgae collection in Brazil, holding around 700 strains of freshwater microalgae, mostly isolated from diverse water bodies in Sao Paulo State, Brazil. This collection has been the source of samples for work undertaken by both public and private institutions, across a wide range of academic and biotechnological projects. Furthermore, it has also been responsible for the specialized training of personnel, including researchers which went to initiate other algae culture collections in Brazil. Recently, renewed efforts were concentrated in the expansion of the collections holdings, as part of the BIOTA-Fapesp network.

Traditionally, strains at CCMA-UFSCar were maintained in replicate as metabolically active cultures under suboptimal growth conditions, which must be sub-cultured every 2-3 months (Vieira, pers. comm) to maintain their viability. These cultures are readily available for further application and distribution; however, for their successful maintenance, conditions must be carefully considered and met, such as the culture medium, light, temperature and the intervals between transfers (Lorenz et al. 2005). Also, species may greatly differ in their needs for growth, with specifications and requirements for their successful maintenance (Brand et al. 2013). Thus, the maintenance of metabolically active cultures is a laborious activity which requires a well-designed space and dedicated highly trained personnel. This necessitates significant investment in facilities, personnel and consumables for the upholding of the collection. Also, the sub-culturing process carries several risks for the cultures, mostly related to the transferring activities, as it opens possibilities for cross-contamination of samples, mislabeling, or contaminations from outside organisms, which can lead to the loss of strains when not carefully monitored (Brand et al. 2013; Day & Brand 2005; Lorenz et al. 2005), or raise the requirements for their maintenance with the need for re-isolation and decontamination over time. Besides the trained personnel to perform these functions associated with the maintenance of metabolically active cultures, a further requirement is a dedicated infrastructure, with controlled temperature and illumination and sufficient space to allow the further expansion of the collection holdings.

In order to reduce the costs of maintenance and the exposure of strains to sources of contamination and deterioration of samples, cryopreservation is recommended for the long term maintenance of samples in an arrested state (OECD 2007). This type of methodology reduces the routine costs for the culture collection, increasing the capacity of expansion of the holdings while insuring the stability and purity of samples and reducing the risks of contamination during the handling of samples. The recent expansion of the CCMA-UFSCar holdings has increased the volume of work associated with the maintenance of active cultures, which is rapidly reaching unsustainable levels under the current regime in the terms of investment and resource requirements. Furthermore, recently, contamination events by fungus and other organisms have occurred, which in turn increased the workload associate with the collections curation. Therefore, efforts were raised and focused on the establishment of a cryopreserved biobank for the culture collection, in order to secure the collection holdings, which are the focus of much of the research work of the laboratory and source of samples for many research groups in Brazil.

Material and Methods

1. Establishment of the cryobank infrastructure

The CCMA-UFSCar has been established as a culture collection for almost 40 years at its current location, maintaining metabolically active cultures. It is registered at the World database for Culture Collections with the reference WDCM 835. However, cryopreservation efforts were only recently initiated. Thus, adjustments of the laboratory infrastructure were necessary to facilitate the successful implementation of cryopreservation protocols for the maintenance of existing and recently incorporated microalgae strains from the culture collection. The acquisition of the necessary equipment was funded by the FAPESP Thematic Project (2011/50054-4), and included: a controlled rate freezer (CRF) Planer Kryo 360 1.7 (Planer PLC, UK) with associated software which allows the control of the cryopreservation process; an Ultra-Low temperature freezer (Revco Value Series, Thermo Scientific, USA); sample storage dewar SC-47 R (Sempercrio, Brazil) and nitrogen transportation dewar SC-60/I (Sempercrio, Brazil).

2. Training on cryopreservation techniques

As part of the initial activities of the project, the training on the required techniques was undertaken at the Culture Collection of Algae and Protozoa (CCAP), based at the Scottish Association for Marine Science (SAMS), Oban, UK. The CCAP has over 40 year's expertise on the maintenance of frozen strains of marine and freshwater algae and protozoa. The training included basic theory and practical protocols for the successful maintenance of strains at ultra-low temperatures, which were further adapted for applicability using the Brazilian laboratory facilities. These experiences were applied for the development of the standard operating procedure (SoP) for cryopreservation of freshwater strains at CCMA-UFSCar.

3. Collection Holdings

CCMA-UFSCar currently holds almost 700 strains of freshwater microalgae, from a wide range of groups. From these, 133 strains are currently maintained in axenic cultures, which are periodically checked for bacterial proliferation using WC medium (Guillard & Lorenzen 1972) modified by the addition of glucose and peptone (250 $mg.L^{-1}$ each), in a visual analysis. The remaining cultures of the collection are maintained as uni-algal cultures after isolation from environmental samples.

For the initial establishment of the cryopreserved biobank, efforts were focused on the maintenance of the axenic strains, which are currently the main focus of further research at the Phycology Laboratory at UFSCar. The tests included 93 strains of freshwater microalgae, mostly Chlorophyceae (62 strains), but also including organisms from Conjugatophyceae (11 strains), Trebouxiophyceae (7 strains), Cryptophyceae (1 strain), Synurophyceae (1 strain), Xantophyceae (2 strains) and Bacillariophyceae (9 strains). The effects of the presence of a contaminant heterotrophic community within the microalgae cultures on the viability and recovery of post-thaw cultures were also analyzed using 18 strains of non-axenic microalgae.

4. Freezing Protocol

Initially, a commonly employed protocol was applied for all the tested organisms, to establish the main database for the maintenance of cryopreserved organisms at CCMA-UFSCar. For this, a cryoprotectant solution/Cryoprotective Additive (CPA) of dimethyl sulfoxide (DMSO) was prepared in the appropriate culture medium at 10% concentration (v/v). All strains of microalgae were cryopreserved using a two-step, controlled rate cooling protocol as previously described by Day & Brand (2005). Briefly, an aliquot of 0.5 mL 10% (v/v) cryoprotectant solution was added to 0.5 mL of a dense microalgal culture in a cryovial, which was incubated for 15 min at room temperature, out of direct light to ensure the cryoprotectant action. Samples were then transferred to a controlled rate cooler (Planer, UK) at a start temperature of 20 ^{o}C. Cultures were then cooled at a cooling rate of $-1^{o}C.min^{-1}$ to -40 ^{o}C, held at this temperature for 15 min and then plunged into liquid nitrogen. Samples were transferred to the local cryostorage facility and three samples of each cryopreserved strain were thawed for viability analysis. Where this conventional approach proved unsuccessful a series of empirical studies were performed to optimize the applicability of the approach to the broader holdings of CCMA-UFSCar (Figure 1).

5. Toxicity of the CPA solutions

Simultaneously to testing the standard freezing protocols, the toxicity of DMSO 5% (final concentration during cryopreservation) was assessed for several strains of microalgae. This test consisted in the mixture equal parts of CPA solution (10%) and microalgae culture, which were incubated for 15 min at room temperature. Aliquots (1 mL) of the samples were then diluted in 10 mL of fresh medium, and maintained to the standard culturing/ incubation conditions of the bank: 100 $\mu mol\ m^{-2}\ s^{-1}$ (photosynthetically active radiation), with a 12:12h dark:light cycle and at a temperature of $23 \pm 1^{o}C$ for growth. The viability of these samples was determined analyzing culture growth after 2-3 weeks of incubation under the above regime, using Chl-a fluorescence (see 8).

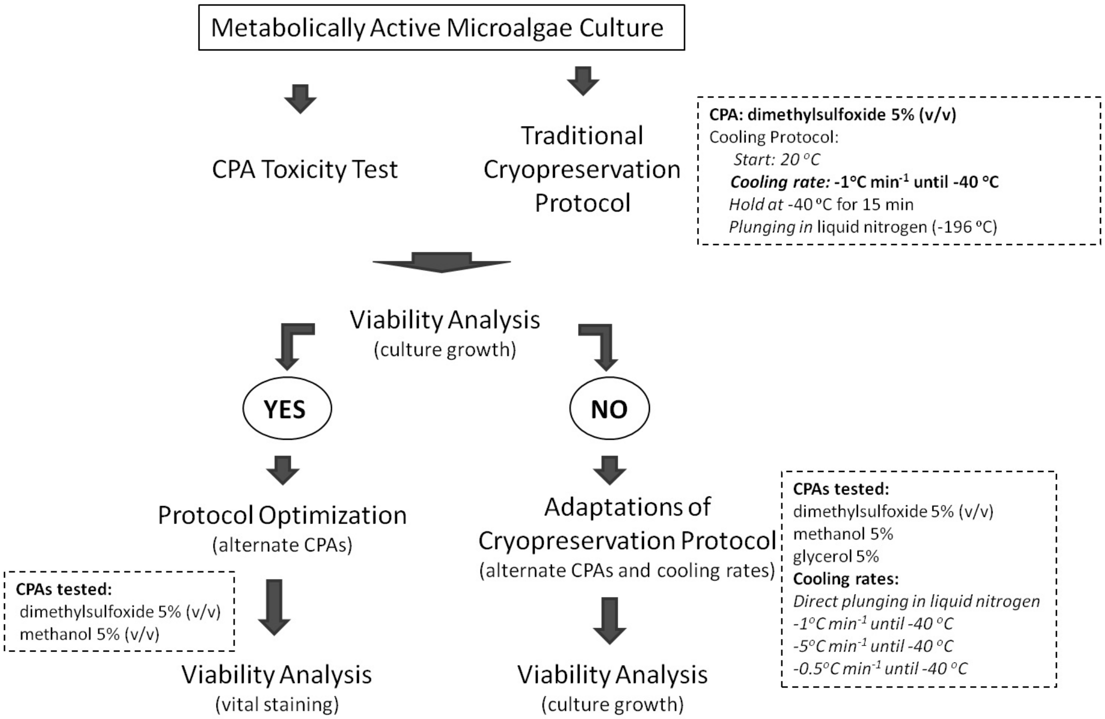

Figure 1. Flowchart of empirical development of effective cryopreservation protocols.

6. Alternate freezing protocols: analyzing the success rates of dimethylsulfoxide (DMSO) and methanol (MeOH) for the cryoprotection of axenic and non-axenic samples.

The possibility of employing MeOH as an alternative to DMSO for the cryopreservation of 18 strains of microalgae was verified. In this test, the cooling protocol previously described was employed, exchanging the CPA solution from DMSO 10% for a methanol 10% solution, prepared under the same conditions. For this study, viability measurements were performed using vital staining, with erythrosine-b (see 8).

7. Cryopreservation of Chlamydomonas chlorastera: assessment of different cooling rates and cryoprotectant agents.

The initial attempt to cryopreserve *Chlamydomonas chlorastera* (CCMA-UFSCar 009) with the previously described two-step protocol using DMSO 5% as CPA was unsuccessful, with no recovery of viable algae from the samples. Thus, several adaptations on the cooling rates and CPA employed for cell protection during the process were tested in order to obtain the most successful protocol for the maintenance of this species in liquid nitrogen for long term storage. Combinations of three different CPAs (methanol, DMSO and Glycerol) and four different cooling rates were tested for cryopreservation of a *C. chlorastera* culture while in exponential growth phase, generating 20 different freezing treatments. The toxicity of the CPA in the samples was also assessed by the exposure of samples to higher concentrations of these compounds, with subsequent dilution to "innocuous" concentrations. The viability of samples was assessed using the absorbance of samples after recovery and monitoring of subsequent growth.

8. Thawing of samples and viability analysis

The stored cryovials were transferred from the cryostore to a small Dewar containing liquid nitrogen. These were thawed in a pre-heated water bath (40^{o}C) until all visible ice had melted (Day & Stacey 2007). They were then rapidly transferred to a laminar flow cabinet, the outside of the cryovial wiped with 70% (v/v) ethanol, to reduce the risk of contamination by adhering bacteria, caps aseptically removed and using a sterile pipette, the contents (0.5 ml) from each of the three thawed replicate samples were transferred into culture tubes with 10 mL of WC medium to dilute the potentially toxic characteristics of the cryoprotectant. Cultures were maintained for 24-36h in the dark and then incubated under the standard culturing conditions of the bank: 100 µmol m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark:light cycle and at a temperature of 23 ± 1^{o}C. Assessment of viability was performed after 2- 3 weeks of incubation under the above cultivation regime, with analysis of the Chl-a fluorescence using a Trilogy Laboratory Fluoremeter (Turner Designs, USA) equipped with a chlorophyll *in vivo* Module (7200-043).

Alternatively, for the comparison of the efficacy of using DSMO or MeOH as CPA, the viability of samples was assessed using microscopy analysis of samples stained with erythrosine-b (SIGMA). This stain can only permeate dead cells, which will acquire the red color, while the living (viable) cells maintain their natural green color. The viability of samples before cryopreservation was assessed for control and standardization of results. After cryopreservation, 100 µL of the culture were separated in a 200 µL PCR tube, to which 30 µL of a saturated erythrosine solution was added and mixed. After 15 min, samples were analyzed by optical microscopy and the number of cells stained (red) and not stained (green) annotated, (a minimum of 50 cells were counted on each slide). The viability of the samples was calculated dividing the number of living cells (green) by the total amount of cells counted (red+green), multiplying the results by 100 to obtain the percentage. In order to avoid the sub estimation of viability due to the presence of dead cells in the original cultures (prior to freezing), the viability of these samples was also calculated and considered for the final viability results. When applicable, comparisons between treatments with the different CPAs were performed using t-tests to determine the species in which significant differences of viability were observed.

Results

1. Traditional cryopreservation protocol

The applicability of the conventional two-step protocol tested in this project generated a range of results, considering the wide range of groups in the 93 strains analyzed. The distribution of these results has been summarized in Figure 2. The group with the highest success rates i.e. highest levels of recovery after freezing was the Chlorophyceae, with 58 strains successfully cryopreserved (92%). The application of the standard method to the Conjugatophyceae and diatoms was the least successful, with success in 9% and 22% of the strains tested, respectively.

For the Chlorophyceae, the family Selenastraceae was the most intensively studied group, with 44 strains (Table 1). Most species of this group successfully recovered after cryopreservation; however, a few exceptions were observed in strains of *Selenastrum bibraianum* and two strains of *Kirchneriella*. Similar results were observed for the family Scenedesmaceae, where comparably high viability levels after application of the standard cryopreservation protocol were observed, with all the samples tested able to regenerate robust cultures on transfer to fresh medium (Table 2).

Table 1. Viability of samples from the family Selenastraceae (Chlorophyceae) after recovery from cryopreservation protocol.

Accession number		Strain	CPA	r ABS (%)	
CCMA-UFSCar	003	*Ankistrodesmus densus*	DMSO 5%	81	✓
CCMA-UFSCar	005	*Messastrum gracile*	DMSO 5%	89	✓
CCMA-UFSCar	024	*Monoraphidium arcuatum*	DMSO 5%	95	✓
CCMA-UFSCar	047	*Selenastrum bibraianum*	DMSO 5%	52	✓
CCMA-UFSCar	048	*Raphidocelis subcapitata*	DMSO 5%	47	✓
CCMA-UFSCar	063	*Selenastrum bibraianum*	DMSO 5%	7	✗
CCMA-UFSCar	083	*Ankistrodesmus flexuosus*	DMSO 5%	73	✓
CCMA-UFSCar	123	*Kirchneriella aperta*	DMSO 5%	33	✓
CCMA-UFSCar	125	*Selenastrum bibraianum*	DMSO 5%	55	✓
CCMA-UFSCar	128	*Ankistrodesmus densus*	DMSO 5%	100	✓
CCMA-UFSCar	137	*Chlorolobion cf. braunii*	DMSO 5%	89	✓
CCMA-UFSCar	168	*Selenastrum bibraianum*	DMSO 5%	76	✓
CCMA-UFSCar	174	*Kirchneriella irregularis var. spiralis*	DMSO 5%	85	✓
CCMA-UFSCar	176	*Monoraphidium griffithii*	DMSO 5%	100	✓
CCMA-UFSCar	230	*Kirchneriella irregularis*	DMSO 5%	84	✓
CCMA-UFSCar	234	*Kirchneriella irregularis var. spiralis*	DMSO 5%	100	✓
CCMA-UFSCar	239	*Ankistrodesmus densus*	DMSO 5%	100	✓
CCMA-UFSCar	241	*Selenastrum bibraianum*	DMSO 5%	62	✓
CCMA-UFSCar	278	*Ankistrodesmus stipitatus*	DMSO 5%	95	✓
CCMA-UFSCar	306	*Monoraphidium contortum*	DMSO 5%	100	✓
CCMA-UFSCar	325	*Monoraphidium pseudobraunii*	DMSO 5%	61	✓
CCMA-UFSCar	333	*Monoraphidium kormakovae*	DMSO 5%	100	✓
CCMA-UFSCar	345	*Kirchneriella obesa*	DMSO 5%	75	✓
CCMA-UFSCar	346	*Kirchneriella pseudoaperta*	DMSO 5%	63	✓
CCMA-UFSCar	348	*Kirchneriella irregularis*	DMSO 5%	94	✓
CCMA-UFSCar	349	*Monoraphidium contortum*	DMSO 5%	100	✓
CCMA-UFSCar	350	*Curvastrum pantanale*	DMSO 5%	92	✓
CCMA-UFSCar	353	*Monoraphidium kormakovae*	DMSO 5%	90	✓
CCMA-UFSCar	423	*Ankistrodesmus bernardii*	DMSO 5%	82	✓
CCMA-UFSCar	447	*Kirchneriella contorta var. elegans*	DMSO 5%	74	✓
CCMA-UFSCar	455	*Chlorolobion braunii*	DMSO 5%	100	✓
CCMA-UFSCar	462	*Chlorolobion braunii*	DMSO 5%	92	✓
CCMA-UFSCar	470	*Messastrum gracile*	DMSO 5%	94	✓
CCMA-UFSCar	476	*Chlorolobion braunii*	DMSO 5%	100	✓
CCMA-UFSCar	478	*Raphidocelis subcaptata*	DMSO 5%	89	✓
CCMA-UFSCar	492	*Pseudokirchneriella elongata*	DMSO 5%	100	✓
CCMA-UFSCar	498	*Pseudokirchneriella elongata*	DMSO 5%	81	✓
CCMA-UFSCar	516	*Kirchneriella obesa*	DMSO 5%	93	✓
CCMA-UFSCar	549	*Monoraphidium indicum*	DMSO 5%	100	✓
CCMA-UFSCar	604	*Chlorolobion lunatum*	DMSO 5%	100	✓
CCMA-UFSCar	606	*Monoraphidium irregularis*	DMSO 5%	79	✓
CCMA-UFSCar	609	*Desmodesmus comunis*	DMSO 5%	96	✓
CCMA-UFSCar	611	*Ankistrodesmus fusiformis*	DMSO 5%	93	✓
CCMA-UFSCar	622	*Messastrum gracile*	DMSO 5%	100	✓

(The analysis was based on the growth rate calculated using the optical density of samples at two different points in time, compared with the control cultures (non-cryopreserved). The symbols and colors indicate the relative success of growth: green – good growth, yellow – medium success and red – poor growth.)

Table 2. Viability of samples from the family Scenedesmaceae (Chlorophyceae) after recovery from cryopreservation protocol.

Accession number		Strain	CPA	r ABS (%)	
CCMA-UFSCar	**029**	*Scenedesmus bijugus*	DMSO 5%	80	✓
CCMA-UFSCar	**030**	*Desmodesmus comunis*	DMSO 5%	86	✓
CCMA-UFSCar	**046**	*Desmodesmus spinosus*	DMSO 5%	75	✓
CCMA-UFSCar	**060**	*Coelastrum sphaericum*	DMSO 5%	78	✓
CCMA-UFSCar	**062**	*Desmodesmus spinosus*	DMSO 5%	88	✓
CCMA-UFSCar	**088**	*Scenedesmus ecornis*	DMSO 5%	82	✓
CCMA-UFSCar	**244**	*Verrucodesmus verrucosus*	DMSO 5%	79	✓
CCMA-UFSCar	**326**	*Hariotina reticulata*	DMSO 5%	100	✓
CCMA-UFSCar	**493**	*Hariotina reticulata*	DMSO 5%	81	✓
CCMA-UFSCar	**609**	*Desmodesmus comunis*	DMSO 5%	96	✓

(The analysis was based on the growth rate calculated using the optical density of samples at two different points in time, compared with the control cultures (non-cryopreserved). The symbols indicate the relative success of growth: ✓ – good growth, ✗ – poor growth.)

The bigger and more complex organisms had lower rates of survival, with only one of the desmid strains (Conjugatophyceae) tested demonstrating successful regrowth after the cryopreservation protocol was completed: *Spondylosium pygmaeum* (CCMA-UFSCar 014). The remaining 10 strains of this group tested had no detectable viability, i.e. they were not able to form new cultures (Figure 2). The experiments with diatoms also had limited success, with only 2 of the 9 strains tested able to regrow after undergoing the cryopreservation protocol (Figure 2).

2. Toxicity and cryopreservation effects of CPA on axenic strains

The potential toxicity of DMSO at 5% as a CPA was not a determinant factor for the cryopreservation of most microalgae strains tested. Measurements of Chl-a fluorescence of samples after 2-3 weeks of growth showed similar patterns for samples with and without treatment with DMSO for 15 min, before dilution in fresh medium (Figure 3).

Cryopreservation using DMSO or MeOH as CPA had similar results for the majority of the strains tested (15 strains) (Figure 4A). However, for *K. obesa*, *K. lunaris* and *P. duplex,* cryopreservation with methanol resulted in slightly better levels of success, although materials subjected to both treatments were able to form new robust cultures after 2 weeks growth.

3. Post-thaw viability: non-axenic cultures

Post-thaw viability levels observed for non-axenic cultures were, in general, high and similar to the responses reported for axenic cultures of the same strain frozen employing the same protocol (Figure 4A). Comparisons for the viability of non-axenic strains frozen with MeOH and DMSO as CPA are detailed in Figure 4B. It was noted that for a few strains, mostly of *Kirchneriella* spp., significantly ($p<0.05$) higher viability was observed with samples treated with MeOH for cryopreservation.

4. Cryopreservation of Chlamydomonas chlorastera

For the study on cryopreservation of *Chlamydomonas chlorastera* a total of 20 different combinations of CPA and cooling rates were tested for cryopreservation of an actively growing culture (vegetative cells). The objective was to maximize viability levels, as demonstrated by the ability to re-grow robust cultures that were comparable to control cultures (i.e. not frozen sample(s)). MeOH was found to be an effective CPA, capable of protecting the cells from the freezing damage (Table 3). However, the use of DMSO or Glycerol as CPAs was not effective for the protection of cultures during the process and no re-establishment of cultures was observed for these samples. It was noted that, cultures that were exposed to the CPA for a short period of time before dilution and subsequent incubation under standard conditions showed no inhibitory effects that could be attributed to these compounds at the concentrations tested. This led to the conclusion

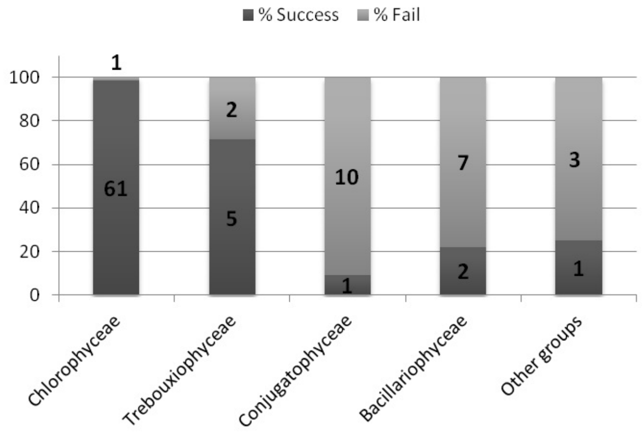

Figure 2. Success and failure percentages for the cryopreservation protocol applied for different groups of freshwater microalgae. The numbers inside the bars are the number of strains tested in each taxonomic group.

Table 3 - Viability of samples *Chlamydomonas chlorastera* (CCMA-UFSCar 009) after recovery from different cryopreservation protocols, using DMSO, MeOH or Glycerol as CPA and frozen at three different cooling rates.

Treatment	CPA	Cooling rate	
T01	no	not frozen	✓
T02	no	LN	✗
T03	no	-1 ^{o}C min^{-1}	✗
T04	no	-5 ^{o}C min^{-1}	✗
T05	no	-0,5 ^{o}C min^{-1}	✗
T06	DMSO 5%	not frozen	✓
T07	DMSO 5%	LN	✗
T08	DMSO 5%	-1 ^{o}C min^{-1}	✗
T09	DMSO 5%	-5 ^{o}C min^{-1}	✗
T10	DMSO 5%	-0,5 ^{o}C min^{-1}	✗
T11	MeOH 5%	not frozen	✓
T12	MeOH 5%	LN	✗
T13	MeOH 5%	-1 ^{o}C min^{-1}	✓
T14	MeOH 5%	-5 ^{o}C min^{-1}	✓
T15	MeOH 5%	-0,5 ^{o}C min^{-1}	✓
T16	Glycerol 5%	not frozen	✓
T17	Glycerol 5%	LN	✗
T18	Glycerol 5%	-1 ^{o}C min^{-1}	✗
T19	Glycerol 5%	-5 ^{o}C min^{-1}	✗
T20	Glycerol 5%	-0,5 ^{o}C min^{-1}	✗

(The symbols indicate the relative success of growth after visual analysis: ✓ – good growth and ✗ – no growth.)

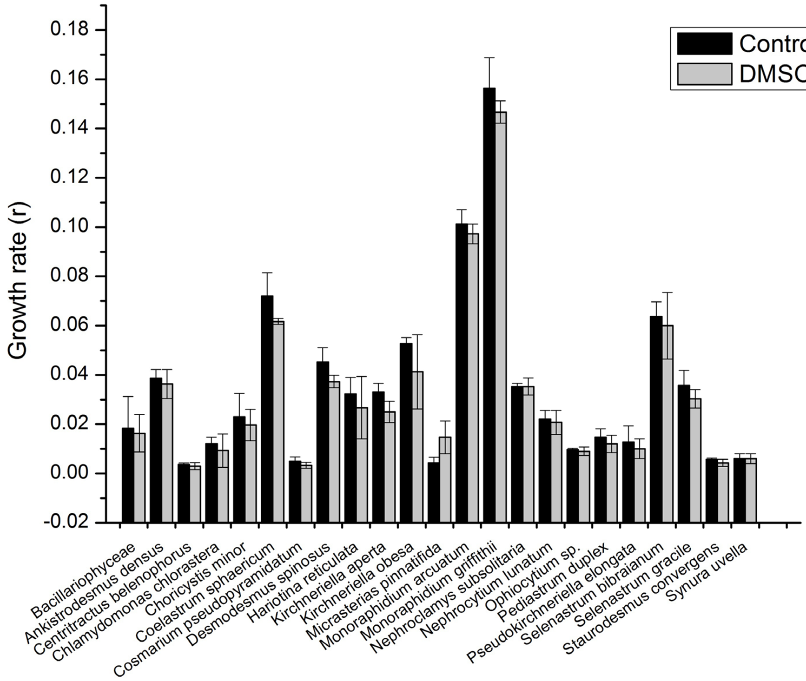

Figure 3. Comparison of growth (Chl-a fluorescence) between samples untreated (Control) and treated (DMSO 5%) with CPA and incubated for 15 min before dilution in fresh medium. The cultures were grown for 2-3 weeks.

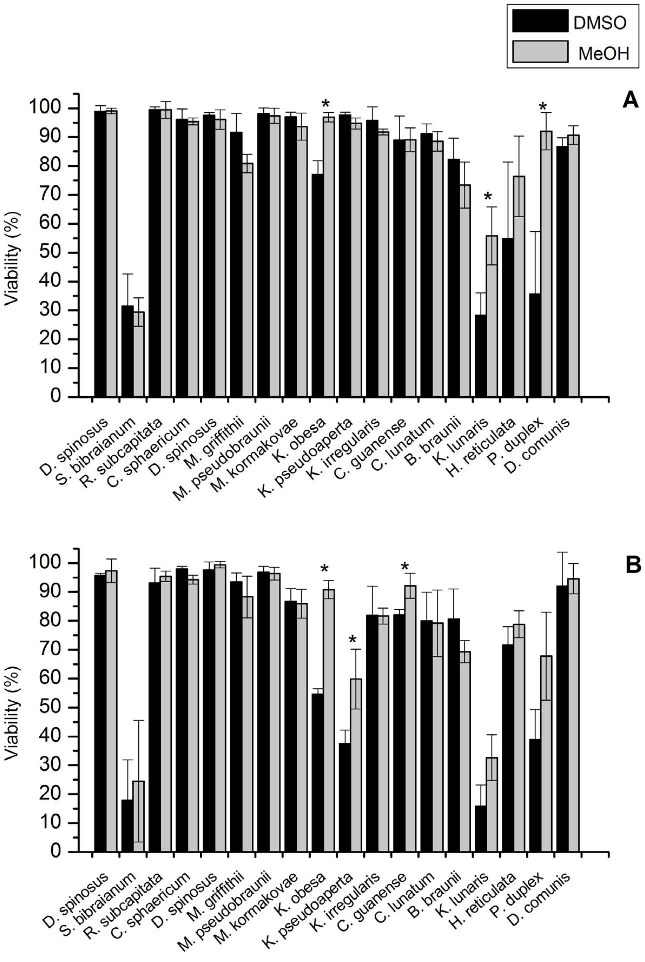

Figure 4. Viability (%) of samples cryopreserved using different CPAs (DMSO and MeOH) for protection of the cultures. A – axenic cultures and B – non-axenic cultures. * = statistically significant differences in recovery after cryopreservation with MeOH or DMSO ($p<0.05$).

that the damage happened during the freezing/thawing of cultures and that these compounds had little or no colligative cryoprotective effect for this alga under the cooling regime employed.

Discussion

1. CPA: toxicity & protective effect

The cryopreservation process results in a range of complex physical, biophysical and chemical changes in both the cells being cryopreserved and their immediate environment, thus careful consideration is required in order to achieve the highest post-thaw viability for samples (Day & Fleck 2015). One critical point for the success of the process is the addition of a cryoprotective additive (CPA), which is almost always necessary for organisms to survive the lethal consequences of the freezing/thawing process (Doebbler 1966). Penetrative CPAs, such as MeOH and DMSO can rapidly permeate cell membranes, which in addition to their colligative capabilities of keeping salts in solution, affect the ice crystal nucleation by forming strong hydrogen bonds with water molecules (Weng et al. 2011; Yu & Quinn 1994). However, altering the solute composition and concentration might have extremely toxic effects, inhibiting the growth of cultures (Hubalek 2003; Yu & Quinn 1994).

The basic two-step cryopreservation protocol tested for the CCMA-UFSCar microalgae required the exposure of cultures for a minimum 15 min to DMSO 5% concentrations. As observed for the growth of cultures treated with CPAs (CPA control cultures), for most samples no inhibitory effects were observed from this step and cultures maintained their culture formation ability. Indeed, this approach adheres to this recommended use of DMSO concentrations lower than 15% to avoid the effects on microalgae cultures, and to carefully consider the temperature of reaction and time of exposure (Hubalek 2003).

2. Cryopreservation of Chlorophyceae

Over 50% of the CCMA-UFSCar biobank holdings are composed of strains belonging to the Phylum Chlorophyta, of which almost 80% are Chlorophyceae strains, which can be further divided into divided in 20 families. These organisms are the focus of the most recent publications from the research group (Garcia et al. 2017; de Moraes & Vieira 2014; Vieira et al. 2016), more specifically taxa belonging to the order Sphaeropleales and its families Selenastraceae and Scenedesmaceae. These groups are composed mostly of small organisms, which can be unicellular, or in many cases form small colonies, and thus present a wide range of phenotypic forms (Garcia et al. 2017). Successful cryopreservation of organisms from these groups was verified employing the classic cryopreservation protocol in this study.

Organisms with reduced size and morphological simplicity are generally expected to be the most tolerant to the cryopreservation process (Day & Brand 2005). The success of the freezing/thawing process is entirely dependent on the equilibrium of water loss by cells, during the cooling process to avoid the formation of intracellular ice, while maintain the necessary conditions for the survival of cells (Day & Fleck 2015; Mazur 1984). The morphological simplicity of these organisms, coupled with their cell wall characteristics help to maintain viability during the process. This was reflected by the high viability levels observed after the freezing, thawing and recovery cycle.

However it was observed that some strains of *Selenastrum bibraianum* had more limited levels of recovery (Table 1) from the process. This demonstrated that even organisms very closely related may have specific requirements to ensure the complete success of the protocol (Brand et al. 2013). This was also the case observed for some strains of *Kirchneriella,* specifically *K. aperta* and *K. pseudoaperta*, where somewhat limited success was observed on application of the standard two-step cryopreservation protocol using DMSO as CPA. *Kirchneriella pseudoaperta* (CCMA-UFSCar 346) when further tested demonstrated that the ability to form new cultures was maintained; however, the *lag* phase of the growth curve was a few days longer (data not shown). Furthermore, a change in the protocol, i.e. employing MeOH as CPA during the two-step cryopreservation protocol, resulted in slightly better recovery of viability (Figure 4). Once again demonstrating the specific requirements which must be met for each culture to be cryopreserved. However, for most strains tested, no significant ($p>0.05$) differences were found in the protection provided by the CPAs tested (Figure 3), which can make them interchangeable for these organisms, giving priority to other aspects to be considered for the long term maintenance of frozen microalgae.

The post-thaw viability levels obtained for the non-axenic strains tested were generally high (Figure 4B) and directly comparable to their axenic counterparts. For *Kirchneriella obesa*, *K. pseudoaperta* and *C. guanense,* viability was higher for samples cryopreserved using MeOH as CPA ($p<0.05$), rather than DMSO. The process of cryopreservation of non-axenic strains, beyond the viability of samples assessed immediately after the freezing/thawing cycle, must consider the ability of samples to recover and overcome competition with co-culture organisms. The propagation of the heterotrophic associated community, thus, may lead to inhibition of microalgae growth (Day & Harding 2008). The choice of CPA, although with limited effect on the viability of samples post-thaw, had direct effect on the recovery of robust cultures of microalgae of non-axenic strains. The utilization of MeOH as cryoprotectant during this process might be problematic, as although it results in higher viability rates as demonstrated for some strains of *Kirchneriella* (Figure 4B), it also favors the proliferation of bacteria, verified by the opacity developed in the medium (data not shown), which could inhibit the development of the microalgal culture (Amaral et al. 2013).

3. Cryopreservation of desmids (Conjugatophyceae) and diatoms (Bacillariophyceae)

The organisms belonging to the Classes Conjugatophyceae (desmids) and Bacillariophyceae (diatoms) were some of the most recalcitrant groups for the protocols tested, with over 75% of the strains showing no post-thaw viability of samples. These groups have already been described as problematic for the maintenance in liquid nitrogen, with very few positive results for post-thaw viability (Day 2007). Algae with larger cell sizes and most filamentous algae still cannot be cryopreserved yet, and the higher rates of success were found for smaller, morphologically non-complex algae (Day & Brand 2005).

Successful cryopreservation protocols are known to reduce osmotic stress, cold shock and potential damage by ice formation, highlighting the importance of the cooling regime (Day et al. 2000) and must be usually empirically determined for the more sensitive strains (Taylor & Fletcher 1998). In a study with two strains of *Micrasterias,* Morris et al. (1986) reported that the cell walls did not contract to accompany the shrinkage of the protoplast, which led to the plasmolysis. This was not observed in similar studies with *Cylindrocystis brebissonii*, emphasizing the importance of cell volume and surface area, cell wall composition, as well as interactions between the wall and cell membrane, and their influence on the cryopreservation process (Morris et al. 1986).

4. Cryopreservation of Chlamydomonas chlorastera: a protocol study

The initial negative results for cryopreservation of *Chlamydomonas chlorastera* (CCMA-UFSCar 009) using DMSO as CPA in a two-step protocol indicated that further research was necessary for the successful long term maintenance of this strain. Thus, tests were performed using

MeOH 5% and Glycerol 5%, as well as different cooling rates in order to obtain the highest viability results for these organisms. Although the cooling rates tested were not relevant for this algae, with the exception of the direct plunging of samples in liquid nitrogen, which was lethal independently of CPA, differences were found between CPAs, with MeOH being the only effective compound for the cyoprotection of this organism. Similar results were previously reported for tests with *Chlamydomonas reinhardtii* (Crutchfield et al. 1999; Scarbrough & Wirschell 2016; Yang & Li 2016).

Neither of the CPAs tested had direct inhibitory effects on the growth of *C. chlorastera*, which leads to the indication of lethal damaging occurring at some point during the freezing/thawing of samples. Indeed, Yang and Li (2016) described, in tests with *C. reinhardtii*, that methanol was effective in attenuating the degradation of membrane lipids during the process, results that were not replicated with DMSO. As the complexity of the organisms to be maintained in liquid nitrogen storage increases, specific tests of protocol are necessary to reach the most adequate protocol, to avoid cryo-injury and other damaging effects of this stressful process for cultures.

5. Consequences of a cryopreserved biobank: perspectives for CCMA-UFSCar

The necessity for the establishment of a cryopreserved biobank is linked to several practical considerations including: the reduction of costs associated with routine transfers and the space necessary for the maintenance of cultures, the reduced risks of contamination and the maintenance of genetic integrity (Brand et al. 2013). Thus, it is recommended that culture collections/ Biological Resource Centers should maintain most of their holdings in cryopreserved stocks (OECD 2007), to avoid the occurrence of genetic mutations, which will directly alter the characteristics of the cultures (Brand et al. 2013; Grout 1995). This is specifically true for strains of high commercial or scientific value, as only cryopreservation has the potential to guarantee their long term stability, including their potential to produce biotechnologically interesting products (Day 2004; Hipkin et al. 2014). CCMA-UFSCar microalgae are distributed as the starting point for research projects throughout Brazil, and much data can be found on the importance and applicability of the maintained strains. Thus, the guaranteed stability offered by cryopreservation can provide insurance for the continuity of academic activities and possible applications of microalgae-derived compounds. Furthermore, as the collection is part of a laboratory in a Federal Institution, and not individually institutionalized, the maintenance personnel are mostly undergraduate and post-graduate students, who have to work in tandem with the development of their own research projects. Thus, the reduction of the cost and effort necessary for the constant sub-culturing is essential for the possibility of expansion of the culture holdings which was a result of the FAPESP Project efforts on the study of Brazilian biodiversity. Also, for the maintenance of cryopreserved stocks in liquid nitrogen dewars, the space required is reduced, another positive point in a university laboratory.

The positive results obtained for the initial pilot study on establishment of a cryopreserved biobank for CCMA-UFSCar illustrated the feasibility of introducing these practices for microalgae culture collections in Brazil, successfully conserving the largest part of the collection stocks, which as currently small unicellular organisms. However, it must be noted that some strains of microalgae are apparently truly cryopreservation-recalcitrant due to the nature of their susceptibility to injury (Day 2004). Also, some organisms require the dedicated studies in order to obtain the highest viability rates, due to specificities in their biochemical composition and physical cell characteristics. Therefore the success observed using the protocols outlined in this study, whilst important, shows that a conventional cryopreservation protocol cannot be applied to the full range of algal taxa held in the collection and further developmental work is needed to maximize the applicability of this approach to algal curation at CCMA-UFSCar.

Acknowledgements

We thank the São Paulo Research Foundation (FAPESP) for financial support and scholarships. We are also grateful to the reviewers for comments which helped to improve the manuscript.

Author Contributions

Armando Augusto Henriques Vieira: substantial contribution in the concept and design of the study; contribution to manuscript preparation and critical revision.

John Godfrey Day: substantial contribution in the concept and design of the study; contribution to data analysis and interpretation; contribution to manuscript preparation and critical revision.

Leticia Piton Tessarolli: substantial contribution in the concept and design of the study; contribution to data collection; contribution to data analysis and interpretation; contribution to manuscript preparation and critical revision.

Conflicts of interest

The authors declares that they have no conflict of interest related to the publication of this manuscript.

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Received: 11/11/2016
Revised: 14/02/2017
Accepted: 05/03/2017

Biota Neotropica 17(2): e20160274, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
Inventory

Composition and diversity of mosquitoes (Diptera: Culicidae) in urban parks in the South region of the city of São Paulo, Brazil

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Carvalho, G. C., Ceretti Junior, W., Barrio Nuevo, K. M., Wilk-da-Silva, R., Christe, R. O., Paula, M. B., Vendrami, D. P., Multini, L. C., Evangelista, E., Camargo, A. A., Souza, L. F., Wilke, A. B., Medeiros-Sousa, A. R., Marrelli, M. T. **Composition and diversity of mosquitoes (Diptera: Culicidae) in urban parks in the South region of the city of São Paulo, Brazil. Biota Neotropica.** 17(2): e20160274. http://dx.doi.org/10.1590/1676-0611-BN-2016-0274

Abstract: Many parks in the city of São Paulo contain remnants of Atlantic Forest. Of the 30 municipal parks in the South of the city, we investigated two in this study (Santo Dias Park and Shangrilá Park) in order to survey their mosquito fauna and investigate the presence of potential bioindicators of environmental conditions and vectors of human pathogens. Mosquitoes were collected monthly between March 2011 and February 2012 using aspirators, Shannon and CDC traps for adult mosquitoes and larval dippers and suction samplers for immature forms. Sampling effort was evaluated by plotting a species accumulation curve, and total richness was estimated using the first-order jackknife. To compare the diversity between the two parks Shannon and Simpson diversity indexes were calculated. Species similarity was compared by the Sorensen similarity index. In all, 8,850 specimens were sampled in both parks. Collections in Santo Dias Park yielded 1,577 adult mosquitoes and 658 immature individuals distributed in seven genera (*Aedes, Anopheles, Culex*, *Limatus*, *Mansonia*, *Toxorhynchites* and *Wyeomyia*) and 27 taxonomic units. Among the adult mosquitoes collected, *Culex nigripalpus* .and *Aedes fluviatilis* were the most abundant, while the most abundant immature forms were *Cx. imitator*, *Wy. davisi*, *Wy. galvaoi* and *Ae. albopictus.* Collections in Shangrilá Park yielded 4,952 adult specimens and 1,663 immature forms distributed in eight genera (*Aedes*, *Anopheles*, *Culex*, *Limatus*, *Mansonia*, *Toxorhynchites*, *Uranotaenia* and *Wyeomyia*) and 36 taxonomic units. Species accumulation curves in both parks were close to the asymptote, and the total richness estimate was close to the observed richness. Although the observed species richness was higher in the Shangrilá Park, there was no statistically significant difference between the diversity indexes measured. Regarding species composition, the two sites shared 16 species, including those of epidemiological importance such as *Culex nigripalpus*, *Cx. quinquefasciatus*, *Aedes albopictus* and *Ae. aegypti*. As some of the mosquito taxa found are bioindicators of environmental conditions and have epidemiological potential to carry pathogens, we recommend that urban parks should be included in official mosquito surveillance programs, and regular surveys carried out to detect circulating arboviruses.

Keywords: *Diversity, mosquitoes, composition, urban parks*

Composição e diversidade de mosquitos (Diptera: Culicidae) em parques urbanos localizados na região Sul do município de São Paulo, Brasil

Resumo: Parques urbanos do município de São Paulo contêm remanescentes de Mata Atlântica. No sul da cidade há 30 parques municipais, sendo os parques Santo Dias e Shangrila alvos deste estudo. Este estudo teve a proposta de levantamento da fauna de culicídeos desses dois parques no sul da cidade de São Paulo e avaliar a presença de potenciais bioindicadores e espécies vetoras de patógenos aos seres humanos. Os mosquitos foram coletados mensalmente entre março de 2011 e fevereiro de 2012, com aspiradores, armadilhas de Shannon e CDCs para mosquitos adultos e conchas entomológicas e bombas manuais de sucção para os imaturos. O esforço amostral foi avaliado por traçar uma curva de acumulação de espécies, e a riqueza total foi estimada pelo método jackknife de primeira ordem. Para comparar a diversidade entre os dois parques, foram calculados os índices de diversidade de Shannon e de Simpson. A similaridade na composição de espécies foi comparada pelo índice de similaridade de Sorensen. Foram coletados um total de

8.850 espécimes de culicídeos em ambos os parques. Coletas no parque Santo Dias renderam 1.577 mosquitos adultos e 658 imaturos, distribuídos em sete gêneros (*Aedes, Anopheles, Culex, Limatus, Mansonia, Toxorhynchites* e *Wyeomyia*) e 27 unidades taxonômicas: *Culex nigripalpus*, e *Aedes fluviatilis* foram as mais abundantes unidades taxonômicas coletadas como adultos, enquanto em formas imaturas, as espécies mais abundantes coletadas foram *Cx. imitator*, *Wy. davisi, Wy. galvaoi* e *Ae. albopictus*. Coletas no parque Shangrilá renderam 4.952 espécimes como adultos e 1.663 formas imaturas, distribuídas em oito gêneros (*Aedes*, *Anopheles*, *Culex*, *Limatus*, *Mansonia*, *Toxorhynchites*, *Uranotaenia* e *Wyeomyia*) e 36 unidades taxonômicas.. As curvas de acúmulo de espécies em ambos os parques ficaram perto da assíntota, e as estimativas de riqueza total foram próximas às riquezas observadas. Apesar da riqueza observada ter sido maior no parque Shangrilá, não houve diferença estatisticamente significante entre os índices de diversidade mensurados. Em relação à composição de espécies os dois locais compartilharam 16 espécies, incluindo as de maior importância epidemiológica como *Culex nigripalpus, Cx. quinquefasciatus, Aedes albopictus* e *Ae. aegypti*. Alguns táxons de culicídeos são bioindicadores de condições ambientais nas áreas ou possuem potencial para veicular patógenos. Atenção deve ser dada a parques urbanos, com inclusão destes locais nos programas oficiais de vigilância entomológica e investigações periódicas na circulação de arbovírus.

Palavras-chave: *Diversidade, mosquitos, composição, parques urbanos*

Introduction

São Paulo, in southeast Brazil, is the largest city in South America and one of the largest megalopolises in the world. It is characterized by an extensive urban sprawl and has a population of around 12 million people (IBGE 2016). The municipal parks in the city contain remnants of Atlantic Forest (including lakes and springs), which provide shelter for mammals, birds and arthropod vectors (Medeiros-Sousa et al. 2013), and are intended to meet the human population's need for pleasant leisure spaces and compensate for the massiveness of urban structures (Kliass 1993).

According to the 2014 São Paulo Municipal Guide, there are 30 municipal parks in the south of the city. Two of these, Santo Dias Park and Shangrilá Park, were selected for this study. Both provide refuge for several species of mosquito and suitable conditions for maintenance of their populations (Medeiros-Sousa et al. 2013, 2015, Ceretti-Junior 2016), allowing human-mosquito contact and increasing the risk of the emergence of infectious diseases, especially those caused by arboviruses (Fernandes et al. 2016).

Currently, there are about 3,552 valid species of mosquitoes described worldwide, some of which are of public health importance as they can act as vectors of pathogens to the human and animal population (Harbach 2016). In Brazil, approximately 500 mosquito species have already been described (Harbach 2016). A descriptive study of blood meal sources in mosquitoes collected in municipal parks in the city of São Paulo showed that several species of vertebrates (birds, dogs, cats, rodents, humans and other primates) are a source of blood for mosquitoes and may sustain transmission chains for pathogens of public health importance (Carvalho et al. 2014).

Because of their epidemiological importance, a knowledge of mosquito species in these locations and assessment of their potential as vectors of pathogens are crucial. In addition, surveys of mosquito species composition in urban parks highlights the need to preserve these spaces in urban areas and identify their biological richness in order to help find potential bioindicator species, which are useful for assessing environmental quality (Montes 2005, Anjos and Navarro-Silva 2008, Medeiros-Sousa et al. 2015). The present study therefore sought to survey the mosquito (Diptera: Culicidae) composition and diversity of two parks in the south region of the city of São Paulo and investigate the presence of potential bioindicators and vector species that can transmit pathogens to humans.

Material and Methods

1. Study area

The study areas are fragments of Atlantic Forest in two urban parks in the outh of the city of São Paulo. Santo Dias Park (23°39'47"S, 46°´21'46"W) and Shangrilá Park (23°76'11"S, 46°66'43"W) were chosen for the study because of their size and the large number of people who visit them every month (Figure 1).

Inaugurated in November 1992, Santos Dias Park extends over 134,000m^2 and is home to a spring as well as remnants of the Atlantic Forest. Lying on a steep slope covered by woods, it has a central clearing and is bordered by houses, a food-manufacturing company, an educational institution (São Paulo Adventist University Center) and a stream (Takahashi et al. 1993). Eighty-four species of vertebrates have been reported in the park, of which seventy-five are birds (Prefeitura de São Paulo 2014).

Shangrilá Park is located near the Billings reservoir and extends over 75,000m^2 in the Bororé-Colônia environmental protection area. The park was created to preserve the city's environmental heritage through the acquisition of green areas, to preserve and enrich the city's biodiversity and to protect the reservoir. Between invertebrates (mosquitoes, butterflies and spiders) and vertebrates (frogs, reptiles, marsupials, primates and 90 species of birds) there are 109 species of fauna. There are various gardens and eucalyptus trees with an understory where tree seedlings have been planted (Prefeitura de São Paulo 2014).

2. Collection methods

Mosquito collections were carried out once a month in each park from March 2011 to February 2012. Four different collection methods were used: (i) Shannon traps, from which specimens were collected for two hours starting at evening twilight by two individuals wearing personal protective equipment; (ii) CDC light traps (baited with 200 g of dry ice) placed 1 m above the ground (two traps) and in the canopy 5 m above the ground (two traps) for three hours; (iii) three 12V battery-powered aspirators used in a standardized 20-minute collection effort (Nasci 1981) and (iv) an active search for immature specimens in breeding sites using a 400 mL larval dipper or suction samplers or by pipetting/emptying containers, depending on where the collection was being carried out. Sampling effort throughout the study period totaled about 240 hours in each park, divided as follows: 12 hours of aspiration, 24 hours of Shannon traps, 144 hours of CDC light traps and 60 hours of active search for immature forms.

3. Identification and data analysis

After the collections, the adult mosquitoes were killed using chloroform and stored in labeled plastic pots containing silica gel. Immature specimens were transported in labeled 200 mL plastic pots containing water from the breeding site. All the sampling material was transported to the Entomology Laboratory at the Faculty of Public Health, University of São Paulo, where the specimens were identified. Immature individuals were kept in the laboratory until they reached the adult stage. Morphological identification

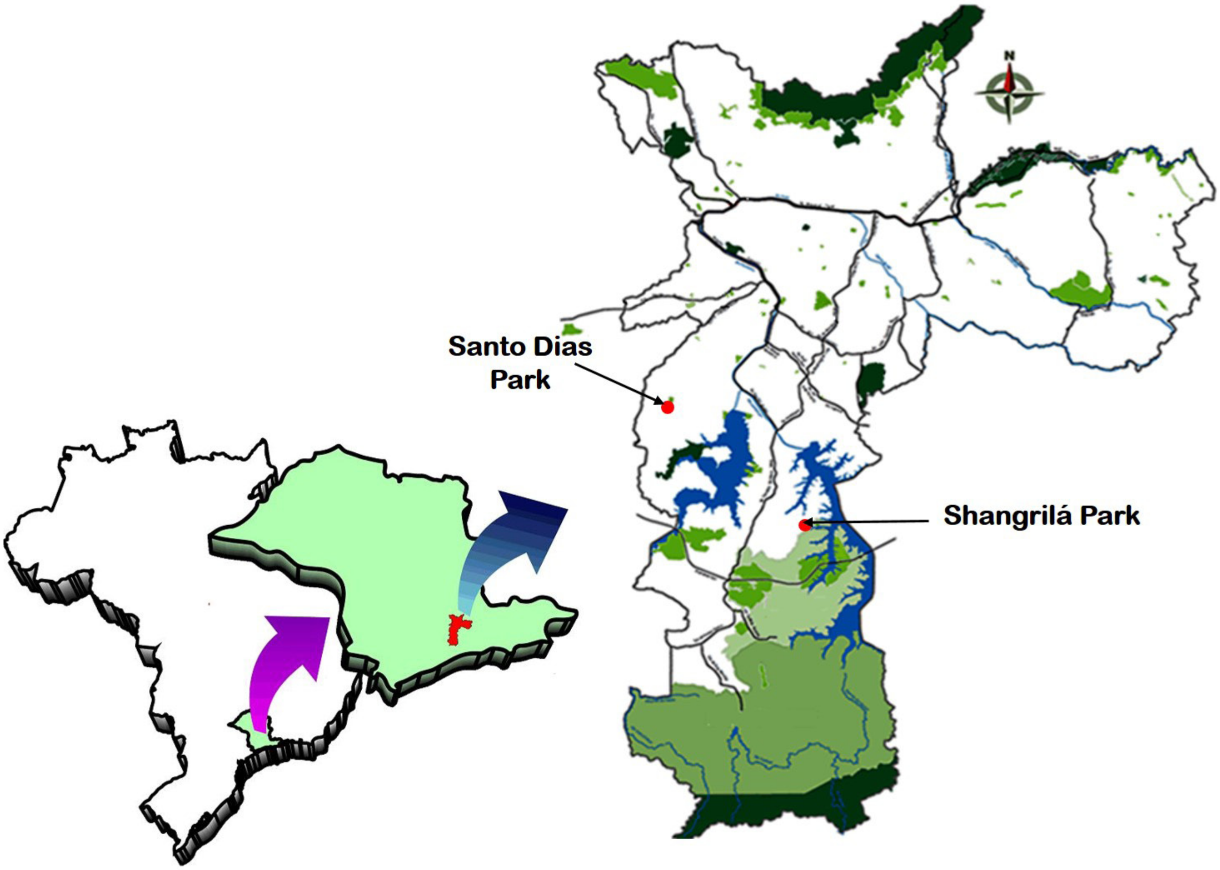

Figure 1. Location of Santo Dias Park and Shangrilá Park in the South of the city of São Paulo, SP, Brazil.

was based on Lane (1953) and Forattini (2002). The abbreviations for genera and subgenera used here follow the standardization proposed by Reinert (2001).

Sampling effort was estimated by plotting a species accumulation curve, and the first-order jackknife method (Burnham and Overton 1979) was used to estimate total richness. In both cases, EstimateS (Colwell et al. 2004) was used with 1,000 randomizations without replacement and a confidence interval of 95%. To compare the diversity between the two parks, the Shannon's (H ') and Simpson's (D) diversity indices were calculated for each monthly sample (N = 12 samples / park). Since the data did not showed evidence of normal distribution deviation and heteroscedasticity, it was applied a T-test to verify if the mean diversity observed for the two areas differ statistically. Species composition similarity was measured by the Sorensen's similarity index.

Results

We collected 8,850 mosquito specimens in both parks (Tables 1 and 2). Collections in Santo Dias Park yielded 1,577 adult mosquitoes and 658 immature forms (a total of 2,235 individuals) distributed in 7 genera (*Aedes* Meigen, *Anopheles* Meigen, *Culex* Linnaeus, *Limatus* Theobald, *Mansonia* Blanchard, *Toxorhynchites* Theobald, *Wyeomyia* Theobald) and 27 taxonomic units. *Culex* (*Cux.*) *nigripalpus* (34.12%), and *Aedes* (*Och.*) *fluviatilis* (14.65%) were the most abundant taxonomic units among the adults, while *Cx.* (*Mcx.*) *imitator imitator* (67.33%), *Wy.* (*Pho*) *davisi* (8.97%), *Wy.* (*Pho.*) *galvaoi* (8%) and *Ae.* (*Stg.*) *albopictus* (7.75%) were the most abundant among the immature forms (Table 1).

Collections in Shangrilá Park yielded 4,952 adult specimens and 1,663 immature forms (a total of 6,615 individuals) distributed in 8 genera (*Aedes*, *Anopheles*, *Culex*, *Limatus*, *Mansonia*, *Toxorhynchites*, *Uranotaenia* Lynch Arribálzaga and *Wyeomyia*) and 36 taxonomic units. The most abundant taxonomic units among the adult specimens were, *Culex* (*Cux.*) *nigripalpus* (27.87%) and *Cx.* (*Cux.*) *chidesteri* (14.30%), while *Ae.* (*Stg.*) *albopictus* (34.16%), *Cx.* (*Cux.*) *quinquefasciatus* (32.23%) and *Li. durhami* (18.34%) were the most abundant among the immature specimens (Table 2).

Mosquito species accumulation curves for both parks were close to an asymptote at the end of the 12-month collection period. The first-order jackknife total richness estimator indicated that the curve stabilized at between 20 and 30 species for Santos Dias Park (Figure 2A) and 30 to 50 species for Shangrilá Park (Figure 2B). The average Shannon (H') diversity index for Santo Dias Park was 1.691 (1.565-1.817 CI 95%) and for Shangrilá Park was 1.690 (1.534-1.845). The T-test indicated no statistically significant difference between the two areas (t=0.015, p=0.988). Similarly, there was also no statistically significant difference between the mean Simpson's dominance index (t =-0.480, p=0.636), as the mean value presented by the Santo Dias Park was D=0.258 (0.219-0.298 95% CI) and Shangrila Park was D=0.272 (0.223-0.321). Regarding species composition, the two sites shared 16 species, including those of greater epidemiological importance such as *Culex nigripalpus*, *Cx. quinquefasciatus*, *Aedes albopictus* and *Ae. aegypti*. Another 24 species occurred only in one of the two parks. Sorensen's similarity index between the mosquito assemblages of the parks was 0.571.

Table 1. Distribution of adult and immature mosquitoes collected in Santo Dias Park in the city of São Paulo between March 2011 and February 2012 by collection technique.

Taxonomic units	Adults					Immature individuals				Total
	Aspiration	**Shannon traps**	**CDC traps in canopy**	**CDC traps on ground**	**Subtotal**	**Dipping**	**Pipetting/ emptying**	**Suction**	**Subtotal**	
Cx. (*Cux.*) *nigripalpus* Theobald	94	147	234	63	538					538
Culex (*Cux.*) spp. Linnaeus	105	31	218	105	459					459
Ae. (*Och.*) *fluviatilis* (Lutz)	43	116	8	64	231					231
Cx. (*Cux.*) *chidesteri* Dyar	28	2	67	8	105					105
Ae. (*Och.*) *scapularis (*Rondani*)*	21	33	2	13	69					69
Cx. (*Cux.*) *declarator* Dyar & Knab	56				56					56
Cx. (*Cux.*) *quinquefasciatus* Say	37	2	7		46		22		22	68
Cx. (*Cux.*) *bidens* Dyar	18				18					18
Ae. (*Och.*) *albopictus (*Skuse)	8			5	13		2	49	51	64
Cx. (*Mcx.*) *imitator* group Theobald	9	1		1	11	3		440	443	454
Cx. (*Cux.*) *eduardoi* Casal & Garcia								2	2	2
Cx. (*Cux.*) *lygrus* Root	5	3			8					8
Cx. (*Cux.*) *coronator* group Dyar & Knab	1	2	3	1	7					7
Wy. (*Pho.*) *galvaoi* Corrêa & Ramalho	3				3			53	53	56
Li. durhami Theobald	1	1		1	3		3	4	7	10
Cx. (*Cux.*) *dolosus/ eduardoi/bilineatus*	2				2					2
Cx. (*Cux.*) *saltanensis* Dyar	1				1					1
Wy. serratoria Lutz								1	1	1
Wy. (*Pho.*) *davisi* Lane & Cerqueira	1				1			59	59	1
Ma. (*Man.*) *indubitans* Dyar & Shannon	1				1					
Ae. (*Stg.*) *aegypti (*Linnaeus*)*			1		1			13	13	14
Cx. (*Cux.*) *coronator* Dyar & Knab	1				1					1
Wyeomyia (*Pho.*) spp. Theobald	1				1					1
Cx. (*Mel.*) *Melanoconion* section	1				1					1
Toxorhynchites spp.								7	7	7
An. (*Nys.*) *evansae* (Bréthes)	1				1					1
Total	**438**	**338**	**540**	**261**	**1,577**	**3**	**27**	**628**	**658**	**2,235**

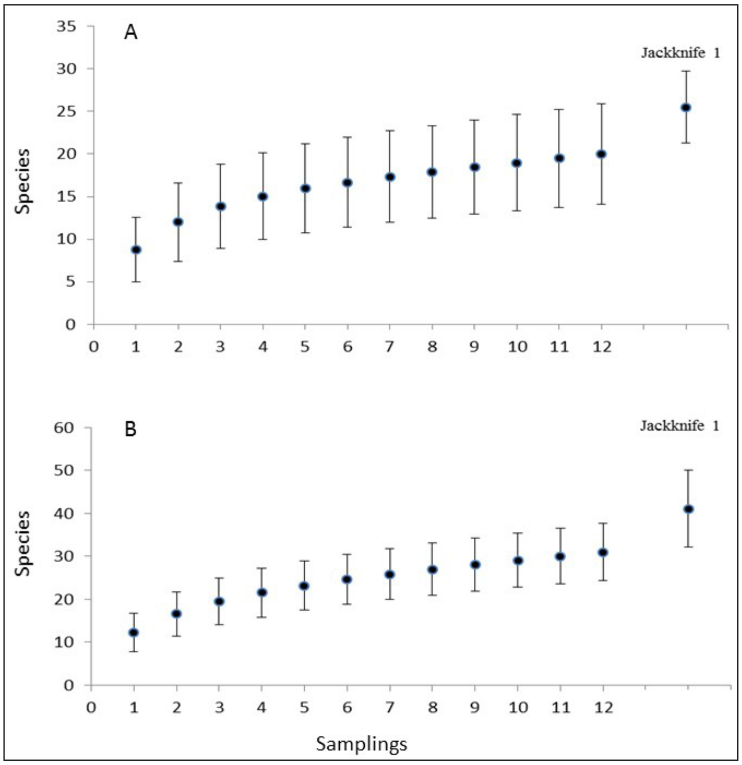

Figure 2. Sample-based species accumulation curves (jackknife 1) with a 95% confidence interval for Santo Dias Park (A) and Shangrilá Park (B) based on collections between March 2011 and February 2012.

Table 2. Distribution of adult and immature mosquitoes collected in Shangrilá Park in the city of São Paulo between March 2011 and February 2012 by collection technique.

Taxonomic units	Adults					Immature individuals				Total
	Aspiration	Shannon traps	CDC traps in canopy	CDC traps on ground	Subtotal	Dipping	Pipetting/ emptying	Suction	Subtotal	
Culex (*Cux.*) spp. Linnaeus	632	57	424	478	1,591					1,591
Cx. (*Cux.*) *nigripalpus* Theobald	768	242	183	127	1,320	31			31	1,351
Cx. (*Cux.*) *chidesteri* Dyar	281	19	233	175	708	26		6	32	740
Cx. (*Cux.*) *declarator* Dyar & Knab	487			2	489					489
Cx. (*Cux.*) *bidens* Dyar	169				169	3			3	172
Ae. (*Och.*) *fluviatilis* (Lutz)	99	36	6	9	150	3		11	14	164
Ae. (*Stg.*) *albopictus* (Skuse*)*	113	2	2	1	118	193	37	338	568	686
Cx. (*Cux.*) *quinquefasciatus* Say	77		6	22	105	58	5	536	599	704
Cx. (*Mel.*) *aureonotatus* Duret & Barreto	13	23	9	13	58					58
Cx. (*Mel.*) *vaxus* Dyar		2	5	44	51					51
Ae. (*Och.*) *scapularis* (Rondani)	32	4	2	4	42					42
Ma. (*Man.*) *wilsoni* (Barreto & Coutinho)	15	10	1	5	31					31
Cx. (*Cux.*) *dolosus/ eduardoi/ bilineatus*	22			1	23					23
Cx. (*Mel.*) *ribeirensis* Forattini & Sallum	1	17			18					18
Ma. (*Man.*) *titilans* (Walker)	2	3	1	9	15					15
Cx. (*Cux.*) *saltanensis* Dyar	15				15					15
Cx. (*Cux.) lygrus* Root	10				10					10
Wy. (*Prl.*) *confusa* Lutz	1		3	3	7					7
Ur. (*Ura.*) *nataliae* Lynch Arribálzaga								1	1	1
Ur. (*Ura.*) *lowii* Theobald	4			2	6					6
Li. durhami Theobald	4		1		5	79	205	21	305	310
Cx. (*Mel.*) *delpontei* Duret	1		2		3					3
Cx.(*Cux.*) *coronator* group Dyar & Knab		3			3	36			36	39
Ma. (*Man.*) *indubitans* Dyar &Shannon	2				2					2
Ad. (*Ady.*) *squamipennis* Lynch Arribálzaga			2		2					2
Cx. (*Phc.*) *corniger* Theobald	2				2					2
Ae. (*Stg.*) *aegypti* (Linnaeus)	2				2					2
Cx. (*Cux.*) *dolosus* Lynch Arribálzaga				1	1					1
Cx. (*Cux.*) *coronator* Dyar & Knab	1				1					1
Ur. (*Ura.*) *calosomata* Dyar & Knab	1				1					1
Cx. (*Mcx.*) *pleuristriatus* group Theobald								1	1	1
Cx. (*Mel.*) *melanoconion* section	1				1					1
An. (*Nys.*) *strodei* Root	1				1					1
Ae. (*Pro.*) *terrens* (Walker*)*	1				1					1
Cx. (*Cux.*) *eduardoi* Casal & Garcia						30	0	33	63	63
Toxorhynchites spp.						7		3	10	10
Cx. (*Cux.*) *brami* Forattini, Rabelo & Lopes	1				1					1
Total	**2,758**	**418**	**880**	**896**	**4,952**	**466**	**247**	**950**	**1,663**	**6,615**

Discussion

Ecological changes produced by human activities in recent decades have been identified as one of the causes of the geographic expansion of vectors and arboviruses (Daszak et al. 2001, Vasconcelos et al. 2001, Kruse et al. 2004, Medeiros-Sousa et al. 2015, Fernandes et al. 2016). In light of this, the present inventory of mosquito species in two parks was undertaken to investigate the presence of species that are of medical importance and have an impact on public health, whether because they transmit pathogens to humans and animals or because they represent a nuisance to the human population. The results of the inventory are expected to help public health authorities in the planning and implementing of surveillance, monitoring and control measures for mosquito-borne diseases.

Medeiros-Sousa et al. (2013) performed specific single mosquito collections in a preliminary investigation of Culicidae diversity in parks in the city of São Paulo and the epidemiological role of these species in pathogen transmission. While they identified four genera of Culicidae in Santo Dias Park and Shangrilá Park together, we found seven genera in the former and eight in the latter. This indicates that monthly monitoring using a range of strategies to collect adult specimens and active searches for immature forms in breeding sites in different collection points in these parks is essential for a more complete sampling of their mosquito fauna.

Comparing our findings with those of Medeiros-Sousa et al. (2013), in twelve months of collections in Santo Dias Park, 27 mosquito species were identified, of which 13 were reported by Medeiros-Sousa et al (2013). In Shangrilá Park, 36 species were found, of which 14 were already recorded by Medeiros-Sousa et al (2013). This difference in the number of species between the two studies shows the advantages of studying this type of environment for longer periods. As significant seasonal changes occur during the year in the city of São Paulo and these are reflected in mosquito richness and abundance, surveys over longer periods are needed to gain a better understanding of the Culicidae fauna in the city.

The species accumulation curve for Shangrilá Park showed that the sampling effort was sufficient and the collection points suitably located to estimate total species composition in the area, whereas for Santos Dias Park the estimators indicated that the collection points and the same sampling effort used in Shangrilá Park were insufficient for total coverage of the species that inhabit the park. The difference in species richness found in both areas may be due to the fact that Shangrila Park is on the edge of the Billings reservoir and Environmental Protection Area Bororé Colonia (which extends about 90 km^2), and there are fewer houses in its vicinity than in the vicinity of Santo Dias Park.

Despite the difference in species richness, diversity (ie, the balance between richness and equability in species abundances) was similar between the two parks, as shown statistically for the Shannon and Simpson indices. The species similarity between the assemblages was moderate with the two areas sharing mainly species of greater abundance or better adapted to the urban environment, it is necessary to emphasize the abundant presence of *Cx. nigripalpus* in the two parks, besides other epidemiologically important species as *Cx. quinquefasciatus*, *Ae. albopictus* and *Ae. aegypti*.

The finding of *Cx. nigripalpus* colonizing both parks has epidemiologic relevance, because t this species has been incriminated as the vector of St. Louis encephalitis virus (Foratinni 2002), which has been found in birds living in green areas in the Tietê Ecological Park in the city of São Paulo (Pereira et al. 2001). In addition, Carvalho et al. (2014) described a range of hosts that serve as blood-meal sources for this species in parks in the city, and in the two parks studied here there are plentiful blood-meal sources such as birds, humans, dogs and rodents.

Another species of epidemiological importance found in both parks in the present study is *Cx. quinquefasciatus*, which is recognized as the primary and main vector of bancroftian filariasis and secondary vector of Oropouche virus (which causes Oropouche fever) in Brazil. The following species were found in both parks: *Cx. bidens* Dyar, *Cx. brami* Forattini & Rabello, *Cx. coronator* Dyar & Knab, *Cx. coronator* group, *Cx. chidesteri* Dyar, *Cx. declarator* Dyar & Knab, *Cx. dolosus* Lynch Arribálzaga *eduardoi* Casal & Garcia, *Cx. lygrus* Root and *Cx. saltanensis* Dyar, among other taxa from the genus *Culex*. These mosquitoes are adapted to a wide range of breeding sites and shelters. Because of the feeding habits of this eclectic group, these species are considered potential vectors of human and animal arboviruses as well as a nuisance to people in the parks because of their bite. Also found in both parks were species of the subgenus *Melanoconion* of *Culex* (Tables 1 and 2), which can colonize natural and artificial breeding sites and exhibit eclectic blood-feeding behavior (Montes 2005). Of particular note is their potential for involvement in the natural cycles of some arboviruses, such as the Venezuelan equine encephalitis virus (Forattini 2002).

Four species of the genus *Aedes* were collected in both parks: *Ae. albopictus* Skuse, *Ae. aegypti* Linnaeus, *Ae. fluviatilis* Lutz and *Ae. scapularis* Rondani. Although these species were found in much smaller numbers than *Cx. nigripalpus* and *Culex* (*Cux.*) spp., this finding is very important for vector monitoring and control, since the first two *Aedes* species have been incriminated as potential vectors of dengue-causing arboviruses and Zika virus and Chikungunya virus, both recently introduced in Brazil. *Aedes scapularis* is a useful indicator of environmental changes (Dorvillé 1995) and has competitive advantages over other species of mosquitoes because it can colonize small temporary breeding sites in soil and its immature forms develop faster than other species of mosquitoes. The distribution of this species in the parks studied here is related to the blood-feeding habit of females on mammals (Forattini 2002).

Although Santo Dias Park and Shangrilá Park contain remnants of native forests, the fact that they are located within an urban environment where they suffer constant anthropogenic impacts means that the fauna and flora in them are subjected to significant environmental pressures. This is reflected in the finding of mosquitoes of the tribe Mansoniini, indicating highly degraded environments. When in their immature stages, these mosquitoes live in close association with macrophytes in lakes, obtaining air from their aerenchyma, and are therefore indicators of the presence of these plants, which in turn indicate the presence of eutrophied water (Consoli and Lourenço-de-Oliveira 1994).

Two anopheline species, *Anopheles evansae* Brethes and *An. strodei* Root were found in Santo Dias Park and Shangrilá Park, respectively. These species are generally associated with wild or rural environments (Forattini 2002) and are considered secondary or potential vectors of human malaria, despite having exophilic and zoophilic behavior (Consoli and Lourenço-de-Oliveira 1994).

Specimens of the subgenera *Microculex* Theobald, which belong to the genus *Culex*, were collected in Santos Dias Park, and individuals of the *Sabethini* tribe were captured in both parks. These species use tree holes, bromeliads and palm leaves, which are common in wild environments, as breeding sites. The small number of specimens of these species collected in our study indicates that anthropogenic changes and environmental degradation have occurred in both parks, adversely affecting survival of these mosquitoes (Forattini 2002, Medeiros Sousa et. al. 2015).

Some native species of the Atlantic forest from the genera *Aedeomyia*, *Uranotaenia* and *Wyeomyia* were found in both parks. The finding of indigenous Culicidae fauna in densely populated areas is important, as some of these species have shown vector competence and capacity for important pathogens (Smith et al. 2004, Guedes 2012). For example, *Ad. squamipennis*, has been proven to be a vector of Gamboa virus (*Orthobunyavirus*) and a wild vector of an avian malaria agent in Venezuela (Galbadon et al. 1977, Bicudo and Gomes 2007).

The observations of this research demonstrate the importance of including urban parks in official mosquito surveillance programs and regular arbovirus surveys, as they can develop epidemiological conditions that favor the spread of vector-borne diseases. On the other hand, although many mosquitos of degraded environments have been found, it is noted that these areas may harbor a relevant richness of species, functioning as biological diversity islands in the cityscape.

Acknowledgements

We would like to thank the State of São Paulo Research Foundation (FAPESP: BIOTA Program: Project 2010/51230-8) for providing funding. We are also grateful to the staff at the Department of Epidemiology in the Faculty of Public Health at the University of São Paulo, the Zoonosis Control Center, COVISA, SMS, PMSP and the Department of Parks and Green Spaces, SVMA, São Paulo for providing help with the field and laboratory work.

Author Contributions

Karolina Morales Barrio-Nuevo, Ramon Wilk da-Silva, Rafael de Oliveira Christe, Daniel Pagotto Vendrami, Laura Cristina Multini, André Barretto Bruno Wilke, Eduardo Evangelista, Amanda Alves Camargo and Laura de Freitas Souza: substantial contribution to the field work and data collection and contribution to manuscript preparation.

Marcia Bicudo de Paula: substantial contribution in the species identification and contribution to critical revision of the manuscript.

Mauro Toledo Marrelli, Antônio Ralph Medeiros de Sousa, Walter Ceretti Junior and Gabriela Cristina de Carvalho: substantial contribution in the concept and design of the study, contribution to data analysis and interpretation, contribution to manuscript preparation and contribution to critical revision, adding intelectual content.

Conflicts of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Received: 11/10/2016
Revised: 04/06/2017
Accepted: 22/06/2017
Published online: 13/07/2017

Biota Neotropica 17(2): e20170358, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
short communication

Re-discovering jaguar in remaining coastal Atlantic Forest in southeastern Brazil by non-invasive DNA analysis

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SOUZA, A. S. M. C., SARANHOLI, B. H., CRAWSHAW JR., P. G., PAVIOLO, A. J., RAMPIM, L. E., SARTORELLO, L., GALETTI Jr., P. M. **Re-discovering jaguar in remaining coastal Atlantic Forest in southeastern Brazil by non-invasive DNA analysis**. Biota Netropica. 17(2): e20170358. http://dx.doi.org/10.1590/1676-0611-BN-2017-0358

Abstract: Jaguar populations have been declining in Brazil mostly due to habitat loss and fragmentation, conflict with humans, poaching and reduction of prey. This is dramatically true in the Atlantic Forest, where occurrence of this large felid is now restricted to very few remaining areas. We used a non-invasive DNA analysis to search through felid scats collected in the Santa Virginia Unit (SVU) of the Serra do Mar State Park, to test for the potential presence of jaguar there. Our results indicated at least three individuals (two females and one male) inside SVU, thus confirming at least temporary presence of this top predator in this important protected area. It is now crucial to intensify studies in that area and surroundings, to evaluate the status of these individuals and identify conservation needs to urgently improve the prospects for the establishment of a resident population, allowing it to expand to adjoining units of the Serra do Mar State Park and Serra da Bocaina National Park.

Keywords: *Panthera onca, fecal DNA, Santa Virginia Unit, Serra do Mar State Park*

Redescobrindo a onça-pintada em um remanescente de Mata Atlântica costeira no sudeste do Brasil por análise não-invasiva de DNA

Resumo: As populações de onça-pintada têm sofrido declínio populacional devido a perda e fragmentação de habitat, por conflito com humanos, através da caça e pela redução da disponibilidade de suas presas. Isso é particularmente drástico no bioma Mata Atlântica, onde a ocorrência desse grande felino está atualmente restrita a poucos remanescentes. Utilizamos análise de DNA a partir de amostras de fezes para verificar a pontencial presença da espécie no Núcleo Santa Virgínia (NSV) do Parque Estadual da Serra do Mar. Os resultados indicaram a ocorrência pelo menos esporádica de três indivíduos (duas fêmeas e um macho) no interior do NSV. Com isso, se torna crucial agora intensificar estudos naquela unidade e em áreas adjacentes, para avaliar a situação desses indivíduos e identificar as necessidades mais urgentes de conservação para melhorar a probabilidade de estabelecimento de uma população residente da espécie, permitindo que ela possa expandir para unidades de conservação vizinhas, como outros núcleos do Parque Estadual da Serra do Mar e o Parque Nacional Serra da Bocaina.

Palavras-chave: *Panthera onca, DNA fecal, Núcleo Santa Virgínia, Parque Estadual da Serra do Mar*

Introduction

Jaguar (*Panthera onca,* Linnaeus 1758) populations have been steadily declining in Brazil, mainly since the mid-1900, due to habitat loss and fragmentation and because of conflicts with humans, from poaching (Zeller 2007, Beisiegel et al. 2012) and reduction of its prey (Sanderson et al. 2002, Beisiegel et al. 2012). Although currently considered near-threatened by IUCN (2016), the jaguar has been categorized as vulnerable in Brazil (Morato et al. 2013) and critically-endangered in the state of São Paulo (Bressan et al. 2009), where the largest contiguous portions of Atlantic Forest (AF) remain (Beisiegel et al. 2012). Originally distributed in several biomes in the Neotropical region, the jaguar is the only representative of the *Panthera* genus in the American continent (Sunquist & Sunquist 2002) and has had its historic distribuiton reduced by 50% during the last 50 years (Sanderson et al. 2002, Zeller 2007).

Because of the high level of threat, of the high endemism rate and species richness, the Atlantic Forest is considered one of the five hotspots of biodiversity in the world (Myers et al. 2000), and is now reduced to about 16% of its original area, distributed in thousands of small remaining fragments (Ribeiro et al. 2009). According to Galetti et al. (2013), the Atlantic Forest can be the first tropical biome to lose its largest top predator, if the jaguar becomes extinct. At the moment, the species is present in only a few remainings areas (Beiseigel et al. 2012), with an estimated total population of less than 250 adult individuals, mostly living inside protected areas (Galetti et al. 2013). The largest remaining continuous block of Atlantic Forest is situated in the mountains of the Serra do Mar along the Atlantic coast, which in the state of São Paulo, is under protection of Serra do Mar State Park (PESM, acronym in Portuguese). The park encompasses a landscape mosaic of 332,000.00 hectares (from 23°17' to 23 24'S and 45°03' to 40°11'W). Representing about 7% of the original extent of AF (Ribeiro et al. 2009), the park is responsible for maintaining a rich biodiversity of this biome (São Paulo 2006).

Because of its large size, the park was divided into ten administrative units, which include the Santa Virginia unit, where the study was conducted and which is recognized for its high biodiversity (Galetti et al. 2009, Rocha-Mendes et al. 2015). However, although the jaguar is believed to occur there (Rocha-Mendes et al. 2015), its presence has not been confirmed, after extensive camera trapping efforts between the years 2008-2011 (Crawshaw Jr., pers. commun.). In a recent camera trapping-based large scale survey on the jaguar occurrence, no individual was accounted in SVU (Paviolo et al. 2016).

In this study, we used a non-invasive DNA analysis to identify felid scats collected in the SVU, a recognized method for species identification (Farrel et al. 2000, Haag et al. 2009, Chaves et al. 2012, Miotto et al. 2014). Our results indicated at least three individuals (two females and one male) inside SVU, thus confirming the occurrence of this key top predator in this important protected area.

Material and Methods

1.Fecal samples

Twelve fecal samples were collected during 2012–2013 along roads and trails in the Santa Virginia unit of PESM; GPS coordinates were recorded for each sample. A small portion of each was preserved in 96% ethanol and kept in a -20 °C freezer until DNA extraction, which was later carried out using the QIAamp DNA Mini kit (Qiagen), following its specific protocol. Felid tissue samples obtained from the bank maintained by the Centro Nacional de Pesquisa e Conservação de Mamíferos Carnívoros – CENAP/ICMBIO were used as control and the DNA extraction followed Sambrok et al. (1989).

2. Molecular species identification

DNA amplification of two mitochondrial genes was used for species identification of each faeces sample. A Cyt*B* primer pair developed by Farrel et al. (2001) was used to amplify a fragment of the Cytochrome *B* gene, and the ATP6-DF3; ATP6-DR2 primer pair (Chaves et al. 2012) was used to amplify a fragment of the ATP6 gene, following the respective PCR protocols given by the already cited authors. All PCRs were carried out using a Veriti 96 Well Thermal Cycler (Applied Biosystems) and tissue sample DNA as a positive control.

The PCR products were sequenced in an ABI3730XL sequencer (Applied Biosystems). All the sequences obtained, as well as reference sequences obtained from GenBank, were analysed and aligned using the Geneious v.7.1.7 software (Kearse et al. 2012). Genetic distances between sequences were obtained by MEGA v.6 (Tamura et al. 2013), using Kimura 2 Parameters model (Kimura 1980). A Neighbor-Joining tree (Saitou & Nei 1987), using 1000 replicates bootstrap, was constructed.

3. Individual identification

Ten microsatellite loci (FCA742, FCA146, FCA98, FCA740, FCA723, FCA453, FCA441, FCA391, FCA53 e FCA42) obtained from Menotti-Raymond et al. (1999, 2005) were used for the individual identification, following PCR protocols given in Haag et al. (2010).

The PCR products were genotyped using an ABI 3730xl sequencer (Applied Biosystems). The fecal samples were genotyped at least in five independent rounds for each microsatellite locus, and only those with five confirmed genotypes were considered. Genotyping used the Geneious v. 7.1.7 software (Kearse et al. 2012).

Based on the obtained genotypes, the individual identification was carried out using GIMLET v.1.3.3 (Valière 2002). The probability of two individuals in the population randomly sharing identical genotypes for all of the analyzed loci was analysed assuming the presence of siblings ($P_{(ID)sibs}$), following Waits et al. (2001).

In addition, the presence of melanic individuals was tested using the Melacortin 1 receptor gene (MC1R), in which melanic individuals are homozygous for a 15bp delection while the wild body pattern could be either wild allele homozygous or heterozygous (Eizirik et al. 2003, Haag et al. 2010). The allele pattern was identified in the Geneious software (Kearse et al. 2012).

4.Sexing and Kinship

The amelogenin gene was used to identify sex of the identified individuals, following Pilgrim et al. (2005). Each sexed individual was confirmed through three independent PCRs.

Kinship was analysed using the ML-Relate software (Kalinowski et al. 2006), in which unrelated (UN), half-sibling (HS), full-sibling and/or parental-offspring (FS/PO) individuals were categorized.

Results and Discussion

Both fragments from ATP6 and Cyt*B* gene were successfully amplified for all 12 fecal samples. Blasting these sequences against the reference sequences obtained from blood samples and from GenBank, three of them were identified as belonging to jaguar, and nine from *Puma concolor*. The neighbor-joining-tree recovered these three jaguar samples within the group of *Panthera onca* (Figure 1), reinforcing that the method used was able to correctly identify the species who deposited the faeces collected. The ATP6 and Cyt*B* gene have been previously used for felid identification (Chaves et al. 2012, Miotto et al. 2014, Wultsch et al. 2016), and proved to be a powerful molecular marker for this group of carnivores.

The microsatellite analysis of the three jaguar samples identified three different individuals, based on the genotypes produced by the ten loci used, with significant probability ($P_{(ID)SIBS}$=0,0096). All three individuals showed at least one different allele for each locus analysed (Table 1). These results are quite surprising and indicate that three individuals are at least temporarily using the SVU (Figure 2). The presence of this large carnivore highlights the importance of this conservation unit and that it maintains a high degree of habitat integrity, with its high species diversity (Ripple et al. 2014, Rocha-Mendes et al. 2015). Moreover, this fact likely ensures more stability to this ecosystem since the jaguar has an important role on a top-to-down population control (Terborgh et al. 2001). Testing for body color pattern, no mutational signal (15 bp deletion) in the melanism-related MC1R gene (Eizirik et al. 2003) was observed, suggesting the absence of melanism among the identified individuals.

Two females and one male were identified, after sexing with the amelogenin gene. Although the presence of two females may suggest some level of residence in the area, this remains to be investigated. The partnership analysis showed no kin relation among these animals, being not able to detect philopatry which might support an idea of residence. Therefore, the presence of jaguar in Santa Virginia increases the importance of this area for conservation of PESM, with a possibility, if this incipient potential population is well protected, to serve as a source area for adjacent conservation units, including other sectors of PESM (Cunha, Picinguaba, Caraguatatuba) and the Serra da Bocaina National Park. Thus, it is our recommendation that more efforts are concentrated in the SVU and neighboring areas, using all methods available, including track and scat surveys, camera traps, and, as possible, GPS-satellite telemetry to collect more information on the status of jaguar in the area, and direct conservation efforts to ensure the presence

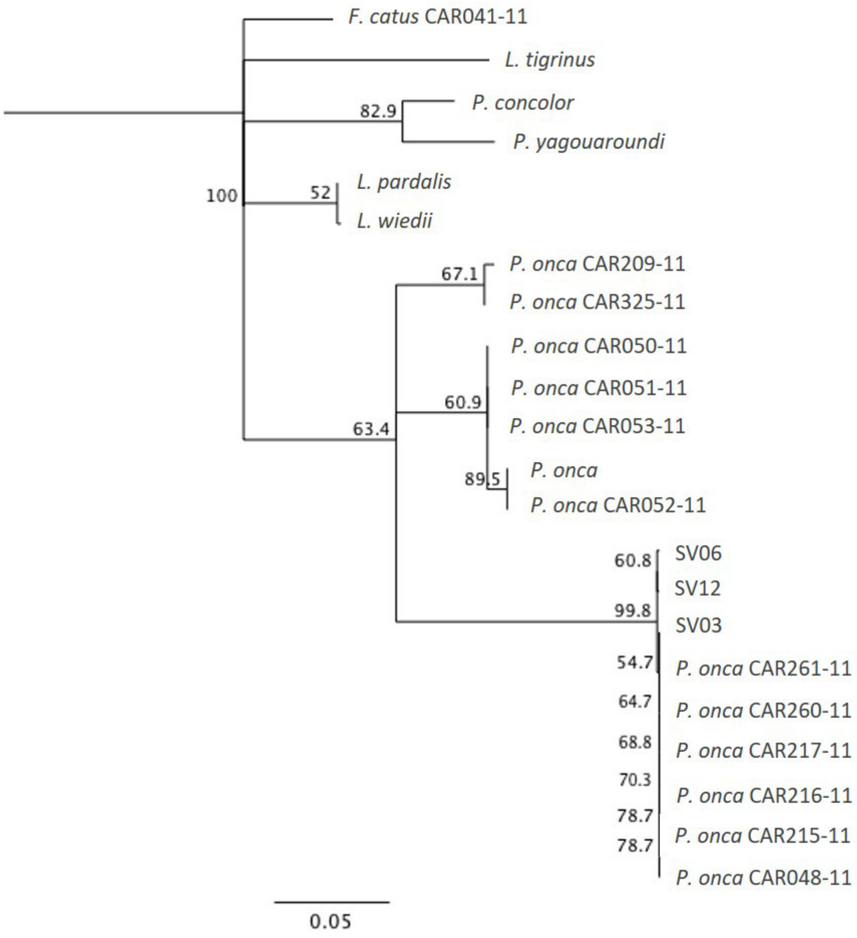

Figure 1 - Neighbor Joining tree highlighting the *Panthera onca* group. SV03, SV06 and SV12 sequences obtained from three fecal samples in the Santa Virginia Unit (SVU); *P. onca* (CAR048-11, CAR050-11- CAR053-11, CAR215-11- CAR217-11, CAR209-11, CAR260-11, CAR261-11 e CAR325-11) and *F. catus* (CAR051-11) sequences obtained in the BOLD systems; *L. trigrinus*, *P. concolor*, *P. yagouaroundi*, *L. pardalis*, *L. wiedii* and *P. onca* sequences obtained here from tissue samples.

Table 1 – Individual microsatellite alleles from the 10 loci genotyped. The "-" representes non-confirmed genotype.

		Microsatellite loci									
		FCA441	**FCA391**	**FCA98**	**FCA42**	**FCA53**	**FCA723**	**FCA740**	**FCA742**	**FCA453**	**FCA146**
Sample	**Sex**	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype
SM3	F	161/161	-	189/189	276/276	214/218	-	246/258	127/205	186/210	185/185
SM6	F	153/153	197/269	183/186	-	218/226	177/177	206/258	127/197	210/210	164/164
SM12	M	157/157	237/241	183/183	244/248	-	-	190/190	133/137	210/226	149/149

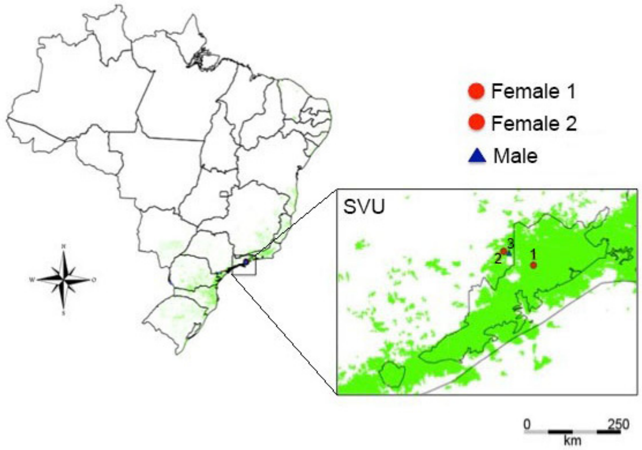

Figure 2 – Santa Virgínia Unit (SVU) in the Brazilian southeastern coast (São Paulo state), and the location of the three DNA identified jaguar faeces samples: (1) Female 1; (2) Female 2; (3) Male.

of the species in the region. This continuum of the Serra do Mar Atlantic Forest is considered high priority for conservation of this species (Zeller 2007, Paviolo et al. 2016) and our results can provide new momentum to implement an efficient management plan for the UC - SVU, taking into consideration all the requirements for the long term persistence of the jaguar.

Acknowledgements

The authors thank João Paulo Villani, Director of Núcleo Santa Virgínia and COTEC for collection permission. CENAP (Centro Nacional de Pesquisa e Conservação de Mamíferos Carnívoros) for providing samples used in control tests and as reference samples. This work is due to Sisbiota network - Top Predators, and the authors are thankful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - 2010/52315-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 563299/2010-0) for financial support. ASMCS thanks FAPESP (2012/00534-2), BHS thanks FAPESP (2013/24453-4) and PMGJr thanks CNPq (308385/2014-4).

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a) Substantial contribution to the conception and design of the work: Andiara Silos Moraes de Castro e Souza and Pedro Manoel Galetti Jr.;
b) Contribution to data acquisition: Andiara Silos Moraes de Castro e Souza, Bruno Henrique Saranholi and Pedro Manoel Galetti Jr.;
c) Contribution in the analysis and interpretation of data: Andiara Silos Moraes de Castro e Souza, Bruno Henrique Saranholi, Peter G. Crawshaw Jr. and Pedro Manoel Galetti Jr.;
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Conflicts of interest

The author(s) declare(s) that they have no conflict of interest related to the publication of this manuscript.

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Received: 04/04/2017
Revised: 24/04/2017
Accepted: 01/05/2017
Published online: 22/05/2017

Biota Neotropica 17(2): e20160252, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
inventory

Amphibians found in the Amazonian Savanna of the Rio Curiaú Environmental Protection Area in Amapá, Brazil

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LIMA, J. R. F., LIMA, J. D., LIMA, S. D., SILVA, R. B. L., ANDRADE, G. V. **Amphibians found in the Amazonian Savanna of the Rio Curiaú Environmental Protection Area in Amapá, Brazil.** Biota Neotropica. 17(2): e20160252. http://dx.doi.org/10.1590/1676-0611-BN-2016-0252

Abstract: Amphibian research has grown steadily in recent years in the Amazon region, especially in the Brazilian states of Amazonas, Pará, Rondônia, and Amapá, and neighboring areas of the Guiana Shield. Even so, few data are available for the Amazonian savannas of Brazil. To contribute to the understanding of the diversity of the amphibians of these savannas, we surveyed 15 temporary ponds, six located in open areas, seven on the edge of savanna forest, and two within the forest, in the savanna of the Rio Curiaú Environmental Protection Area (EPA) in Macapá, in the state of Amapá, northern Brazil. Sampling occurred from May 2013 to August 2014 during periods when the ponds contained water. Amphibians were sampled through visual and auditory surveys conducted during both the day- and the nighttime periods on seven days each month over a total of 84 days. A total of 1574 individuals belonging to four families, 12 genera, and 28 species were recorded during the surveys. The cumulative species curve reached the asymptote, indicating that sampling effort was adequate. The number of species was 25% higher than that reported in other studies in Amazonian savannas. Twelve species were recorded for the first time in the savanna of Amapá. *Lysapsus boliviana* was the most common species (n = 332 of the individuals recorded). The greatest amphibian species richness found in the Rio Curiaú EPA was associated with the savanna mosaic, forest patches, lowland swamp, and temporary ponds. The results of this study contribute to the understanding of the diversity of amphibian species in the Guianan area of endemism in northern Brazil, and also the Amazonian savannas.

Keywords: *Amphibians, Amazonian, Amapá, Savanna, savanna forest, species list.*

Anfíbios em Savana Amazônica na Área de Proteção Ambiental do Rio Curiaú, Amapá, Brasil

Resumo: Existe um crescimento constante em estudos sobre os anfíbios Amazônicos especialmente nas regiões do Amazonas, Pará, Rondônia, Amapá e outras áreas no Escudo das Guianas. Porém, poucos estudos são realizados em Savana Amazônica no Brasil. Para diminuir esta lacuna, amostramos anfíbios em 15 lagoas temporárias, sendo seis em área aberta, sete com a borda próxima a mata de savana e 2 com borda interna em contato com a mata de savana na Área de Proteção Ambiental (APA) do Rio Curiaú em Macapá no estado do Amapá, norte do Brasil. As amostragens ocorreram de maio de 2013 a agosto de 2014, nos períodos em que as lagoas continham água, sete dias por mês, totalizando 84 dias de amostragem, através de procura visual e auditiva no período diurno e noturno. Os 1574 indivíduos registrados pertenciam a quatro famílias, 12 gêneros e 28 espécies. A curva de acumulação de espécies atingiu a assíntota, indicando que o esforço amostral foi suficiente. O número de espécies foi 25% maior que o registrado nos outros estudos em Savanas Amazônicas. Doze espécies foram registradas pela primeira vez em Savana Amapaense. *Lysapsus boliviana* contribuiu com o maior número de indivíduos (n=332). A maior riqueza de anfíbios nesta savana foi associada ao mosaico de savana, ilhas de matas de savana, florestas de várzea e lagoas temporárias existentes na APA do Curiaú. Os dados apresentados contribuem para preencher a lacuna de conhecimento para o Centro de Endemismo Guiana no norte do Brasil e em Savana Amazônica.

Palavras-chave: *Anfíbios, Amazônia, Amapá, Savana, mata de savana, lista de espécies.*

Introduction

A total of 1080 amphibian species are currently known to occur in Brazil (Segalla et al. 2016), of which, 308 anurans, 18 caecilians, and five caudatans are found in Amazonia (Hoogmoed 2016a, b, Hoogmoed & Galatti 2016). Studies of the diversity, geographic distribution, and ecology of Amazonian amphibians have increased considerably over the past decade, especially in the Brazilian Amazon basin. These studies have provided a great deal of valuable data, especially for the Brazilian states of Amazonas (Zimmerman & Rodrigues 1990, Tocher et al. 2001, Gordo 2003, Neckel-Oliveira & Gordo 2004, Vogt et al. 2007, Menin et al. 2008, Ilha & Dixo 2010, Lima et al. 2012, Prudente et al. 2013, Waldez et al. 2013), Pará (Ribeiro-Junior et al. 2008, Avila-Pires et al. 2010), Rondônia (Bernarde 2007, Turci & Bernarde 2008), and Amapá (Lima 2008, Queiroz et al. 2011, Araujo & Costa-Campos 2014), as well as the countries of the Guiana Shield (Hoogmoed & Avila-Pires 1991, Lescure & Marty 2000, MacCulloch & Reynolds 2012, Reynolds & MacCulloch 2012, Cole et al. 2013).

Despite these advances, very few data are available on the amphibians of the Amazonian savannas of Brazil. In the state of Pará, Ávila-Pires et al. (2010) compiled a list of the amphibian species of the Grão-Pará Ecological Station, which encompasses both *terra firme* forests and savannas. Neckel-Oliveira et al. (2000) analyzed the distribution and diversity of amphibians in a savanna area at Alter do Chão, Pará state. Other savanna studies include that of Barbosa et al. (2007), who conducted an inventory in Roraima, and two recent studies that focused on the amphibian community of Amapá (Pereira-Junior et al. 2013, Costa-Campos 2015).

The savanna of Amapá is considered to be the most recent formation of the Amazonian savannas, and the most similar to those of the Cerrado of the ancient crystalline formations of the central Brazilian plateau (see Costa-Campos 2015). However, Silva et al. (2013) concluded that, while the arboreal stratum of this savana is composed of typical savanna plant species, the vegetation of the herbaceous-shrubby stratum is influenced by the anthropogenic impacts that are widespread in the region. The Amazonian savannas expanded through the region during the Cenozoic (Costa-Campos 2015), and are characterized by complex and heterogeneous phytophysiognomies influenced by the vegetation of neighboring biomes, accentuated by anthropogenic impacts (Sugai et al. 2014, Bitar et al. 2015).

Given this, inventories in areas of Amazonian savanna, such as that found in the Rio Curiaú Environmental Protection Area (EPA) are essential for the understanding of the formation of these environments, and the fauna that occupy them. In the specific case of this EPA, the heterogeneity of the habitats found around the ponds surveyed appears to be highly favorable to amphibian diversity (Souza & Eterovick 2011, Fahr & Kalko 2011).

Another important aspect of the biogeography of Amapá is that it is located in the Guianan area of endemism (Silva et al. 2005). Avila-Pires et al. (2010) emphasized the need to inventory this area due to the many new species found in the neighboring region of northern Pará, including the expansion of the known ranges of many species in this state and Brazil as a whole.

The savanna is the second most extensive landscape in Amapá, and is the dominant vegetation in the Rio Curiaú EPA, with a total area of 9861.92 ha. Data from the Amapá State Environment Secretariat (SEMA 2003) show that the most common anthropogenic pressures in this environment are burn-off for cassava (*Manihot esculenta* Crantz) plantations and the renovation of pasture for livestock, as well as squatting on private landholdings. In this context, the present study inventoried the amphibian fauna of the Amazonian savanna found in a conservation unit under anthropogenic pressure, with the aim of providing guidelines for the conservation of amphibians in this EPA, and in the Guianan area of endemism as a whole.

Material and Methods

1. Study area

The Rio Curiaú Environmental Protection Area (established through State Law No. 431 of August 15th, 1998, 0º10'0"N and 51º2'0"W) is located north of the municipality of Macapá, Amapá, and encompasses approximately 21,676 hectares (Figure 1). Lowland marshes and swamps (*várzeas*) are the predominant environments in the EPA, although approximately 35% of the area is covered in savanna (SEMA, 2003).

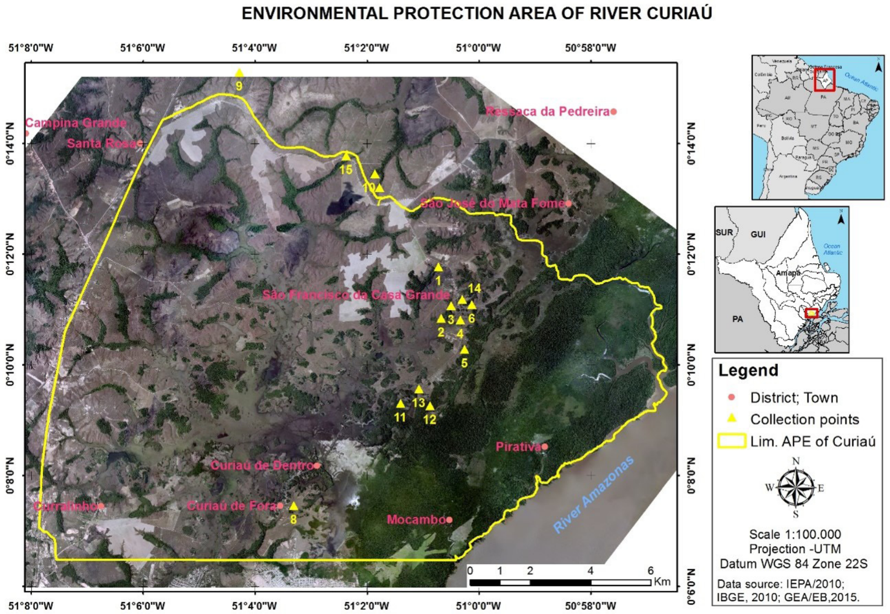

Figure 1. Location of the 15 ponds sampled in the Amazonian savanna of the Rio Curiaú Environmental Protection Area (including the surrounding area) in Amapá, northern Brazil.

According to the Köppen classification, the predominant climate in the EPA is Tropical savanna (Aw, Peel et al. 2007). The rainy season usually lasts from January to June and is characterized by high precipitation (2500 mm, Silva et al. 2013). Average monthly temperature range from 24.4°C to 28.4°C (Costa-Campos 2015).

2. Data collection

A total of 15 temporary ponds, six of which were located in open areas, seven at the edge of the forest, and two within the savanna forest (Figure 2) were sampled between May and September 2013 and in February to August 2014. Data were collected by two researchers on seven days per month, for a total sample of 84 field days, during both the daytime (09:00–15:00 h) and at night (18:00–00:00 h). The sampling period lasted until the complete drying of these temporary aquatic environments. All of the temporary ponds occupied by breeding anurans in the Rio Curiaú EPA were sampled, although small ponds (with an area of less than 100 m^2) that lasted less than 15 days were excluded from the analyses due to time constraints. The amphibian species present at each site were recorded during visual and auditory surveys (Heyer et al. 1994). Searches were conducted in the different types of habitat and microhabitat found within each area.

Figure 2. An example of the diversity of environments found in the temporary ponds sampled in the Amazonian savanna between May 2013 and August 2014 in the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil.

The voucher specimens were deposited in the Collection of the Fauna of Amapá at the Amapá State Institute for Scientific and Technological Research (IEPA, Appendix 1). Sampling was authorized by the Chico Mendes Institute for Biodiversity Conservation (SISBIO permit 38641-4/2014).

The amphibian species richness was determined by the total number of species recorded during the 12 months of sampling. The Chao 2 and Jackknife 2 estimators of species richness were also applied to the data. The Chao 2 is the most appropriate for relatively small samples, such as that of the present study (n = 12 months), while Jackknife 2 is less conservative (Magurran, 2004). The analyses were run in EstimateS, version 9.1 (Colwell 2013).

Results and Discussion

A total of 1574 individuals belonging to four families, 12 genera, and 28 species were recorded in the study (Table 1, Figures 3-6). The cumulative species richness curve (Figure 7) reached the asymptote. However, the number of species observed (28) was lower than that estimated by Chao 2 (29.96±2.3 species) and Jackknife 2 (33.96±3.1 species). A similar pattern was observed by Gondim-Silva et al. (2016) in a study of anurans in the Brazilian state of Bahia.

Table 1. Amphibians recorded in temporary ponds in the Amazonian savanna of the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil, between May 2013 and August 2014.

Family	Total number of individuals	First record in Amapá savanna
Bufonidae		
Rhinella granulosa (Spix, 1824)	114	
Rhinella marina (Linnaeus, 1758)	11	
Hylidae		
Dendropsophus nanus (Boulenger, 1889)	95	X
Dendropsophus sp	63	X
Hypsiboas boans (Linnaeus, 1758)	6	X
Hypsiboas geographicus (Spix, 1824)	7	
Hypsiboas multifasciatus (Günther, 1859″1858″)	70	
Hypsiboas punctatus (Schneider, 1799)	81	
Hypsiboas raniceps Cope, 1862	12	
Lysapsus boliviana Gallardo, 1961	332	
*Osteocephalus taurinus S*teindachner, 1862	3	
Phyllomedusa hypochondrialis (Daudin, 1800)	8	
Pseudis paradoxa (Linnaeus, 1758)	23	
Scinax boesemani (Goin, 1966)	27	X
Scinax fuscomarginatus (A. Lutz, 1925)	31	
Scinax garbei (Miranda-Ribeiro, 1926)	69	X
Scinax nebulosus (Spix, 1824)	91	
Scinax ruber (Laurenti, 1768)	65	
Sphaenorhynchus carneus (Cope, 1868)	16	
Sphaenorhynchus lacteus (Daudin, 1800)	19	X
Leptodactylidae		
Hydrolaetare schmidti (Cochran & Goin, 1959)	5	X
Leptodactylus fuscus (Schneider, 1799)	15	
Leptodactylus longirostris Boulenger, 1882	98	X
Leptodactylus macrosternum Miranda-Ribeiro, 1926	22	
Leptodactylus mystaceus (Spix, 1824)	14	X
Leptodactylus petersii (Steindachner, 1864)	15	X
Pseudopaludicola sp	259	X
Microhylidae		
Hamptophryne boliviana (Parker, 1927)	3	X
TOTAL	1574	12

The number of amphibian species recorded in the Rio Curiaú EPA was 33% greater than that reported in other studies in Amazonian savannas. Barbosa et al. (2007) recorded 20 species in Roraima, for example, while Pereira-Junior et al. (2013) found the same number in an area of secondary forest surrounded by savanna and successional habitat on the campus of the Federal University of Amapá. Costa-Campos (2015) recorded 21 species in an area of savanna surrounded by experimental plantations on the grounds of the EMBRAPA campus in Amapá, while Neckel-Oliveira et al. (2000) found 19 species on the margins of the Tapajós River and adjacent lakes, in Pará.

The family Hylidae was represented by the largest number of species (18), followed by the Leptodactylidae (7), Bufonidae (2), and Microhylidae (1). The predominance of the former two families is typical of the Amazon region and other South America biomes (Duelman 1988, Strussmann 2000, Gordo 2003, Neckel-Oliveira & Gordo 2004, Ilha & Dixo 2010, Lima et al. 2012, Waldez et al. 2013).

Lysapsus boliviana (Figure 3F), a semi-aquatic anuran that lives on floating vegetation (Brandão et al. 2003, Furtado, et al. 2013), was the most abundant species (n = 332 individuals). This species was previously recorded at a high density (n = 1240 individuals) in a temporary pond in the Rio Curiaú EPA (Furtado et al. 2014).

Twelve of the 28 species recorded in the Rio Curiaú EPA were recorded for the first time in the savannas of Amapá (Table 1). However, two of these species – *Leptodactylus mystaceus* and *Leptodactylus petersii* – are widespread in the *terra firme* forests of the Amazon basin (Lima 2008, Menin et al. 2008, Avila-Pires et al. 2010, Waldez et al. 2013, Lima et al. 2015). Similarly, *Hypsiboas boans*, *Scinax boesemani*, *Hydrolaetare schmidit*, and *Hamptophryne boliviana* were recorded by Lima (2008) in

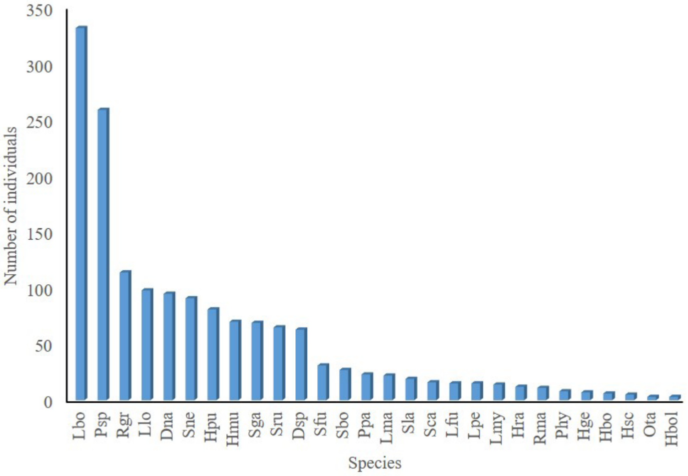

Figure 3. Abundance of the amphibian species recorded in the Amazonian savanna of the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil. Lbo: *Lysapsus boliviana*, Psp: *Pseudopaludicola* sp., Rgr: *Rhinella granulosa*, Llo: *Leptodactylus longirostris*, Dna: *Dendropsophus nanus*, Sne: *Scinax nebulosus*, Hpu: *Hypsiboas punctatus*, Hmu: *Hypsiboas multifasciatus*, Sga: *Scinax garbei*, Sru: *Scinax ruber*, Dsp: *Dendropsophus* sp., Sfu: *Scinax fuscomarginatus*, Sbo: *Scinax boesemani*, Ppa: *Pseudis paradoxa*, Lma: *Leptodactylus macrosternum*, Sla: *Sphaenorhyncus lacteus*, Sca: *Sphaenorhyncus carneus*, Lfu: *Leptodactylus fuscus*, Lpe: *Leptodactylus petersii*, Lmy: *Leptodactylus mystaceus*, Hra: *Hypsiboas raniceps*, Rma: *Rhinella marina*, Phy: *Phyllomedusa hypochondrialis*, Hge: *Hypsiboas geographicus*, Hbo: *Hypsiboas boans*, Hsc: *Hydrolaetare schmidti*, Ota: *Osteocephalus taurinus* e Hbol: *Hamptophryne boliviana*.

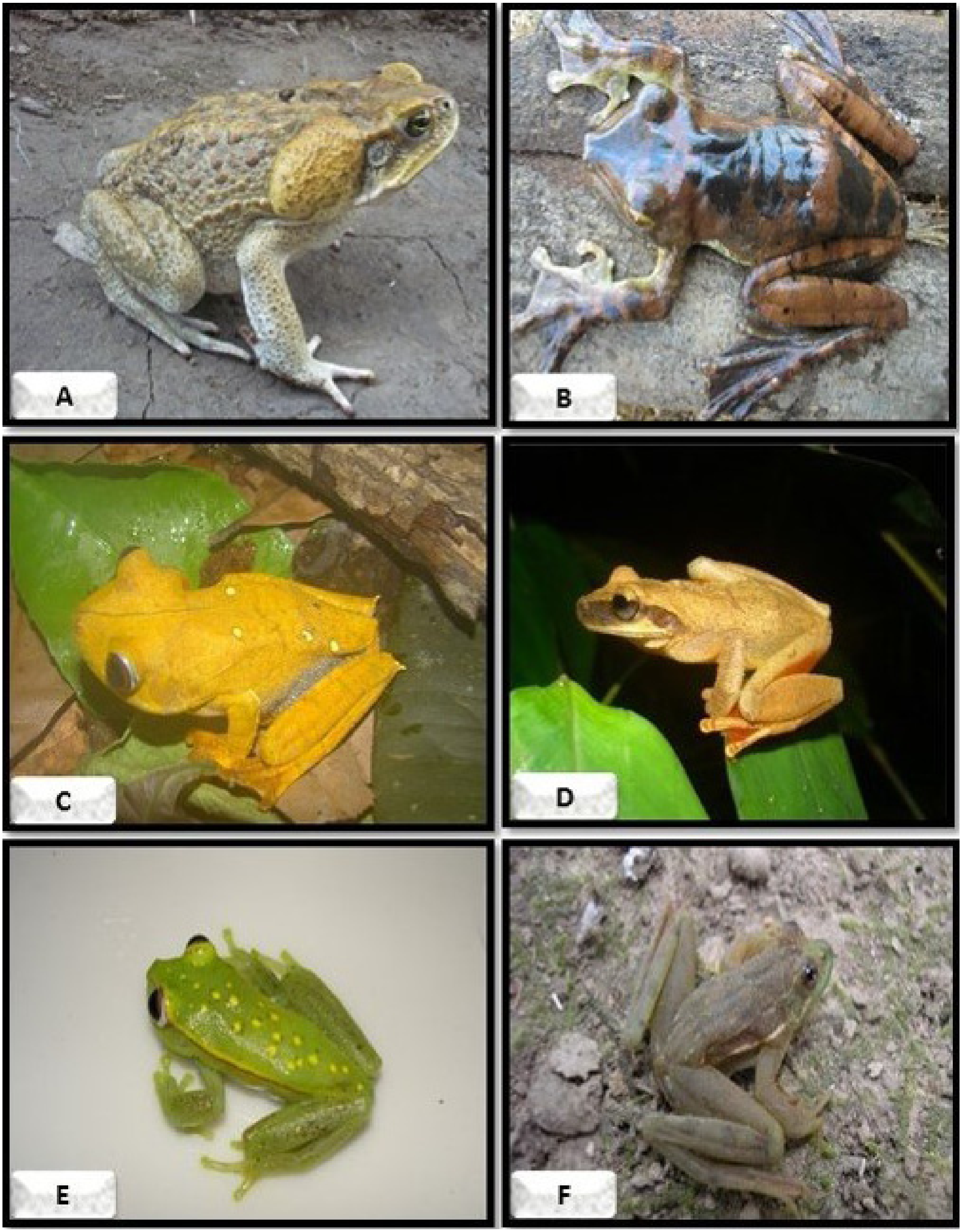

Figure 4. Anuran amphibians recorded in the Amazonian savanna of the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil. A – *Rhinella marina*, B –*Hypsiboas boans*, C – *Hypsiboas geographicus*, D – *Hypsiboas multifasciatus*, E –*Hypsiboas punctatus* and F - *Lysapsus boliviana*.

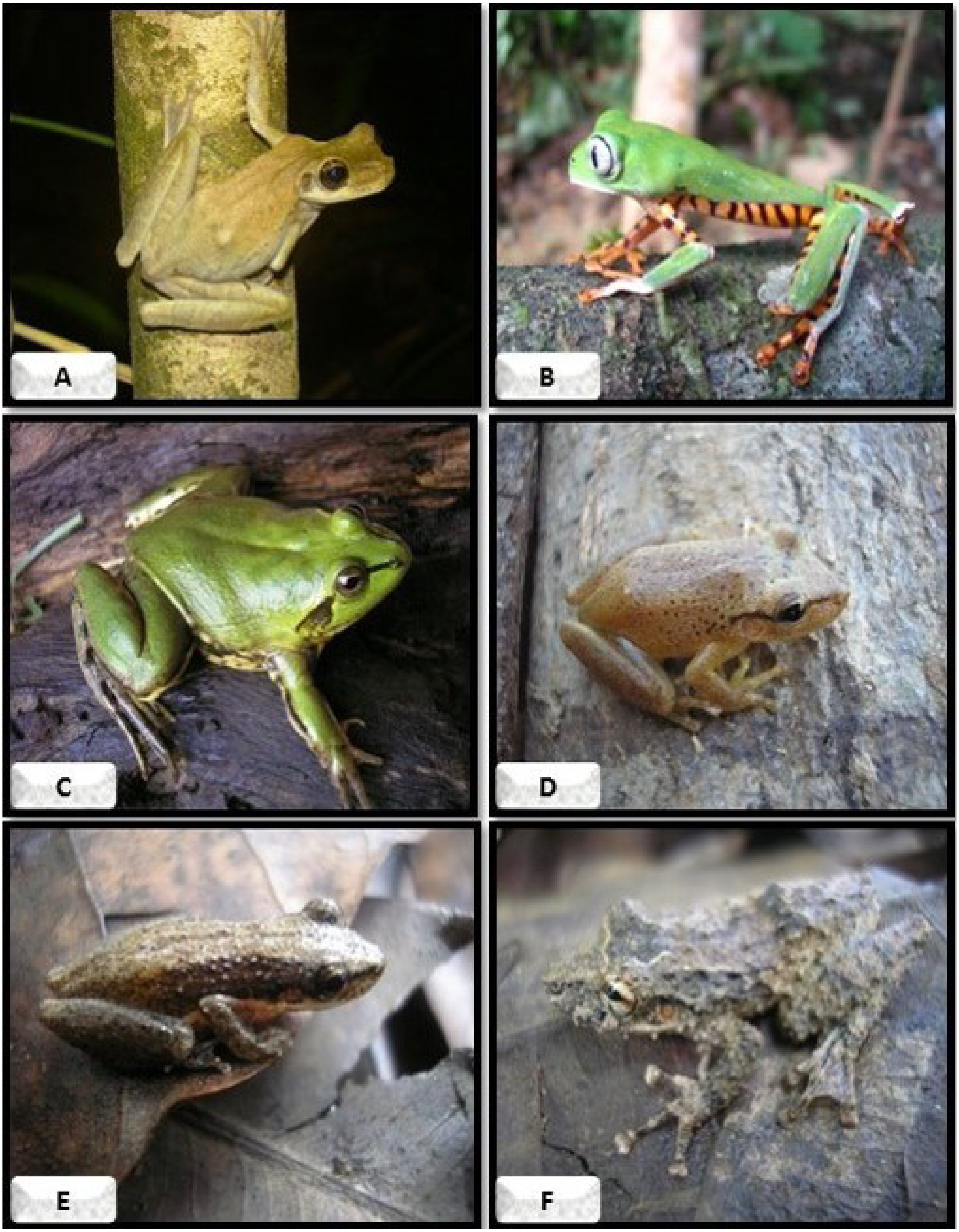

Figura 5. Anuran amphibians recorded in the Amazonian savanna of the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil. A – *Osteocephalus taurinus*, B – *Phyllomedusa hypochondrialis*, C – *Pseudis paradoxa*, D –*Scinax boesemani*, E – *Scinax fuscomarginatus* and F –*Scinax garbei*.

Figura 6. Anuran amphibians recorded in the Amazonian savanna of the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil. A – *Scinax ruber*, B – *Sphaenorhyncus carneus*, C – *Hydrolaetare schmidti*, D – *Leptodactylus fuscus*, E – *Leptodactylus mystaceus* and F – *Leptodactylus petersii*.

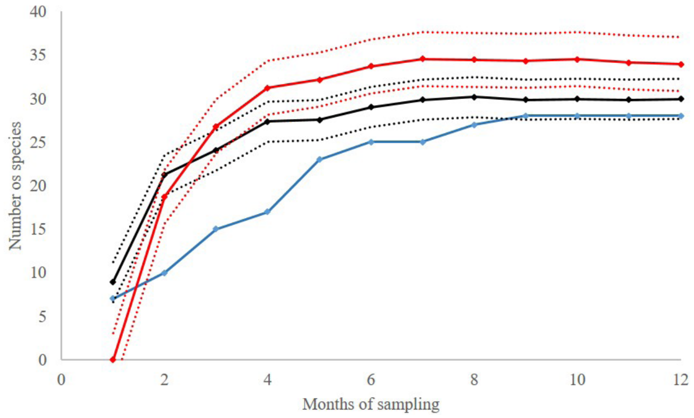

Figure 7. Cumulative and rarefaction curves to amphibian species recorded during 12 months of sampling in the Rio Curiaú Environmental Protection Area in Amapá, northern Brazil. Blue line represents accumulation curve, red line represents species estimates based on Jackknife 2 estimator, black line represents species estimates based on Chao 2; dashed lines represents the 95% confidence intervals.

the dense *terra firme* forests of the Tumucumaque Mountains National Park in northern Amapá, which is considered to be the world's largest park of continuous tropical forest, located on the Guiana Shield. *Scinax garbei*, *Scinax boesemani* and *Sphaenorhyncus lacteus* were also recorded by Waldez et al. (2013) in the region of the lower Purus River in the central Amazonian basin in a vast, continuous mosaic landscape of *terra firme* forests and *várzea*.

The fact that these species (*Leptodactylus mystaceus*, *Leptodactylus petersii*, *Hypsiboas boans*, *Scinax boesemani*, *Scinax garbei, Hydrolaetare schmidit*, *Hamptophryne boliviana*, and *Sphaenorhyncus lacteus*) have been recorded in forest habitats in other studies (Lima 2008, Menin et al. 2008, Avila-Pires et al. 2010, Waldez et al. 2013, Lima et al. 2015) suggests that their presence in the Rio Curiaú study area was due to the proximity of the ponds surveyed to the forest patches found in the local landscape. The phytophysiognomy of the study area in the Rio Curiaú EPA is composed mainly of savanna, with islands of savanna forest, and as some lakes are found in the vicinity of these forests, the anuran species composition may be enriched by this environment, due to the use of these pools by many forest-dwelling species that have aquatic larvae.

Recently, Doria et al. (2015) demonstrated the influence of variables associated with the presence of bodies of water on the distribution of anurans in the transition zone of the Brazilian Cerrado savanna and Caatinga dry forest. Further studies will be necessary to determine which variables favor the occupation of the savannas of the Rio Curiaú by forest-dwelling species.

In this case, it seems likely that amphibian species occurring in the *várzea* swamp forests found within the Rio Curiaú EPA used the nearby temporary ponds to breed, influencing the diversity of species encountered during the surveys. The mosaic of savanna, forest patches, swamps, and temporary ponds found within the Rio Curiaú EPA is thus extremely important for the maintenance of species, and the conservation of local amphibian diversity, given the occurrence in the ponds of species typical of the savanna, *terra firme* forest, and *várzea* swamps. Clearly, effective conservation measures for the EPA should focus primarily on the maintenance of this mosaic of environments, which favors the amphibian diversity of the study area.

Overall, the results of the present study provide important insights into the diversity of the amphibians of the Amazonian savanna, especially considering the scarcity of data for this environment, as highlighted by Azevedo-Ramos & Galatti (2001). In particular, the record of *Sphaenorhynchus carneus* in the EPA (Figura 5A) represents a southward extension of the known distribution of the species in the savannas of Amapá. Despite being widely-distributed in the Amazon basin (Azevedo-Ramos et al. 2004), only a single record of this species exists from Amapá, from an area of savanna in the district of Ariri, Macapá (Correa et al. 2015), and these populations are currently isolated by paved highways and soybean plantations. The results of the present study thus provide important insights into the diversity of amphibians in the Guianan area of endemism in northern Brazil and the Amazonian savannas.

Appendix 1: Voucher specimens of amphibians collected in the Amazonian Savanna in the Environmental Protection Area of Rio Curiaú, Amapá, northern Brazil.

Rhinella granulosa – IEPA4002, IEPA4005; *Rhinella marina* – IEPA4003; *Dendropsophus* sp. – IEPA4004; *Dendropsophus nanus* – IEPA4010, IEPA4015; IEPA4017; *Hypsiboas boans* - IEPA4007; *Hypsiboas geographicus* – IEPA4009; *Hypsiboas multifasciatus* – IEPA4011, IEPA4012; *Hypsiboas punctatus* – IEPA4006, IEPA4008; *Hypsiboas raniceps* - IEPA4013; *Lysapsus boliviana* - IEPA4014, IEPA4016; *Phyllomedusa hypochondrialis* – IEPA4019; *Osteocephalus taurinus* - IEPA 4018; *Scinax boesemani* - IEPA4020, IEPA4021; *Pseudis paradoxa* – IEPA4035; *Scinax fuscomarginatus* – IEPA4029; *Scinax garbei* – IEPA4027, IEPA4030; *Scinax nebulosus* – IEPA4034, IEPA4025; *Scinax ruber* – IEPA4022, IEPA4026; *Sphaenorhynchus carneus* – IEPA4023; *Sphaenorhynchus lacteus* – IEPA4031; *Hydrolaetare schmidti* – IEPA4024; *Leptodactylus fuscus* – IEPA4031, IEPA4033; *Leptodactylus longirostris* – IEPA4032; *Leptodactylus macrosternum* – IEPA4042, IEPA4040; *Leptodactylus mystaceus* – IEPA4036, IEPA4037; *Leptodactylus petersii* – IEPA4038, IEPA4041; *Pseudopaludicola* sp. – IEPA4039, IEPA4044; *Hamptophryne boliviana* – IEPA4043.

Acknowledgements

J. R. F. L thanks CAPES for fellowships and Posgraduate program in Biodiversity and Biotechonoly – BIONORTE Network and Institute of Scientific and Technological Research of the State of Amapá – IEPA. G.V.A., J.R.F.L. and S. D. L thanks SISBIOTA Program (CNPq: 563075/2010-4 and Fapesp: 2010/52321-7). G.V.A. thanks the research fellowship of CNPq (Proc.:312286/2015-5). We are grateful to an anonymous reviewer for helpful comments on the original version of the manuscript.

Authors Contributions

Janaina Reis Ferreira Lima: substantial contribution in the concept and design of the study, contribution to data collection, contribution to data analysis and interpretation, contribution to manuscript preparation and contribution to critical revision, adding intelectual contente.

Jucivaldo Dias Lima: substantial contribution in the concept and design of the study, contribution to data collection, contribution to data analysis and interpretation, contribution to manuscript preparation and contribution to critical revision, adding intelectual contente.

Soraia Dias Lima: contribution to data collection and contribution to manuscript preparation.

Raullyan Borja Lima e Silva: substantial contribution in the concept and design of the study, contribution to data analysis and interpretation, contribution to manuscript preparation and contribution to critical revision, adding intelectual contente.

Gilda Vasconcellos de Andrade: substantial contribution in the concept and design of the study, contribution to data analysis and interpretation, contribution to manuscript preparation and contribution to critical revision, adding intelectual contente.

Conflicts of interest

The authors declares that they have no conflict of interest related to the publication of this manuscript.

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Received: 18/08/2016
Revised: 06/03/2017
Accepted: 07/03/2017
Published online: 27/03/2017

Biota Neotropica 17(2): e20160253, 2017
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
Article

Distribution of the assemblage of phlebotomine sandflies (Diptera: Psychodidae) along an environmental gradient

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ANTONINI, Y., FERNANDES, G. W., BARATA, R. A. **Distribution of the assemblage of phlebotomine sandflies (Diptera: Psychodidae) along an environmental gradient.** Biota Neotropica. 17(2): e20160253. http://dx.doi.org/10.1590/1676-0611-BN-2016-0253

Abstract: The geographical distribution of leishmaniasis has undoubtedly expanded, and is now being reported to be in areas that were previously non-endemic. This study therefore aimed to investigate whether there is any variation in the structure and in the composition of phlebotomine communities along an environmental gradient - from a mesic to a xeric environment, across different climatic periods (rainy and dry seasons). Furthermore, we test whether there were differences in sex ratio of sand flies between wet and dry environments. A total of 535 specimens of sand flies belonging to 18 species were recorded. Richness, abundance, and composition of species were significatively higher in the xeric environment (semidecidual Forest) compared to the mesic environment (Riparina Forest). We unveiled site specific differences in sand fly collections since the community composition changes with the geographic distance among the sampling points. These results led to the conclusion that for sand flies species, the environmental conditions available for each species could be different within the habitat taking into account the degree of humidity in the forest as well as the climatic season (dry or wet). The present study gives a valuable contribution to the knowledge of sand flies, as a group of insects, from the Atlantic Forest biome.

Keywords*: Species composition, ecology, leishmaniasis, Atlantic Forest, Brazil*

Distribuição da assembléia de flebotomíneos (Diptera: Psychodidae) em um gradiente ambiental

Resumo: A distribuição geográfica das leishmanioses, sem dúvida, tem se expandido, e agora está sendo relatada em áreas previamente consideradas como não endêmicas. Assim, este estudo teve como objetivo investigar se existe alguma variação na estrutura e na composição das comunidades de flebotomíneos ao longo de um gradiente ambiental – em habitats classificados como mesicos e xericos, em diferentes períodos climáticos (chuva e seca). Além disso, testamos se havia diferenças na razão sexual entre ambientes mais umidos e mais secos. Um total de 535 espécimes de flebotomíneos pertencentes a 18 espécies foram registradas. Riqueza, abundância e composição de espécies foram significativamente maiores nos habitats xericos (Floresta semidecidual) em comparação com os habitats mesicos (Floresta Ciliar). Revelamos diferenças específicas entre os habitats, nas assembleias de flebotomíneos, uma vez que a composição da comunidade muda com a distância geográfica entre os pontos de amostragem. Estes resultados levaram à conclusão de que as condições ambientais disponíveis para cada espécie de fletomonineo varia dentro do habitat, tendo em conta o grau de umidade na floresta assim como a estação climatica (seca-chuva). O presente estudo dá uma contribuição valiosa para o conhecimento da fauna de flebotomíneos, no bioma Mata Atlântica.

Palavras-chave*: Composição de espécies, ecologia, leishmanioses, Floresta Atlântica, Brasil*

Introduction

Phlebotomine sand flies are vectors of pathogenic organisms, such as *Leishmania,* in many regions of Brazil, which has been a major endemic focus of leishmaniasis for many years. The transmission of the *Leishmania* parasite species to the vertebrate host, occurs when the vertebrate host is bitten by infected female sand flies (Sherlock 2003). The geographical distribution of leishmaniasis has undoubtedly expanded, and is now being reported in areas that were previously known to be non-endemic (McCarthy et al. 2013).

The resting places of the adult sand flies vary, depending on the species and to on the habitat conditions such as forest floor (litter fall), small shrubs and plants, mammal nests and burrows, as well as rock crevices (Young & Duncan 1994). In general, the activity period of sand flies ranges from dusk to dawn, and these insects remain at rest in natural shelters during most part of the day.

Sand flies are very sensitive to environmental changes. In this regard, soil type, land use and modification of natural habitats are critical factors affecting changes in the vector, reservoir population densities, and risks posed by leishmaniasis (Rangel & Lainson 2009).

The understanding of the variation in the community of phlebotomine is of major importante due to the impact it causes in human health and economy. One way of understanding these flies importance is through the description of the variation in their communities in space and time. A better understanding of beta-diversity patterns and their causes, demonstrates that, it has important practical implications for biodiversity conservation as well as the planning and interpretation of ecological research (Tuomisto et al. 2003). Unfortunately, there are very few surveys of the phlebotomine fauna in natural systems that address beta-diversity, despite being fully aware of the importance of these studies in increasing our knowledge of areas where these insects can be found and in minimizing human contact with them.

We evaluated the influence and the relative importance of spatial and environmental factors in explaining the variations on phlebotomine species richness and composition. This study, therefore, aimed to investigate whether there is any variation in the structure and composition of the phlebotomine communities, along an environmental gradient – from Mesic (riparian forest) to a xeric (se midecidual forest) environment and to determine species richness and abundance in those environments. We hipotetize that the richnes and abundance will be higher in mesic enviroments and that the composition of the sand fly assemblage will be different with the distance among the sampling areas.

Material and Methods

1. Study area

The study was carried out in fragments of forest on the Santo Antônio de Pádua municipality, in the Rio de Janeiro state, Brazil (Figure 1). The vegetation in the area consists of fragments of riparian forest (mesic habitat)- along the Rio Pomba River, and small fragments of semidecidual Atlantic forest located at the top of small hills (xeric habitat) (IBGE 1992). The climate is classified into Köppens Aw Tropical (Alvares et al. 2013) with dry winter. Total annual rainfall is high, around 1600 mm. Temperatures are high throughout the year (average temperature is 26°C) with little variation.

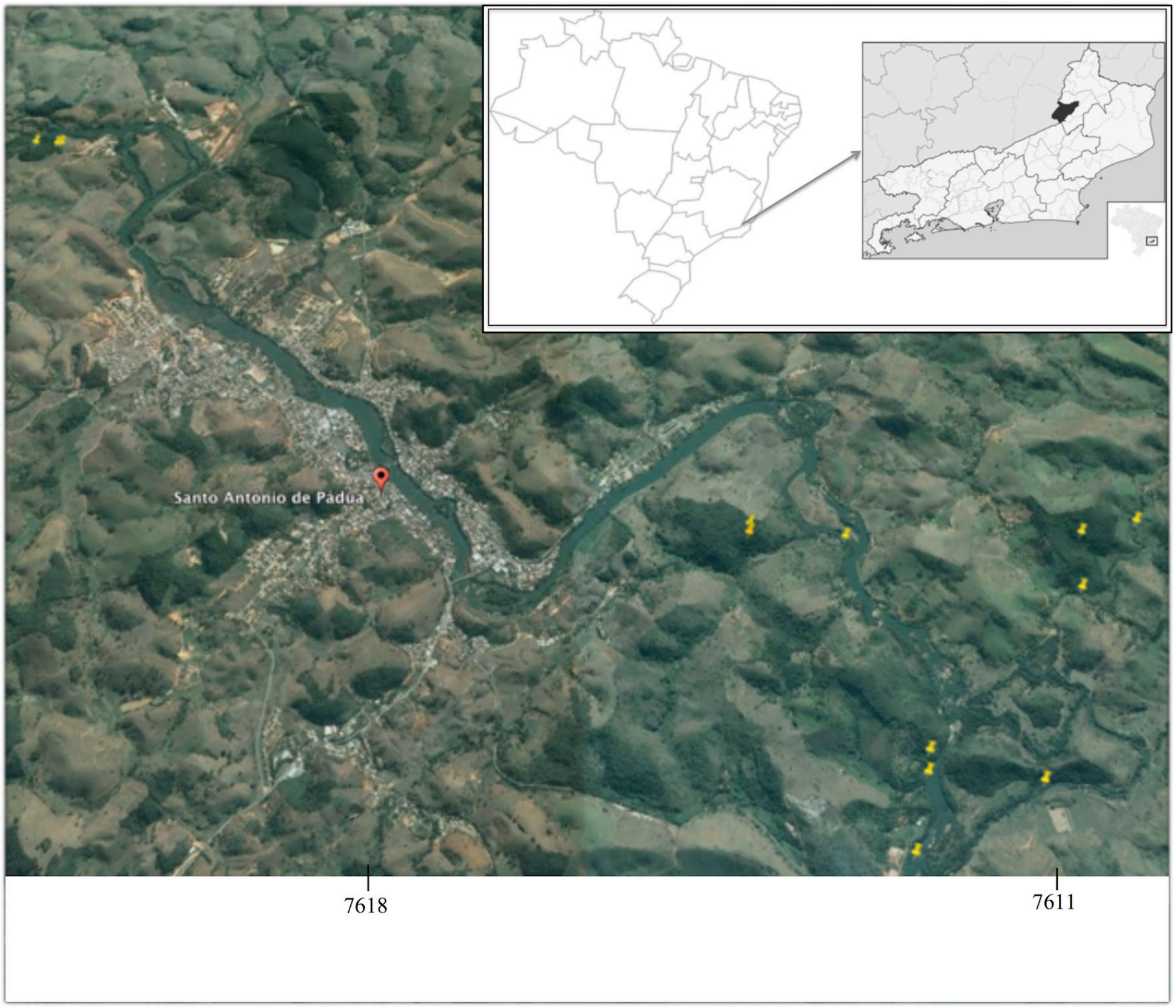

Figure 1. Map showing collecting localities of sand flies at the Santo Antonio de Pádua municipality, State of Rio de Janeiro, southeastern of Brazil. Coordinates in UTM. The yellow pins represent the location for each transect. Source of Map: Google Maps Pro.

http://dx.doi.org/10.1590/1676-0611-BN-2016-0253

2. Sampling

Four fragments of forest were selected for this study, being two of riparian forests and two of semideciduous forests. In each fragment, insects were captured in transects of 100 m (Figure 1). For the purposes of this study, each transect has been categorized according to the vegetation type – wet or mesic (riparian forest – hereafter, referred to as WE) and dry or xeric (semideciduous forest – hereafter, referred to as DE).

Three HP light traps (Pugedo et al. 2005) were installed along each of these transects, at 20 m intervals. The traps were installed at 6 pm and collected at 6 am the following morning, in two sampling events – 14th to 18th February (wet season) and May 30th to June 3rd of 2013 (dry season), giving a total sampling effort of 72h/fragment. The collection was made under ICMBIO permition (10717-1) conceived to Y.A.

The collected phlebotomines were cleared in 10% potassium hydroxide, acetic acid, distilled water and lactophenol, and mounted in Berlese fluid (Langeron 1949). They were later identified according to Young & Duncan (1994) and Martins et al. (1978). Specimens with missing or damaged characters that impaired identification at the specific level were considered as *Lutzomyia* sp. The females belonging to *Brumptomyia* genera were not identified at the specific level and were considered as *Brumptomyia* sp..

3. Statistical analyses

The relationship between the two types of environmental gradients and the species richness and abundance of the specimens collected in the fragments inside each environment was analyzed using a one-way ANOVA. A *t*-test was used to determine whether the average male and female abundance was statistically different. The specimens that where not identified to the species level or morphotype, were considered as belonging to one same species in the model.

Permutational Analysis of Variance (PERMANOVA) was used to test the hypothesis which states that, the composition of sand flies varies between gradients of humidity wet (WE) and dry (DE). The measure of dissimilarity used was that of Bray-Curtis with 1,000 permutations, and in measuring the dispersion of the data, multivariate analyses of Permutation Distance (PERMDISP) were performed. The graphical representation of the variation in the composition of sandflies assemblages between seasons and among sampling units was illustrated by the analysis of Non-metric Multidimensional Scaling (NMDS).

The relationship between geographic distance and similarity in species composition, also known as the distance–decay in similarity relationship, was analyzed using simple linear regressions. A distance-decay plots was built (Nekola & White 1999), in order to analyze the variation in the compositional similarity among pairs of sampling sites, in relation to the geographic distance and to the climatic distance among these sites. The geographic matrix was built using the Euclidean distance between sites, based on their distances (in Km), and also using the Google Maps Pro.

Results

A total number of 535 phlebotomine specimens belonging to 18 species were recorded (Table 1). The following species were captured: *Brumptomyia avellari* (Costa Lima, 1932), *B. guimaraesi* (Coutinho & Barretto 1941), *B. nitzulescui* (Costa Lima 1932), *Lutzomyia capixaba* Dias, Falcão, Silva & Martins 1987, *Lu. cortelezzii* (Brethes 1923), *Lu. christenseni* Young & Duncan 1994, *Lu. edwardsi* (Mangabeira 1941), *Lu. intermedia* (Lutz & Neiva 1912), *Lu. lutziana* (Costa Lima 1932), *Lu. migonei* (França 1920), *Lu. minasensis* (Mangabeira 1942), *Lu. pascalei* (Coutinho & Barretto 1940), *Lu. quinquefer* (Dyar, 1929), *Lu. sallesi* (Galvão & Coutinho 1939), *Lu. sordellii* (Shannon & Del Ponte 1927), and *Lu. tupynambai* (Mangabeira 1942). *Lutzomyia intermedia* accounted for 43% of all specimens sampled, while *Brumptomyia avellari* and *Brumptomyia* sp. accounted for 37% of sampled specimens. The remaining species together, accounted for 20%.

A total number of 294 males (55%) and 241 females (45%) were collected (Table 1). Despite the proportion of male was slightly higher than that of female (Table 1), we did not find any significant difference between male and female abundance. The sexual ratio of the assemblage was 1:0.8. The difference in sex ratio was higher in WE2 – for DE1 and DE3, there is no difference (Figure 2A).

There was significant difference in species richness ($ANOVA_{1,16}= 5.07$, $P=0.025$) and abundance ($ANOVA_{1,16} = 7.34$, $P<0.001$) within the sampling sites (Figure 2B). Higher richness and abundance was found on transects in xeric environments (Figure 2B).

Table 1. Sand flies species composition, total abundance and frequency, total abundance of male and female, of on wet and dry season, sampled in Santo Antonio de Padua Municipality, RJ, Brazil.

Species	Climatic station		SEX		Total (N)	%
	Wet	Dry	♂	♀		
Brumptomyia avellari	80	20	100	-	100	18.7
B. nitzulescui	5	0	5	-	5	0.9
B. guimaraesi	15	3	18	-	18	3.3
Brumptomyia sp.	72	24	3	93	96	17.9
Lutzomyia capixaba	1	0	0	1	1	0.2
Lu. cortelezzii	5	0	2	3	5	0.9
Lu. christenseni	1	0	0	1	1	0.2
Lu. edwardsi	3	1	1	3	4	0.8
Lu. intermedia	226	4	146	84	230	43.0
Lu. lutziana	15	4	8	11	19	3.6
Lu. migonei	5	0	4	1	5	0.9
Lu. minasensis	1	0	1	0	1	0.2
Lu. pascalei	1	0	1	0	1	0.2
Lu. quinquefer	6	1	2	5	7	1.3
Lu. sallesi	2	1	3	0	3	0.6
Lu. sordellii	4	0	0	4	4	0.8
Lu. tupynambai	13	16	0	29	29	5.4
Lutzomyia sp.	4	2	0	6	6	1.1
Total	459	76	294	241	535	100

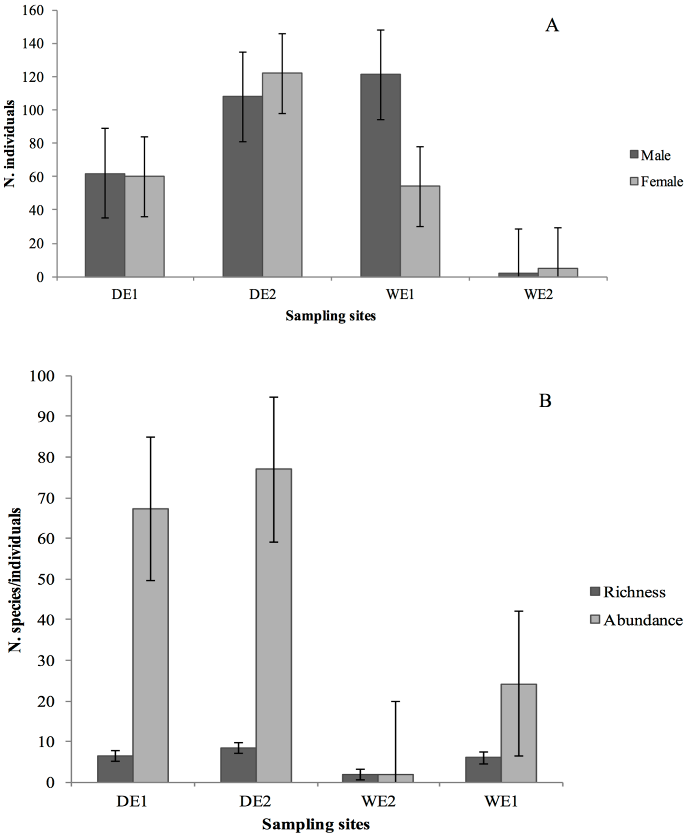

Figure 2. Total abundance of male and female (A) and total richness and abundance (B) of phlebotomine sand flies, and at the four sampling sites, Santo Antonio de Pádua, RJ, Brazil. DE - Dry environment, WE – Wet environment. Bars are means ± SE.

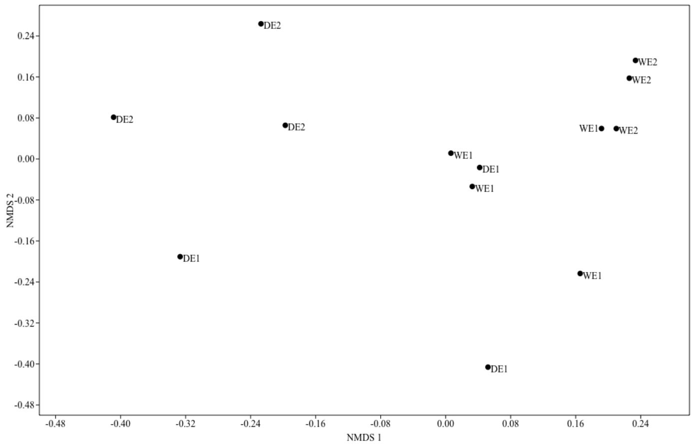

Figure 3. NMDS showing the species composition of phlebotomine sand flies in transcets in Dry Enviroments (DE) and Wet Enviroments (WE), Santo Antonio de Padua, RJ. Brazil.

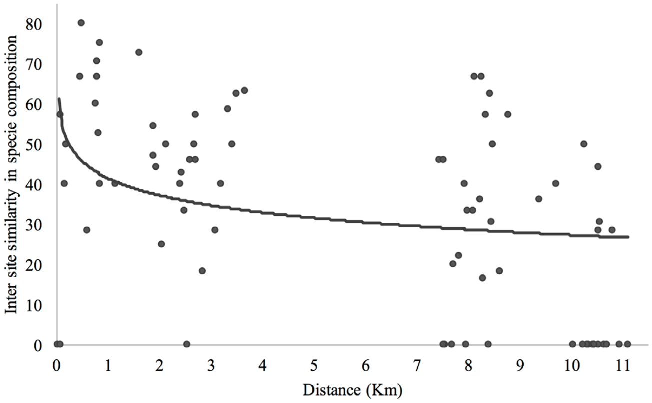

Figure 4. Decay in the similarity of phlebotomine sand flies assemblages in relation to the geographic distance between sampling sites. Geographic distance is the distance in km between pair sites. Similarity in orchid-bee species composition is based on the Bray–Curtis index of similarity. Line represent the logarithmic regression curve.

The level of pairwise similarity among the 11 sand flies communities sampled, was highly variable, ranging from 0% to 73% (Bray–Curtis index, mean = 33.8%), considering the presence or absence of all recorded species. Similarity in the composition of sand flies species among the different pairs of transects decayed as function geographic distances among sites (r = -0.42, P<0.01) (Figure 3).

During the wet season, 459 specimens belonging to all 18 sampling species (85.7%) were collected while during the dry season, 76 individuals (14.3%) belonging to 10 species (Table 1) were collected. The two most abundant species during the wet season were *L. intermedia* and *B. avellari*. In dry season, *Brumptomyia* sp., *B. avellari* and *L. tupynambai* were the most abundant (Table 1).

The composition of sand flies species varied between xeric and mesic habitats (Permanova, R^2=0.2293, P=0.038; Permidisp, F=5.72, p<0.03) (Figure 4). The similarity in species composition between dry and wet climatic period was 55%. The NMDS analyzes show that even the transects on the same area present different composition of species (Figure 4), however, there is a clear spatial separation on species composition, between xeric and mesic environments. The species with the widest distribution, which were found in 10 out of 13 transects, both in mesic and xeric were *B. avellari* and *L. intermedia* are responsible to group sampling sites DE1 with all WE.

Discussion

The number of species found in this study can be considered high. Currently, the sand fly fauna of Rio de Janeiro State has 65 species, belonging to the genera *Brumptomyia* (8 spp.) and *Lutzomyia* (57 spp.) (Carvalho et al. 2014). It is important to notice that the richness could be higher, if the 96 individuals of *Brumptomyia* could be identified to the species level. To the best of our knowledge, only the occurrence of *L. intermedia* had been reported in the study area (Rangel et al. 1984). Yet, the report of *L. intermedia* and *L. migonei* in such areas is important because these species are known to be vectors of cutaneous leishmaniasis agents (Lainson 1985, Pita-Pereira et al. 2005). *Lutzomyia intermedia* seems to play an important role in the transmission mechanism of American Cutaneous Leishmaniasis, due to their high degree of antrophofily and abundance, in many Brazilian regions, including Rio de Janeiro (Rangel & Lainson 2009), where it has been found to be naturally infected with *Leishmania*, presumably *L.* (*V.*) *braziliensis* (Rangel et al. 1984). According to Carvalho et al (2014) the lists of sand flies of the state of Rio de Janeiro, shows records of sand fly species from 46 municipalities. Otherwise the lists corresponds to only 50% of the 92 municipalities of Rio de Janeiro State, and that some other species could certainly be found in other areas with future studies.

The abundance of male and female, considering the whole assemblage, is almost 1:1. However, between sites, and seasons, there were significant differences. On WE1, we found the highest proportion of males, compared to females. The higher proportion of males in the population have been previously reported and this corroborates the results obtained in studies involving sampling of phlebotomines using light trap (Dye et al. 1991, Barata et al. 2004, Rosario et al 2017). For example, male sand flies are attracted to plants as a source of sugar meals. Plants also provide suitable resting, breeding and mating sites for males (Rangel & Lainson 2009).

In the present study, species richness varied a little among different environments on the same category of humidity, although, there were some differences in species composition between them. Indeed, some species are only present in a single site, at a very low density. The capture of certain species in a reduced number and their presence in only one of the studied areas, leads us to believe that among sand flies, differentiated degrees of adaptability might occur in disturbed environments and in isolated habitats due to land use expansion (Ready et al. 1983). Other studies showed a similar pattern; the phlebotomine fauna is composed of a few dominant species and a large number of species with few individuals (see Silva et al 2010, Barata et al 2011, Rosario et al 2017). Further studies must be undertaken, so as to evaluate the impact of anthropogenic environment on the wild flora and fauna of these ecosystems, by considering aspects of behavior, physiology and dispersion of these insects, in order to elucidate the changes, as to the dominance and the composition of the entomological fauna observed in the present study.

It was expected that on WE, the abundance of sand flies would be higher than the driest environment, due to its proximity to the river. While certain sand fly species exhibit local extinctions, others are predicted to adapt successfully as well as increase their relative abundance in modified habitats. That is the case of *L. intermedia* and *L. whitmani*, found in our study. Rangel and Lainson (2009) reported and relate environmental changes to the modification on phlebotomines habitat. All of these factors also affect the spatial and temporal distribution of vectors and reservoirs, which in turn, affect the epidemiology and dynamics of pathogen transmission to the human population. It is important to note that, contrary to the semidecidual fragments of forest sampled in this study, the remnants of Riparian Forest are heavily impacted by human activities and most part of the forest have been completely converted into pastures or agriculture fields (Yasmine Antonini, pers. obs). The same result was found in other studies in forest fragments (Azevedo et al. 2011). According to Martin & Rebêlo (2006), this low frequency may be associated with various factors, such as the absence of a source of blood meals and shelters around the vicinity of the collection points, and the small area of influence of the light traps (5 m) (Dye et al. 1991). Nonetheless, some species exist naturally at very low densities, and their populations can, therefore, suffer considerable reductions in size of small fragments, making them more vulnerable to local extinction.

The species composition changes from the wet to the dry season, and also among sampling sites. The low similarity is an evidence of high seasonality of the assemblage. *Lutzomyia intermedia*, for example, is very seasonal, presenting a higher abundance during wet season. Regarding seasonality, previous studies also reported a higher frequency of *L. intermedia* in wet and warmer months (Gouveia et al. 2012). *Lutzomyia migonei*, *L. lutziana* and *Brumptomyia* sp. were also found in higher abundance during this period. It can be concluded that the seasonal distribution of phlebotominae vectors could be influenced by the characteristics of their environment (Barata et al. 2011).

The low similarity between pairs of habitats, taking into account, the distance, was expected, and our results are in agreement with a growing body of evidence (Ruokolainen et al. 2007; Vasconcelos et al. 2010) which demonstrates that environmental gradients affect the turnover of plant species and invertebrates stronger in the tropical forests than in geographic gradients.

Studies in which the environment was managed to reduce contact with phlebotominae vectors, indicate that the environment can influence seasonal and spatial distributions of vectors (Gouveia et al. 2012, Souza et al. 2015). In the present study, *B. avellari* and *L. tupynambai* are projected to have the greatest increase in range size over time, perhaps due to the higher abundance both in DE and WE. The generalist behavior on habitat selection, which was formerly reported for other sand flies species should also be considered (Souza et al. 2015). *Brumptomyia* species are broadly distributed in America, occurring mainly in forested areas and constitute the group of sand flies commonly associated with armadillo burrows (Martins et al. 1978).

The results presented demonstrated a very dissimilar composition of sand flies in the two habitats (wet and dry). We found that there are site specific differences in sand fly collections. These observations led to a conclusion that environmental conditions available for each species could be different across the environments, taking into account the degree of humidity. The result of this analysis could be explained by the behavioral differences that exist between the most abundant species of the two environments.

The entomologic biodiversity of the Atlantic Forest, the most impacted vegetation type in Brazil, is not completely known and for sand flies, there are few studies. According to the Fundação SOS Mata Atlântica, the remnants of forest in Rio de Janeiro State is less than 10% of the original Biome. The findings presented in this study make up the first information regarding the sand fly fauna found in these forest fragments in the Atlantic Forest and contribute to the knowledge on this insect group distribution, as well as to the better understanding of the leishmaniasis vector epidemiology in the Southeastern region. The present study brings an important contribution to the knowledge of sand flies, as a group of insects and the potential threats imposed by them in animal population due to disease transmission, in the the Atlantic Forest biome. It also creates more awareness on the importance of maintaining these forest areas for the balance of the entomological populations and their interaction with the neighboring community.

Acknowledgments

Roberth Fagundes for their help during field collection. CNPq provided a scholarship to YA and to GWF. The English version was revised by Manuscript Proof Reading and by Erik Wild.

Author Contributions

Y. ANTONINI**:** Contribution in the concept and design of the study, data collection, data analysis and interpretation and manuscript preparation.

R. BARATA: Contribution in the analysis and interpretation and manuscript preparation.

G.W. FERNANDES**:** Contribution to critical revision, adding intelectual content.

Conflict of Interest

The authors declares that they have no conflict of interest related to the publication of this manuscript.

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Received: 29/08/2016
Revised: 25/05/2017
Accepted: 31/05/2017
Published online: 06/07/2017