The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2014, **176**, 369–383. With 5 figures

Osmophore structure and phylogeny of *Cirrhaea* (Orchidaceae, Stanhopeinae)

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Received 7 January 2014; revised 31 May 2014; accepted for publication 1 August 2014

Phylogenetic relationships and osmophore evolution of six *Cirrhaea* spp. were studied. Floral morphology was analysed using fresh flowers, and osmophore anatomy was determined on the basis of fixed flowers. Phylogenetic relationships of *Cirrhaea* were inferred on the basis of internal transcribed spacer (ITS), *matK* and *trnL-F* regions using maximum parsimony and Bayesian analyses. Floral morphology and osmophore structure vary among species. All *Cirrhaea* osmophores have a secretory epidermis without papillae. *Cirrhaea* is monophyletic and includes three subclades: (1) *C. dependens*/*C. nasuta*, with a secretory cylindrical protuberance at the base of the labellar midlobe; (2) *C. fuscolutea*/*C. longiracemosa*, with a secretory tissue at the base of the shell-shaped midlobe; and (3) *C. loddigesii*/*C. seidelii* with secretory tissue on the inner surface of the lateral lobes. The features of the flowers and osmophores in *Cirrhaea* spp. extend our knowledge of the diversity of secretory structures in Stanhopeinae, and demonstrate that floral morphology reflects phylogenetic relationships in *Cirrhaea*. © 2014 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2014, **176**, 369–383.

ADDITIONAL KEYWORDS: anatomy – DNA – flower morphology – fragrance – labellum – light microscopy – orchid – phylogeny – secretory epidermis.

INTRODUCTION

Subtribe Stanhopeinae (Epidendroideae, Cymbidieae) comprises 20 genera distributed throughout tropical America, including *Cirrhaea* L. (Pridgeon *et al*., 2009). Endemic to Brazil, *Cirrhaea* is composed of seven species: *C. dependens* Loudon, *C. fuscolutea* Lindl., *C. loddigesii* Lindl., *C. longiracemosa* Hoehne, *C. nasuta* Brade, *C. seidelii* Pabst and *C. silvana* V.P.Castro Neto & M.A.Campacci (Govaerts, 1999, 2003). Except for *C. silvana*, which is only found in the southern part of the state of Bahia, *Cirrhaea* is distributed across the eastern portion of south-eastern Brazil. These species, which grow as scattered individuals in the Atlantic Forest, are rare in natural habitats (Pansarin, Bittrich & Amaral, 2006).

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Like other genera of Stanhopeinae, *Cirrhaea* flowers produce floral fragrances as a reward for male euglossine bees (Hymenoptera, Apidae; Williams, 1982). In Orchidaceae, beyond Stanhopeinae, floral fragrances as a reward have been reported for Catasetinae and some species of Oncidiinae, Maxillariinae and Zygopetalinae (Williams, 1982). They are produced almost exclusively by glandular or epidermal structures (osmophores) of the labellum (Vogel, 1963a, b), generally formed by a single layer of epidermal cells, or by unicellular or multicellular papillae (Curry *et al*., 1991; Endress, 1994; Ascensão *et al*., 2005; Cseke, Kaufman & Kirakosyan, 2007; Pansarin, Pansarin & Sazima, 2008; Pansarin, Castro & Sazima, 2009; Wiemer *et al*., 2009; Francisco & Ascensão, 2013).

In Stanhopeinae, the diversity and complexity in floral morphology have resulted in some of the most

Table 1. Data on study sites (city/state), geographical coordinates and vouchers of the specimens of *Cirrhaea* collected for morphological and anatomical studies

Species	Locality	Coordinates	Voucher
Cirrhaea dependens Loudon	Ubatuba-SP	23°26′S; 45°04′W	*E.R. Pansarin 95* (UEC)
	Nova Friburgo-RJ	22°16′S; 42°31′ ′W	*E.R. Pansarin & L. Mickeliunas 1057* (UEC)
	Jundiaí-SP	23°11′S; 46°53′W	*E.R. Pansarin & L. Mickeliunas 926* (UEC)
Cirrhaea fuscolutea Hook.	Ubatuba-SP	23°26′S; 45°04′W	*E.R. Pansarin 717* (UEC)
Cirrhaea loddigesii Lindl.	Ubatuba-SP	23°26′S; 45°04′W	*F. Pinheiro & A. Cunha 36* (SP)
	Bertioga-SP	23°51′S; 46°08′W	SP *246824*
Cirrhaea longiracemosa Hoehne	Ubatuba-SP	23°26′S; 45°04′W	SP *24486*
	Santa Teresa-ES	19°56′S; 40°36′W	*L.M. Pansarin & E.R. Pansarin 50* (SPFR)
Cirrhaea nasuta Brade	Santa Teresa-ES	19°56′S; 40°36′W	*L.M. Pansarin & E.R. Pansarin 49* (SPFR)
Cirrhaea seidelii Pabst	Santa Teresa-ES	19°56′S; 40°36′W	*E.R. Pansarin et al. 1320* (SPFR)

elaborate pollination mechanisms among Orchidaceae. However, although all species of Stanhopeinae possess fragrance glands that secrete floral fragrance rewards (see Dressler, 1968; Pansarin *et al*., 2006; Pansarin & Amaral, 2009), the morphology and distribution of osmophores on flowers have been studied rarely. According to Curry *et al*. (1991), the osmophores of *Sievekingia* and *Stanhopea* are diffusely dispersed on the labellum hypochile, and either have flat surfaces or possess uni- to multicellular papillae. In *Stanhopea graveolens* Lindl., the glands are located in the basal part of the labellum, and the osmophore surface is wrinkled or rugose (Antón, Kamińska & Stpiczyńska, 2012). In *Acineta*, *Coryanthes* and *Soterosanthus*, although the osmophores are found on horn-shaped protuberances (Pridgeon *et al*., 2009), their anatomical structure is unknown.

Anatomical studies of the structures attracting pollinators or producing rewards are limited for Orchidaceae (e.g. Stpiczyńska, 1993, 2001; Davies, Turner & Gregg, 2003; Teixeira, Borba & Semir, 2004; Davies & Stpiczyńska, 2008, 2009; Pansarin *et al*., 2008, 2009; Nunes *et al*., 2013). In *Cirrhaea*, such data and those concerning floral morphology and its relation to pollinators and pollination mechanisms are only available for *C. dependens* (Pansarin *et al*., 2006).

In recent years, the use of molecular data has altered the classification of Orchidaceae and has brought several changes in the subtribal and generic concepts within the family (e.g. Neubig *et al*., 2012). Whitten, Williams & Chase (2000) studied phylogenetic relationships in Maxillarieae, emphasizing Stanhopeinae, and showed that *Cirrhaea* is sister to *Gongora*. However, only one species of *Cirrhaea* (*C. dependens*) was included in their analyses, and systematic relationships were therefore not correlated with the anatomical and morphological characters of the flowers.

This study describes the floral morphology and osmophore structure of *C. dependens*, *C. fuscolutea*, *C. loddigesii*, *C. longiracemosa*, *C. nasuta* and *C. seidelii*. As mentioned above, Pansarin *et al*. (2006) studied *C. dependens*, but, although it is the most widely distributed species of the genus, they used plants from a single population; we included more species from a wider geographical range. We demonstrate the relationships of floral morphology and anatomy among *Cirrhaea* spp. in the light of a robust phylogenetic study of this endemic genus. We provide a key to species, and compare osmophore structure among species and discuss osmophores in relation to pollination mechanisms, increasing our knowledge of the diversity of secretory structures in Stanhopeinae.

MATERIAL AND METHODS

Plants of *Cirrhaea* spp. were collected in natural populations occurring in Picinguaba nature reserve, within the boundaries of the city of Ubatuba (state of São Paulo, Brazil, approximately 23°26′02″S, 45°04′16″W), and the Estação Biológica de Santa Lúcia, within the boundaries of the town of Santa Teresa (state of Espírito Santo, Brazil, approximately 19°56′08″S, 40°36′01″W). They were kept at the LBMBP Orchidarium of the Universidade de São Paulo (FFCLRP-USP), in the city of Ribeirão Preto, state of São Paulo, Brazil, approximately 21°10′39″S, 47°48′37″W. Fresh flowers at anthesis were collected in the morning from plants kept at the LBMBP Orchidarium and at the Botanical Garden of the Instituto de Botânica de São Paulo (IBt) (Table 1).

Floral features were analysed with a binocular stereomicroscope. Thirty fresh flowers (three plants, three inflorescences) of each species were examined. The morphological study recorded the shape, colour and size of floral parts and considered possible intraspecific variation (Faegri & van der Pijl, 1979). Vouchers were deposited at the herbaria UEC, SP and SPFR (Table 1).

To determine where the scent was produced, fresh flowers on the first day of anthesis were immersed in 0.1% (w/v) aqueous neutral red for 1 h (Vogel, 1962). Once stained, they were rinsed in tap water and examined. Only floral parts showing the presence of osmophores were used in the histological analysis. To characterize the anatomical structure of odour-producing areas, flowers on the first day of anthesis were fixed in buffered neutral formalin (BNF) for 48 h (Lillie, 1965), left in the fixative under low vacuum and stored in 70% ethanol. Lips were dehydrated through a *tert*-butanol series (Johansen, 1940), embedded in paraffin and sectioned. Then, 10–12-mm-thick longitudinal and transverse serial sections were produced with a rotary microtome. They were stained with Safranin O and Astral Blue (Gerlach, 1969), and permanent slides were mounted in synthetic resin. This methodology is similar to that used to study the secretory structures of *Grobya amherstiae* Lindl. (Pansarin *et al*., 2009). Digital images were captured with a Leica DM500 optical microscope using a Leica ICC50 HD camera attached to a PC running IM50 image analysis software.

For phylogenetic analysis, six *Cirrhaea* spp. were analysed as the ingroup. Members of Stanhopeinae were used to verify the position of *Cirrhaea* in the subtribe; Eulophiinae and Cyrtopodiinae were defined as outgroups. A list of ingroup and outgroup species, voucher and GenBank accession numbers is given in the Appendix.

Total DNA was extracted from fresh or silica gel-dried tissues according to a modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). Amplifications were carried out using 50-μL polymerase chain reaction (PCR) volumes. Betaine (5 M) was added to the PCR for DNA strand relaxation. Primers of internal transcribed spacer (ITS) (Sun *et al*., 1994), *matK* (Johnson & Soltis, 1995) and *trnL-F* (Taberlet *et al*., 1991) were used for amplification and sequencing. Taq DNA polymerase was added to the PCR mix at 80 °C after a period of 10 min of denaturation at 99 °C in the thermocycler. Thirty-five cycles were run according to the following programme: denaturation, 1 min, 94 °C; annealing, 45 s, 51 °C (*matK*), 56–58 °C (*trnL-F*) and 64 °C (ITS); extension, 1 min, 72 °C; final extension, 5 min, 72 °C. Amplified PCR products were purified using GFX PCR columns (GE Health Care). Sequencing reactions were prepared using Big Dye 3.1 (ABI), purified PCR products and the same primers as mentioned above. Samples were dehydrated and resuspended with loading dye. Sequences were obtained with Applied Biosystems automated sequencer model 3100. For sequence editing and assembly of complementary and overlapping sequences, Sequence Navigator and Autoassembler (Applied Biosystems) were used. DNA sequences obtained were aligned with BioEdit version 5.0.9.

Maximum parsimony (MP) analyses were run using PAUP 4.0b.5 (Swofford, 2001) with Fitch parsimony (Fitch, 1971), including autapomorphies. All optimizations were carried out using ACCTRAN, and branches of zero length collapsed. The search strategy used for cladistic analyses was 10 000 replications for each random inclusion of each taxon, with the MULTREES option and tree bisection–reconnection (TBR). Relative support for trees was evaluated with 1000 bootstrap replicates (Felsenstein, 1985). The partition homogeneity test in PAUP*4.0b5 (Swofford, 2001) was employed to measure congruence among the phylogenetic trees, using the following parameters: heuristic search, TBR, with 100 additions of random sequences and 500 replicates to generate the null hypothesis. Heuristic searches were conducted with 27 taxa (674 characters) for ITS, 30 taxa (736 characters) for *matK*, 24 taxa (933 characters) for *trnL-F* and 24 taxa (2343 characters) for the combined data of the three regions.

Bayesian inference (BI) analyses were conducted with MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). A combined data matrix containing 24 taxa (2343 characters) was partitioned into three categories (ITS, *trnL-F* and *mat-K*), and the optimal model of sequence evolution for each partition was selected using jModeltest (Posada, 2008) and under the Bayesian information criterion (BIC). The software selected the evolution model GTR + G for ITS and *trnL-F* partitions and GTR + I + G for *mat-K*. Four Markov chains were run simultaneously for three million generations, with parameters sampled every 100 generations. The consensus tree was calculated after removal of the first 3000 trees, which were considered as 'burn-in'. Posterior probability (PP) values > 0.5 were calculated and mapped onto branches of the consensus tree.

RESULTS

The morphological characteristics of the flowers, the anatomical structure of the secretory tissues and notes on geographical distribution are presented for each species. We also present a dichotomous identification key.

Cirrhaea dependens

Flowers pale green or creamy to red–brown with reddish-purple spots or transverse lines, 2.5 × 3.2 cm (Fig. 1A). Sepals green to red–brown with revolute margins; dorsal sepal linear-lanceolate with acute and revolute apex, 3.1–3.2 × 0.6–0.7 cm; lateral sepals elliptic-lanceolate with an acute apex curved downwards, 2.8–2.9 × 0.6–0.7 cm. Petals pale green to

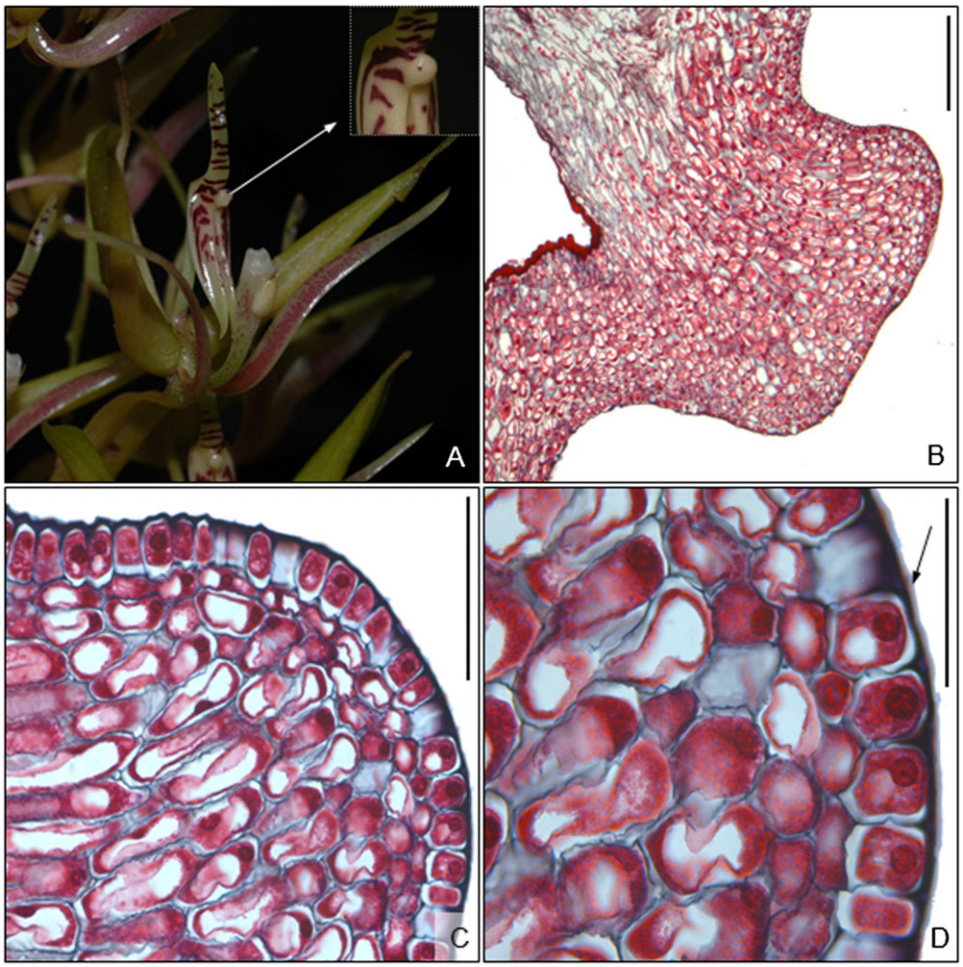

Figure 1. A–D, *Cirrhaea dependens*. A, Flower in frontal view. Note the cylindrical protuberance of the lip (detail). B, Cross-section of the lip protuberance. C, Detail of the lip protuberance showing the secretory epidermis and the underlying parenchyma. D, Detail of the secretory epidermis covered by a thin cuticle (arrow). Scale bars: B, C, 100 μm; D, 50 μm.

red–brown, linear-lanceolate, erect, convex, with acute apex, 2.75 × 0.3 cm. Labellum pale-green with red–brown lines or entirely reddish-purple, fleshy, three-lobed and unguiculate; lateral lobes lanceolate, parallel, 1.1 × 0.4 cm; midlobe narrow, flat and erect, 0.9 × 0.2 cm. Secretory tissue located on a cylindrical protuberance, 0.12 × 0.11 cm (Fig. 1A, inset). Column white-greenish with red–brown dots or completely reddish-purple, curved at base, 1.35 × 0.25 cm; rostellum quadrangular, concave, 0.3 × 0.22 cm; stigma 0.5 × 1 mm; anther white-hyaline, transversely elliptic with rounded apex, 0.45 × 0.2 cm; pollinarium 0.5 cm; pollinia yellow, elliptic-lanceolate, 0.35 × 0.5 mm; stipe white-hyaline, 1.8 × 0.4 mm; viscidium creamy, elliptic, 0.5 × 0.8 mm.

The secretory tissue occurs mainly on the cylindrical protuberance of the labellum, just below the apex of the midlobe (Fig. 1A, arrow, inset), and extends to the basal portion of the inner side of the lateral lobes. This tissue (Fig. 1B) is composed of a single layer of epidermal cells (Fig. 1C), and its secretory function is evidenced by cells with a well-developed nucleus, large vacuoles and densely stained cytoplasm (Fig. 1D). The underlying parenchyma is compact and its cells appear to have a secretory function as they also have conspicuous nuclei and large vacuoles. The secretory tissue is covered by a cuticle (Fig. 1D, arrow).

This species is found in Atlantic Forest in north-eastern (Bahia), south-eastern (Espírito Santo, Minas Gerais, Rio de Janeiro and São Paulo) and southern (Paraná and Santa Catarina) Brazil (Barros *et al*., 2014).

Cirrhaea fuscolutea

Flowers predominantly green-yellowish, 1.5 × 2.2 cm (Fig. 2A). Sepals free, green-yellowish internally and

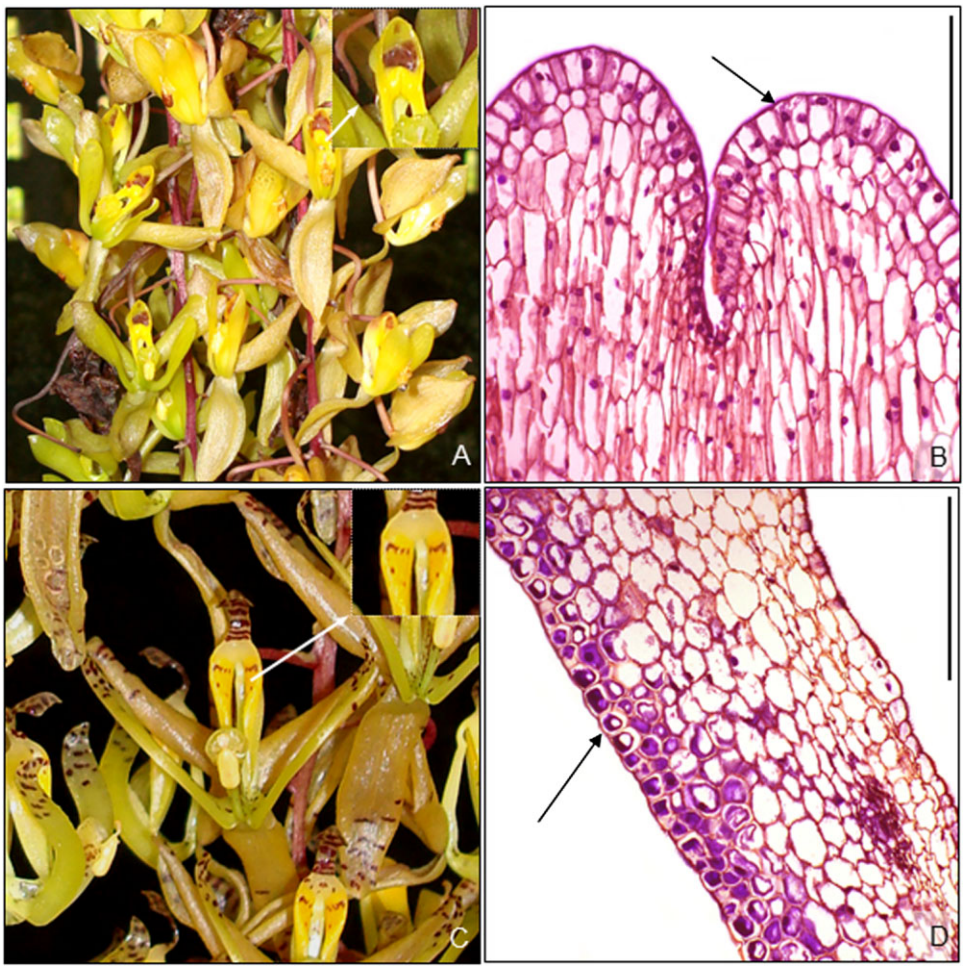

Figure 2. A, Flowers of *Cirrhaea fuscolutea*. Note the secretory region (detail). B, Cross-section of the lip showing the secretory tissue at the apical lobe base (arrow). C, Flowers of *Cirrhaea loddigesii*. Note the secretory region (detail). D, Cross-section of the lip showing the secretory tissue on the inner surface of the lateral lobes (arrow). Scale bars: B, D, 100 μm.

yellow-brownish externally, elliptic-lanceolate with a acuminate apex; dorsal sepal revolute, 2.3–2.4 × 0.8–0.9 cm; lateral sepals facing up forming an angle of 45° with the labellum, 1.9–2.0 × 1.0–1.1 cm. Petals elliptic-lanceolate, yellow to slightly greenish, with rounded apex, 1.8 × 0.55 cm. Lip fleshy, three-lobed and unguiculate; lateral lobes linear-lanceolate, yellow, close to each other, with acute apex, 0.8 × 0.2 cm; midlobe oval, shell-shaped, concave, yellow internally (sometimes with reddish-purple spots) and brownish (or yellow) externally, 0.6 × 0.3 cm; secretory tissue located on a yellow projection at base with a median cleft, 0.2 × 0.15 cm. Column yellow-greenish, curved at base, 1.0 × 0.8 cm; rostellum rounded, plane, with three small projections on the top, 0.35 × 0.4 cm; stigma 0.5 × 2.0 mm; anther lanceolate, white-hyaline, with acute apex, 3.5 × 1.2 mm; pollinarium 0.6 cm; pollinia linear-lanceolate, yellow, 3.1 × 0.9 mm; stipe white-hyaline, 2.0 × 0.5 mm; viscidium white, oval, 0.5 × 0.4 mm.

The secretory cells are mainly located on the protuberance below the apex of the midlobe (Fig. 2A, arrow, inset). The protuberance has an evident slit and comprises a single layer of epidermal cells with a well-developed nucleus and densely stained cytoplasm (Fig. 2B). The underlying parenchyma is made up of elongated cells with large vacuoles and a conspicuous nucleus. The secretory tissue is covered by a cuticle (Fig. 2B, arrow). Secretory cells also occur on the inner surface of the lateral lobes, contributing to fragrance production.

This species is found in Atlantic Forest in north-eastern (Bahia), south-eastern (Rio de Janeiro and São Paulo) and southern (Paraná, Rio Grande do Sul and Santa Catarina) Brazil (Barros *et al*., 2014).

Cirrhaea loddigesii

Flowers predominantly creamy or yellow-pinkish, 1.5 × 2.8 cm (Fig. 2C). Sepals yellow-pinkish with reddish-purple dots at extremities; dorsal sepal elliptic-lanceolate with rounded apex and revolute margins, 2.2 × 0.7 cm; lateral sepals elliptic-lanceolate with subacute and revolute margins, 2.0 × 0.7 cm. Petals yellow-greenish with reddish-purple spots at extremities, linear-lanceolate, erect, with acute apex, 1.7 × 0.3 cm. Labellum three-lobed, fleshy and unguiculate; lateral lobes lanceolate, parallel disposed, slightly spaced at base (*c*. 1.0 mm), yellow at base and creamy towards apex; midlobe arrow-shaped, recurved. Secretory tissue located on lateral lobes, yellow, triangular, 0.4 × 0.2 cm (Fig. 2C, inset). Column white-greenish with some pinkish dots on external surface, 0.9 × 0.3 cm; rostellum transversely oval, concave, 0.27 × 0.35 cm; stigma 0.5 × 1.5 mm; anther elliptic-lanceolate, white-hyaline, with acute apex, 0.4 × 0.17 cm; pollinarium 0.57 cm; pollinia elliptic-lanceolate, yellow, 0.3 × 0.1 cm; stipe white-hyaline, 1.9 × 0.4 mm; viscidium whitish, triangular, 0.7 × 1.0 mm.

The secretory tissue is located on the inner surface of the lateral lobes of the labellum (Fig. 2C, arrow, inset) and comprises a single-layered secretory epidermis and two or three layers of subjacent parenchyma, with anisodiametric, compact cells and small intercellular spaces that accumulate assimilates (Fig. 2D, arrow). The epidermis cells have a dense cytoplasm, a prominent nucleus and large vacuoles (Fig. 2D, arrow).

This species is found in Atlantic Forest in south-eastern (Espírito Santo, Rio de Janeiro and São Paulo) and southern (Paraná, Rio Grande do Sul and Santa Catarina) Brazil (Barros *et al*., 2014).

Cirrhaea longiracemosa

Flowers predominantly green, 1.4 × 3.3 cm (Fig. 3A). Sepals free, green, with slightly revolute margins; dorsal sepal elliptic with rounded apex, 1.85 × 0.9 cm; lateral sepals elliptic with acute apex, 1.8 × 0.9 cm. Petals green-yellowish, linear-lanceolate, erect, forming a 45° angle with the lip, with subacute apex, 1.6 × 0.35 cm. Labellum three-lobed, fleshy and unguiculate (Fig. 3A, inset); lateral lobes elliptic-lanceolate, yellow-whitish with acute apex, spaced *c*. 0.35 cm from each other, 0.6 × 0.3 cm; midlobe oval, broad, shell-shaped, green-yellowish with many reddish-purple striations, concave, 0.3 × 0.35 cm. Secretory tissue at base of midlobe, convex, yellow, 0.3 × 0.3 cm. Column green-yellowish, curved at base, 1.0 × 0.35 cm; rostellum cordate, concave, 0.25 × 0.27 cm; stigma 0.5 × 1.5 mm; anther oblanceolate, white-hyaline, with rounded apex, 3.4 × 1.2 cm; pollinarium 0.58 cm; pollinia linear-lanceolate, yellow, 3.2 × 0.8 mm; stipe white-hyaline, 1.9 × 0.5 mm; viscidium whitish, elliptic, 0.7 × 0.4 mm.

The secretory cells are mainly located on the callosity below the midlobe of the labellum (Fig. 3A, arrow, inset). The secretory tissue below the midlobe possesses a conspicuous hollow and comprises a single layer of epidermal cells (Fig. 3B, arrow). On the inner surface of the lateral lobes, the epidermal cells are also secretory, with six or seven layers of subjacent parenchyma (Fig. 3C). Secretory cells have well-developed nuclei and dense cytoplasm and are covered by a cuticle (Fig. 3D, arrow).

This species is found in Atlantic Forest in south-eastern (Espírito Santo and São Paulo) and southern (Santa Catarina) Brazil (Barros *et al*., 2014).

Cirrhaea nasuta

Flowers predominantly white-pinkish, 2.0 × 2.5 cm (Fig. 4A). Sepals free and white-pinkish; dorsal sepal oval-lanceolate, with acute apex, revolute margins, 2.8 × 1.1 cm; lateral sepals elliptic-lanceolate, with acute, curved apex, 2.6 × 0.9 cm. Petals erect, oblanceolate, forming a 45° angle with the lip, with a falcate base and a subacute apex, 1.9 × 0.5 cm. Labellum three-lobed, fleshy and unguiculate; lateral lobes linear-lanceolate, whitish, close to each other, with acute apex, 1.1 × 0.45 cm; midlobe linear-lanceolate, falcate, with acute apex, whitish with reddish-purple striations, 0.65 × 0.15 cm. Secretory tissue located on a cylindrical protuberance, whitish, oriented towards the column, 0.4 × 0.2 cm. Column whitish with a few pinkish spots laterally, curved at base, 1.4 × 0.3 cm; rostellum oval-cordate, deeply concave, 0.2 × 0.15 cm; stigma 2.0 × 0.5 mm; anther oblanceolate, white-hyaline, with rounded apex, 0.4 × 0.2 cm; pollinarium 0.45 cm; pollinia linear-lanceolate, yellow, 3.5 × 0.5 mm; stipe white-hyaline, 0.4 mm; viscidium whitish, oval, 0.6 × 0.9 mm.

The secretory tissue occurs on the cylindrical protuberance. It is similar to that of *C. dependens*, but about two-fold higher (Fig. 4A, arrow, inset). Epidermal cells are secretory and the underlying parenchyma cells may participate in the production of fragrance because its cells stained strongly with neutral red (Fig. 4B, C). The epidermis is covered by a cuticle (Fig. 4D, arrow).

This species is restricted to Espírito Santo state (south-eastern Brazil) (Barros *et al*., 2014).

Cirrhaea seidelii

Flowers predominantly yellow, 1.1 × 2.0 cm (Fig. 4E). Sepals free, revolute, yellow with reddish-purple dots

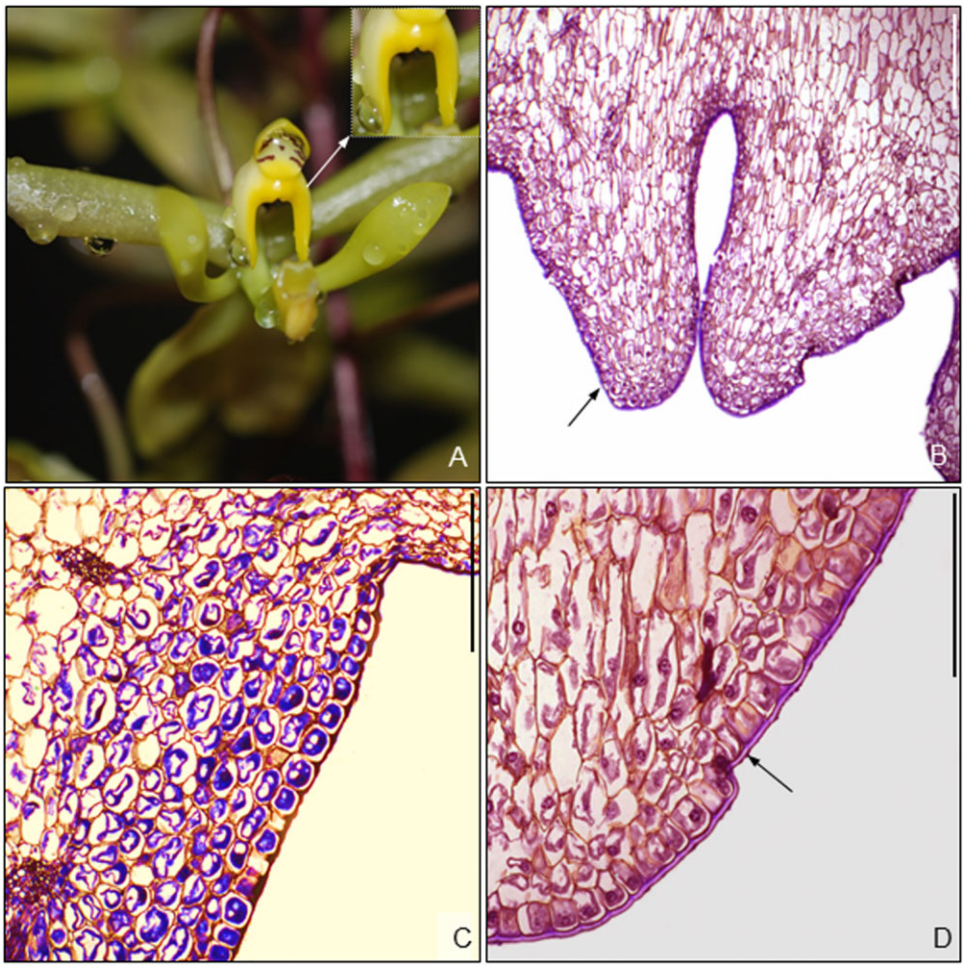

Figure 3. A–D, *Cirrhaea longiracemosa*. A, Flower in frontal view. Note the secretory region (detail). B, Cross-section of the lip showing the secretory tissue at the apical lobe base. C, Cross-section of the lip showing the secretory tissue on the inner surface of the lateral lobes. D, Detail of the secretory epidermis covered by a thick cuticle (arrow). Scale bar: B, D, 100 μm.

at the extremities, elliptic-lanceolate with acute apex; dorsal sepal 1.5 × 0.4 cm; lateral sepals 1.1 × 0.4 cm. Petals linear-lanceolate, concave, yellow with reddish-purple dots at extremities, with rounded apex, 0.9 × 0.15 cm. Labellum three-lobed, fleshy, unguiculate; lateral lobes linear-lanceolate, orange at base and yellow at apex, with a few reddish-purple dots, falcate at base, with acute apex, spaced from each other by *c*. 1.0 cm, 1.1 × 0.3 cm; midlobe rhomboid, yellowish with many reddish-purple striations, curved, forming an angle of almost 90° with the labellum base, with acute apex, 0.35 × 0.15 cm. Column green-yellowish with small reddish-purple spots, curved at base, 0.8 × 0.2 cm; rostellum transversely oval and concave, 0.12 × 0.22 cm; stigma 0.5 × 1.5 mm; anther elliptic, white-hyaline, with rounded apex, 0.28 × 0.13 cm; pollinarium 0.4 cm; pollinia linear-lanceolate, yellow, 2.2 × 0.5 mm; stipe hyaline, 0.5 × 0.2 mm; viscidium whitish, quadrangular, 0.11 × 0.9 mm.

The secretory tissue is located on the inner surface of the lateral lobes (Fig. 4E, arrow, inset), similar to *C. loddigesii*, but is not clearly delimited. Epidermis and subjacent parenchyma (Fig. 4F, arrow) participate in fragrance production. The epidermal cells possess dense cytoplasm, a relatively large nucleus and are covered by a cuticle. Subjacent parenchyma possesses two layers of isodiametric cells with the same characteristics as epidermal cells: the cytoplasm is strongly stained and has large vacuoles, characterizing assimilate accumulation.

This species is restricted to Espírito Santo state (south-eastern Brazil) (Barros *et al*., 2014).

Flowers show some important morphological differences for species identification in *Cirrhaea*. For example, *C. nasuta* is easily distinguishable from

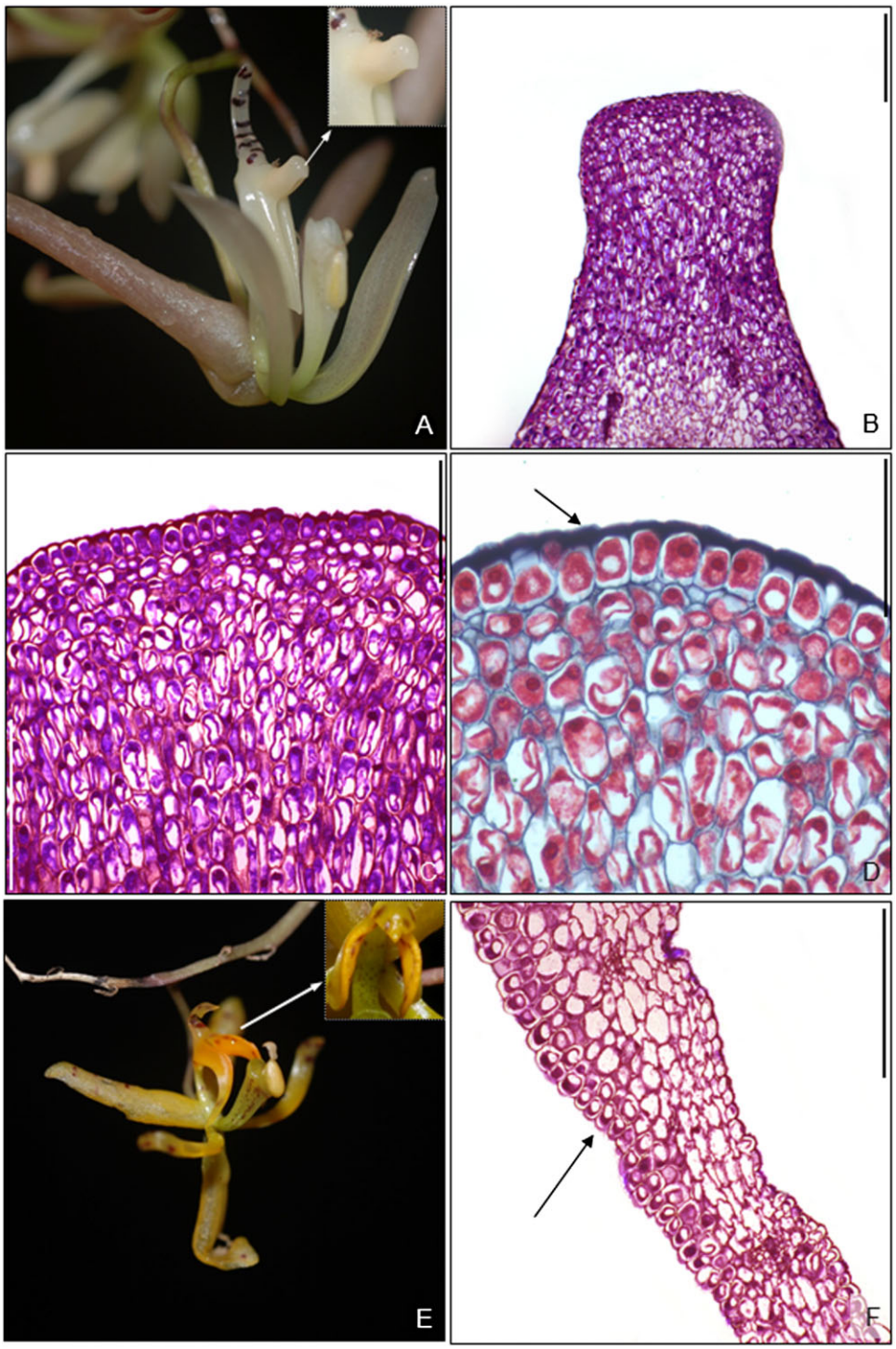

Figure 4. A–D, *Cirrhaea nasuta*. A, Flower in frontal view. Note the secretory region (detail). B, Cross-section of the secretory region showing the secretory epidermis and the underlying parenchyma. C, Detail of the secretory region. D, Detail of the secretory epidermis covered by a cuticle (arrow). E, F, *Cirrhaea seidelii*. E, Flower in lateral view. Note the secretory region (detail). F, Cross-section of the lip showing the secretory region on the inner surface of the lateral lobes (arrow). Scale bar: B, C, F, 100 μm; D, 50 μm.

IDENTIFICATION KEY

1. Flowers with a cylindrical protuberance on midlobe of labellum 2
   Flower without a cylindrical protuberance on midlobe of labellum 3
2. Protuberance of midlobe of labellum 1.0 mm; pollinarium with an elliptic viscidium ..... *Cirrhaea dependens*
   Protuberance of midlobe of labellum 2.0 mm; pollinarium with an oval viscidium ..... *Cirrhaea nasuta*
3. Midlobe of labellum concave and shell-shaped 4
   Midlobe of labellum curved, arrow-shaped or rhomboid 5
4. Inflorescences with 5–25 flowers; pollinarium with an oval viscidium ..... *Cirrhaea fuscolutea*
   Inflorescences with 30–45 flowers; pollinarium with an elliptic viscidium ..... *Cirrhaea longiracemosa*
5. Midlobe of labellum arrow-shaped and recurved; pollinarium with a triangular viscidium ..... *Cirrhaea loddigesii*
   Midlobe of labellum rhomboid, curved, forming an angle of almost 90° with the labellum base; pollinarium with a quadrangular viscidium ..... *Cirrhaea seidelii*

Table 2. Statistical data of phylogenetic trees of the genus *Cirrhaea*, including number of characters used, number of steps, number of variable characters, number of potentially informative phylogenetic characters, number of Fitch trees, consistency index (CI), homoplasy index, excluding uninformative characters (HI), and retention index (RI) for the individual and combined data

Parameter	ITS	*mat-K*	*trnL-F*	Combined
Number of characters	674	736	933	2343
Number of steps	613	237	245	1095
Variable characters	342	138	183	644
Potentially informative characters	172	68	79	315
Number of Fitch trees	9	72	23	3
CI	0.70	0.65	0.81	0.72
HI	0.41	0.50	0.33	0.42
RI	0.71	0.73	0.75	0,67

C. dependens by its more prominent labellum protuberance. In *C. longiracemosa*, the lateral lobes of the labellum are more widely spaced than those of *C. fuscolutea*. Furthermore, *C. fuscolutea* and *C. longiracemosa* differ in number and size of their flowers. *Cirrhaea longiracemosa* possesses inflorescences with 30–45 small flowers, whereas *C. fuscolutea* has 5–25 medium-sized flowers. *Cirrhaea loddigesii* and *C. seidelii* can be separated by their flower size, because those of the latter are smaller, and also by the shape and disposition of their lateral lobes. In addition, the falcate lateral lobes of the labellum of *C. seidelii* are more widely spaced than those of *C. loddigesii*. However, according to the morphological and anatomical features of osmophores, *Cirrhaea* spp. show some similarities that allow them to be grouped into three pairs: *C. dependens* + *C. nasuta*, with a cylindrical protuberance at the base of the midlobe and secretory tissue on the protuberance; *C. fuscolutea* + *C. longiracemosa*, with a shell-shaped midlobe and secretory tissue at the base of it; *C. loddigesii* + *C. seidelii*, with an arrow-shaped midlobe and secretory tissue on the inner surface of the lateral lobes. All secretory tissues are characterized by a single layer of epidermal cells covered by a cuticle. The underlying parenchyma cells are non-isodiametric in all the studied species, except *C. fuscolutea*, which possesses elongated, juxtaposed cells, resembling palisade parenchyma.

Statistical data of the phylogenetic trees for *Cirrhaea*, including the number of characters used, number of steps, number of variable characters, number of trees found, number of potentially informative phylogenetic characters, consistency index (CI), homoplasy index (HI) and retention index (RI) for the individual and combined data, are presented in Table 2.

The MP strict consensus tree of the analysis combining the three regions is almost completely resolved. Furthermore, the topology of the strict consensus tree based on MP and Bayesian analyses is entirely congruent (Fig. 5). The consensus tree for all three regions of DNA is presented in Figure 5.

In all analyses, *Gongora* is sister to *Cirrhaea* (Fig. 5). The *Gongora–Cirrhaea* clade is weakly supported in the *mat-K* [bootstrap support (BS), 63] and *trnL-F* (BS, 65) analyses. However, it is strongly supported in the ITS analysis (BS, 100) and in both

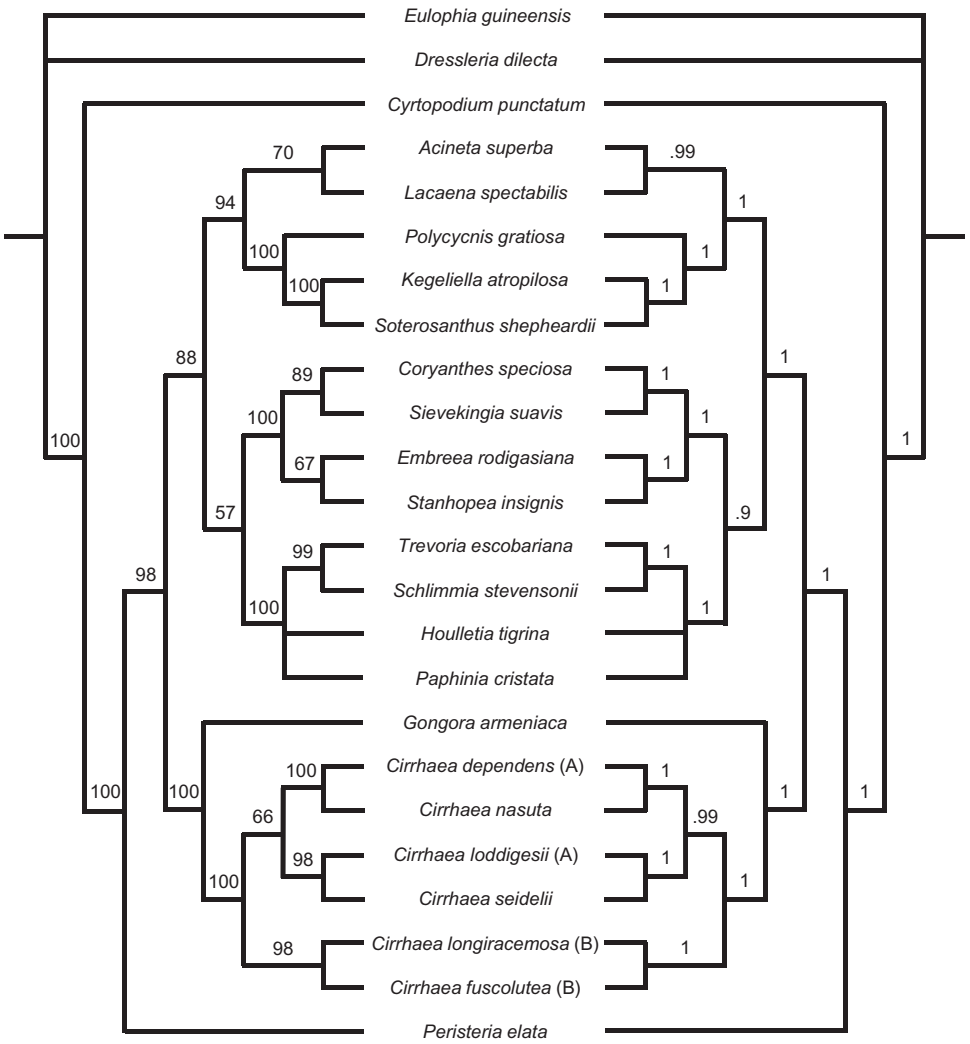

Figure 5. Maximum parsimony (MP, left) and Bayesian inference (BI, right) analyses based on combined internal transcribed spacer (ITS) (nrDNA) and *mat-K* and *trnL-F* (plastid DNA) regions for *Cirrhaea* (Orchidaceae, Stanhopeinae). Bootstrap values > 50 (MP) and posterior probabilities > 0.5 (BI) are given above the branches.

combined MP (BS, 100) and BI (PP, 1) analyses (Fig. 5). In all the trees (individual and combined), *Cirrhaea* is monophyletic (Fig. 5), with good resolution in the ITS (BS, 100), *mat-K* (BS 98) and *trnL-F* (BS, 79) consensus trees, and in both combined MP and BI analyses (BS, 100; PP, 1; Fig. 5).

In the individual analyses of *mat-K* and *trnL-F*, the relationship among *Cirrhaea* spp. is poorly resolved. In the ITS and combined MP and BI analyses, *Cirrhaea* comprises three clades, each one with two species as follows: *C. dependens*/*C. nasuta*; *C. loddigesii*/*C. seidelii*; and *C. fuscolutea*/*C. longiracemosa* (Fig. 5). In the ITS study, these three clades form a polytomy, whereas, in the *mat-K* and combined MP and BI analyses, the clade *C. fuscolutea*/*C. longiracemosa* is sister to a clade including the other species (Fig. 5). In the MP strict consensus tree combining the three regions and in the BI analysis, the subclades are strongly supported: *C. dependens*/*C. nasuta* (BS, 100; PP, 1; Fig. 5); *C. loddigesii*/*C. seidelii* (BS, 98; PP, 1; Fig. 5); and *C. fuscolutea*/*C. longiracemosa* (BS, 98; PP, 1; Fig. 5). The phylogenetic hypotheses based on the combination of the three regions are strongly congruent with the morphology and anatomy of the flowers.

DISCUSSION

It is widely known that members of Stanhopeinae produce floral fragrances that are used in the attraction and reward of male euglossine bees (Williams, 1982). Indeed, members of this subtribe are exclusively pollinated by male Euglossini; the location of the osmophores and floral architecture are fundamen-

tal to the success in the pollination process and the consequent fruit set of the species (Williams & Dodson, 1972). In sympatric Stanhopeinae species sharing the same pollinator species, differences in floral morphology and osmophore location result in the deposition of pollinaria on different parts of the body of the visitor, ensuring reproductive isolation among species (Dressler, 1981; Singer & Sazima, 2004). This is the case in some *Cirrhaea* spp., in which pollinaria are deposited on different sites on the legs of the bees (Pansarin *et al*., 2006; L. M. Pansarin *et al*., unpubl. data). Viscidium size and form (elliptic in *C. dependens* and *C. longiracemosa*, triangular in *C. loddigesii*, oval in *C. fuscolutea* and *C. nasuta*, and quadrangular in *C. seidelii*) may also be important in the identification of sympatric *Cirrhaea* spp., particularly in studies of pollination biology using fragrance baits (Pansarin *et al*., 2006).

Although information on secretory structures, floral rewards and pollination mechanisms is available for several Brazilian orchids (Mickeliunas, Pansarin & Sazima, 2006; Pansarin & Amaral, 2006, 2008; Pansarin *et al*., 2006), detailed studies on the anatomy of secretory tissues are scarce. However, comprehensive data are available for, for example, species of *Bulbophyllum* (Teixeira *et al*., 2004), *Grobya amherstiae* Lindl. (Pansarin *et al*., 2009), *C. dependens* (Pansarin *et al*., 2006) and *Vanilla edwallii* Hoehne (Pansarin, Aguiar & Pansarin, 2014). Davies & Stpiczyńska (2006, 2007, 2008, 2009 and 2012) also studied the anatomy of the secretory structures of Brazilian orchids, but with a greater focus on Oncidiinae and Maxillarinae.

The secretory nature of the epidermal osmophores of *Cirrhaea* spp. was clearly demonstrated by the presence of cells with high metabolic activity (Fahn, 1979), and the possible participation of the underlying parenchyma layers in the production of fragrance could be similar to many reward-producing orchid species (e.g. Davies *et al*., 2003; Davies & Stpiczyńska, 2009; Antón *et al*. 2012). The secretory epidermal cells of the osmophores and their subjacent parenchyma usually possess a thick cellular wall and are often smaller than the cells of inner parenchyma and non-secretory parenchyma cells. The types of epidermal osmophores reported here for *Cirrhaea*, characterized by an underlying parenchyma, have already been documented in other Stanhopeinae, namely *Stanhopea anfracta* Rolfe and *S. pulla* Rchb.f. (Curry, Stern & McDowell, 1988), in *S. graveolens* Lindl. and in the Catasetinae *Cycnoches chlorochilon* Klotzsch (Antón *et al*., 2012). According to Curry *et al*. (1988), the similarities between the epidermal cells and underlying parenchymatous cells result in osmophores with homogeneous patterns of odour-producing tissue with more than one layer of cells. Stpiczyńska (1993) found this same osmophore pattern in *Cymbidium tracyanum* Hort. In *Ophrys fusca* Link. and *O. lutea* Biv., the osmophores are made up of a single and well-differentiated layer of secretory epidermis and two or three layers of subjacent parenchyma (Ascensão *et al*., 2005), and *Bulbophyllum* spp. possess epidermal palisade-like cells with a large central nucleus and strongly stained cytoplasm (Teixeira *et al*., 2004). The underlying tissue of some *Bulbophyllum* spp. has two or three layers of compact parenchymatous cells with a dense cytoplasm; the secretory parenchyma cells are smaller than the inner parenchymatous cells (Teixeira *et al*., 2004), as is the case in *Cirrhaea* spp.

A cuticle covering the osmophores of *Cirrhaea* spp. confers a bright and smooth appearance to the labellum, so that bees slip and fall when they collect volatile oils or abandon the flower (Pansarin *et al*., 2006; L. M. Pansarin *et al*., unpubl. data). In some Stanhopeinae, the cuticle does not block the emission of fragrance, which is released by diffusion (Curry *et al*., 1988). As there are no pores or stomata in the cuticular surface of *Cirrhaea* spp., fragrance release probably occurs through cuticular diffusion. The emission of volatile compounds by cuticular diffusion has also been recorded in some *Stanhopea* spp. (Stern, Curry & Pridgeon, 1987) and in other orchids, such as some species of *Scaphosepalum* (Pridgeon & Stern, 1985).

The secretory surface of the labellum of *Cirrhaea* spp. is a structure similar to that found in other Stanhopeinae, such as *Sievekingia* (Curry *et al*., 1991). Furthermore, other Stanhopeinae possess osmophores with trichomes, such as some *Stanhopea* spp. (Curry *et al*., 1991), or have papillose and multicellular osmophores, such as *Stanhopea lietzei* (Regel) Schltr., *S. insignis* Frost ex Hook. (Pansarin, 2000; Pansarin & Amaral, 2009), *S. tigrina* (Curry *et al*., 1991), *S. graveolens* Lindl. and *Cycnoches chlorochilon* Klotzsch (Antón *et al*., 2012). According to Curry *et al*. (1991), osmophores with a flat surface, such as those of *Cirrhaea* spp., provide a smaller area of fragrance dispersal compared with more elaborate, odour-producing structures with uni- or multicellular papillae or trichomes.

All phylogenetic analyses reveal that *Cirrhaea* is sister to *Gongora*. They share the same spectrum of pollinators (mainly *Euglossa* spp.) and similar vegetative characteristics, as many flowers of *Gongora* resemble those of *C. fuscolutea* and *C. longiracemosa*. However, flowers of the two genera are different morphologically. *Gongora* has backward-facing lateral sepals, petals attached to the side of the column, and an erect and basal protuberance on each lateral lobe of the horizontally disposed labellum (Martini, Schlindwein & Montenegro, 2003). In *Cirrhaea*,

lateral sepals are spreading, and the labellum is erect. Moreover, in *Cirrhaea*, the ovary and pedicel are curved, so that labella are distant from the inflorescence central axis, unlike in some *Gongora* spp. (Gerlach, 1999). With regard to the osmophores, in *G. quinquenervis* Ruiz & Pav., for example, the secretory regions are located mainly at the labellum base (hypochile) and on the lateral sepals (Martini *et al*., 2003). Conversely, the secretory tissue in *Cirrhaea* is predominantly located at the base of the labellum. In other Stanhopeinae, as in some *Stanhopea* spp., the osmophores are located on the sacciform hypochile (Curry *et al*., 1991; Pansarin & Amaral, 2009).

Cirrhaea is monophyletic, forming a well-supported clade in all reconstructed phylogenetic hypotheses. Species are characterized by unifoliate pseudobulbs, plicate and long-pseudopetiolate leaves, lateral and pendent inflorescences, ephemeral flowers with labellum and column erect, and a three-lobed labellum not divided into hypochile, mesochile and epichile (Pridgeon *et al*., 2009). In the cladogram published by Whitten *et al*. (2000), *Cirrhaea* is an early branching member of the base of the Stanhopeinae clade with *Gongora* as its sister group. *Stanhopea* spp. appear as the most derived among Stanhopeinae and have hairy or papillose, multicellular osmophores (Curry *et al*., 1991; Pansarin, 2000; Pansarin & Amaral, 2009).

With regard to the phylogenetic analyses, many studies have reported divergent results from the use of morphological characters with molecular data in phylogenetic reconstructions (Wortley & Scotland, 2006) and how these datasets can be compared (Thiele, 1991; Wiens, 2001). Our data demonstrate that the morphological/anatomical data are congruent with the molecular data in this case.

Morphological and anatomical characteristics of the flowers and osmophores of *Cirrhaea* spp. allow new insights into the floral biology of this rare Brazilian genus. Furthermore, such information represents an additional tool to identify the *Cirrhaea* spp. occurring in south-eastern Brazil, and provides important knowledge on the morphological and anatomical diversity of the secretory structures in Stanhopeinae and their relation to pollination mechanisms.

ACKNOWLEDGEMENTS

We thank the Núcleo Picinguaba, Instituto Florestal for granting permission for field work, Günter Gerlach for providing leaf samples for DNA extraction and Alain François for improving the English. This study is part of the PhD Thesis of LMP at the Departamento de Biologia Vegetal, Pós-Graduação em Biologia Vegetal, Universidade Estadual de Campinas. This research was supported by the State of São Paulo Research Foundation (FAPESP) as part of the Thematic Project Functional Gradient (Process Number 03/12595-7), within BIOTA/FAPESP – The Biodiversity Virtual Institute (http://www.biota.org.br), as well as by FAPESP (grant 07/55591-2), Coordination for the Improvement of Higher Education Personnel (Capes) and National Council for Scientific and Technological Development (Cnpq).

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APPENDIX

SPECIES OF *CIRRHAEA* AND OUTGROUPS INCLUDED IN THE MOLECULAR STUDIES, VOUCHERS, DATA COLLECTED AND GENBANK ACCESSION NUMBERS

Species	Voucher	Data collected	GenBank accession
Acineta superba (Kunth) Rchb.f.	Gerlach 03/1716 (M)	ITS, *mat-K*, *trnL-F*	KM458403, KM458422, M458444
Cirrhaea dependens Loudon	E.R. Pansarin 95 (UEC) **(A)**	ITS, *mat-K*, *trnL-F*	KM458413, KM458432, KM458454
	E.R. Pansarin & L. Mickeliunas 1057 (UEC) **(B)**	ITS, *mat-K*	KM458414, KM458433
	E.R. Pansarin & L. Mickeliunas 926 (UEC) **(C)**	*mat-K*	KM458434
		ITS, *mat-K*, *trnL-F*	AF239381, AF23947, AF239573
	*Whitten,W.M., Williams,N.H. and Chase,M.W. s/n (FLAS) **(D)**		
Cirrhaea fuscolutea Hook.	E.R. Pansarin 717 (UEC) **(A)**	ITS, *mat-K*, *trnL-F*	KM458419, KM458442, KM458457
	E.R. Pansarin & L.M. Pansarin 1065 (UEC) **(B)**	ITS, *mat-K*	KM458420, KM458443
Cirrhaea loddigesii Lindl.	F. Pinheiro & A. Cunha 36 (SP) **(A)**	ITS, *mat-K*, *trnL-F*	KM458415, KM458436, KM458456
	SP 246824 **(B)**	ITS, *mat-K*	KM458416, KM458437
Cirrhaea longiracemosa Hoehne	SP 24486 **(A)**	ITS, *mat-K*, *trnL-F*	KM458417, KM458439, KM458459
	L.M. Pansarin & E.R. Pansarin 50 (SPFR) **(B)**	*mat-K*	KM458440
Cirrhaea nasuta Brade	L.M. Pansarin & E.R. Pansarin 49 (SPFR)	ITS, *mat-K*, *trnL-F*	KM458418, KM458441, KM458455
Cirrhaea seidelii Pabst	E.R. Pansarin et al. 1320 (SPFR)	ITS, *mat-K*, *trnL-F*	KM458421, KM458438, KM458458
Coryanthes speciosa (Hook.) Hook.	Gerlach 09/2718 (M)	ITS, *mat-K*, *trnL-F*	KM458408, KM458426, KM458448
Cyrtopodium punctatum (L.) Lindl.	*Chase O-126 (K)	ITS, *mat-K*, *trnL-F*	AF239412, AF239508, AF239604

APPENDIX *Continued*

Species	Voucher	Data collected	GenBank accession
Dressleria dilecta (Rchb.f.) Dodson	*Whitten F1046 (FLAS)	ITS, *mat-K*, *trnL-F*	AF239411, AF239507, AF239603
Embreea rodigasiana (Claess. *ex* Cogn.) Dodson	*Whitten 90105 (FLAS)	ITS, *mat-K*, *trnL-F*	AF239358, AF239454, AF239550
Eulophia guineensis Lindl.	*Whitten s.n. (FLAS)	ITS, *mat-K*, *trnL-F*	AF239413, AF239509, AF239605
Gongora armeniaca (Lindl. & Paxton) Rchb.f.	*Whitten F1636 (FLAS)	ITS, *mat-K*, *trnL-F*	AF239386, EU214355, AF239578
Houlletia tigrina Linden *ex* Lindl. & Paxton	*Whitten 91354 (FLAS)	ITS, *mat-K*, *trnL-F*	AF239370, AF239466, AF239562
Kegeliella atropilosa L.O. Williams & A.H. Heller	Gerlach 08/0896 (M)	ITS, *mat-K*, *trnL-F*	KM458406, KM458424, KM458446
Lacaena spectabilis (Klotzsch) Rchb.f.	Gerlach 03/2470 (M)	ITS, *mat-K*, *trnL-F*	KM458411, KM458429, KM458451
Paphinia cristata (Lindl.) Lindl.	*Singer *et al*., 2008 Gerlach 1285 (M)	ITS *mat-K*, *trnL-F*	EU441207 KM458430, KM458452
Peristeria elata Hook.	Gerlach 1289 (M)	ITS, *mat-K*, *trnL-F*	KM458404, KM458423, KM458445
Polycycnis gratiosa Endrés & Rchb.f.	*Whitten 93178 (FLAS)	ITS, *mat-K*, *trnL-F*	AF239373, AF239469, AF239565
Schlimmia stevensonii Dodson	*Whitten 94107 (FLAS)	ITS, *mat-K*, *trnL-F*	AF239367, AF239463, AF239559
Sievekingia suavis Rchb.f.	Gerlach 99/0838 (M)	ITS, *mat-K*, *trnL-F*	KM458410, KM458428, KM458450
Soterosanthus shepheardii (Rolfe) Jenny	Gerlach 1299 (M)	ITS, *mat-K*, *trnL-F*	KM458407, KM458425, KM458447
Stanhopea insignis Frost *ex* Hook.	Pansarin ER 212, 01/2000, Ubatuba-SP (UEC)	ITS, *mat-K*, *trnL-F*	KM458412, KM458431, KM458453
Trevoria escobariana Garay	Gerlach 1326 (M)	ITS, *mat-K*, *trnL-F*	KM458409, KM458427, KM458449

* Sequences taken from GenBank