Osmophore structure and phylogeny of Cirrhaea (Orchidaceae, Stanhopeinae)

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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.


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 southeastern Brazil. These species, which grow as scattered individuals in the Atlantic Forest, are rare in natural habitats (Pansarin, Bittrich & Amaral, 2006).

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 Cata-setinae 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 papil-lae (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
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 Soterosanthes, although the osmophores are found on horn-shaped protuberances (Pridgon 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. loddigesi, 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 Espirito 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 SPF (Table 1).

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

<table>
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<th>Species</th>
<th>Locality</th>
<th>Coordinates</th>
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<td></td>
<td>Nova Friburgo-RJ</td>
<td>22°16′S; 42°31′′W</td>
<td>E.R. Pansarin &amp; L. Mickeliunas 1057 (UEC)</td>
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<td></td>
<td>Jundiaí-SP</td>
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<td>23°26′S; 45°04′W</td>
<td>E.R. Pansarin 717 (UEC)</td>
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<tr>
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<tr>
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<td>23°26′S; 45°04′W</td>
<td>SP 24486</td>
</tr>
<tr>
<td><em>Cirrhaea nasuta</em> Brade</td>
<td>Santa Teresa-ES</td>
<td>19°56′′S; 40°36′′W</td>
<td>L.M. Pansarin &amp; E.R. Pansarin 50 (SPFR)</td>
</tr>
<tr>
<td><em>Cirrhaea seidelii</em> Pabst</td>
<td>Santa Teresa-ES</td>
<td>19°56′′S; 40°36′′W</td>
<td>L.M. Pansarin &amp; E.R. Pansarin 49 (SPFR)</td>
</tr>
</tbody>
</table>

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 31000. 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*4.0b.5 (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
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

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**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.
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 northeastern (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 linear-lanceolate, white-hyaline, with acute apex, 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 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 sepals 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 oblong-lanceolate, 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, oblong-lanceolate, 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 oblong-lanceolate, 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
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, *Cirrhea 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, *Cirrhea 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.
OSMOPHORES AND PHYLOGENETICS OF CIRRHAEA

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 .................................................. Cirrhaea seidelii
   Midlobe of labellum curved, arrow-shaped or rhomboid .................................................. 4
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 ........ 4
   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

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<td>0.73</td>
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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
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-
OMPHORES AND PHYLOGENETICS OF CIRRHAEA 379

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. seideli) 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 & Stpiczynska (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 & Stpiczynska, 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 Cynoches 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. Stpiczynska (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 Cynoches 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 (Gerlach, 1999). With regard to the osmophores, in Gongora spp. (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-pseudopetiolute 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

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REFERENCES


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## APPENDIX

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

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<th>GenBank accession</th>
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