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Floral micromorphology of the genus *Restrepia* (Orchidaceae) and the potential consequences for pollination

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ABSTRACT

Restrepia is a small Pleurothallid genus, comprising 57 species, 44 of which were discovered since 1970. These species are indigenous to Central and South America, where their montane forest habitats are under increasing pressure from changes in land use. With resulting increasingly fragmented habitats and dwindling numbers, the pollination systems of obligate out-breeding genera, such as *Restrepia*, may no longer function efficiently which could potentially lead to their extinction. As such, the main aim of the current study was to perform an in-depth investigation of floral structures in the genus, using SEM and photographic technology to formulate a putative pollination mechanism for these species.

The floral micromorphology of dorsal sepal and lateral petal osmophores, synsepal, labellum, cirrhi and calli were investigated by scanning electron microscopy (SEM), macro-photography and quantitative analyses of some floral proportions.

The secretory nature of the labellum, synsepal and osmophore papillae were established and the calli were shown to possess a unique papillate, non-secretory structure. A pollination mechanism for the genus was proposed which includes the role of the scent trails produced by the osmophores and the 'trapping' role of the cirrhi. A 'functional fit' between the flower and the pollinator is suggested. In conclusion, we consider *Restrepia* to represent a non-nectar rewarding and 'deceptive' orchid genus and that this pollination mechanism may be directly linked to the breeding system (gametophytic self-incompatibility) in this genus.

1. Introduction

The genus *Restrepia* belongs to the Pleurothallidinae, the largest sub-tribe within the Orchidaceae. This small Pleurothallid genus currently comprises 57 (WCSP, 2015) exclusively Neotropical species (Millner, 2013), many of which are narrow endemics, indigenous to the montane forests of Venezuela, Colombia, Ecuador, Peru and Bolivia (Luer, 1996), with a small number of species originating in Central America (Luer, 1996). In common with other genera located in these habitats, these species face increasing pressure from habitat degradation through deforestation, fragmentation and changes in land use (Millner, 2013; Millner et al., 2008, 2015).

The largest change to this habitat resulted from the completion of the Pan American Highway throughout the countries of Central and South America. This improved road infra-structure made access to previously remote areas possible and, as a consequence, has led to the discovery of 44 new *Restrepia* species since 1970 (WCSP, 2015), together with many discoveries in other orchid genera. However, the accompanying changes in land use alongside the highway have also served to put many species at risk (Millner, 2013). The long-term survival of any species ultimately depends on its ability to reproduce. For obligate outbreeding genera, such as *Restrepia*, (Millner et al., 2015), dwindling numbers and habitat mean that the chances of successful cross-pollination and thereby their survival are decreased. An understanding of the breeding system and its related pollination mechanism is therefore of great importance for the future conservation of the genus.

Although floral structure and micromorphology are crucial to the pollination biology of any angiosperm, little is known of these in *Restrepia* (Luer, 1996). Studies of pollination within the Pleurothallidinae have not included this genus (Blanco and Barboza, 2005; Borba and Semir, 2001; Borba et al., 2001, 2002; Endara et al., 2010). Consequently, the micromorphology and pollination biology of *Restrepia* remain poorly understood and it was for this reason that the current study was initiated.

The main distinguishing floral characteristics of the genus were first documented by Humboldt (Humboldt et al., 1816) and were

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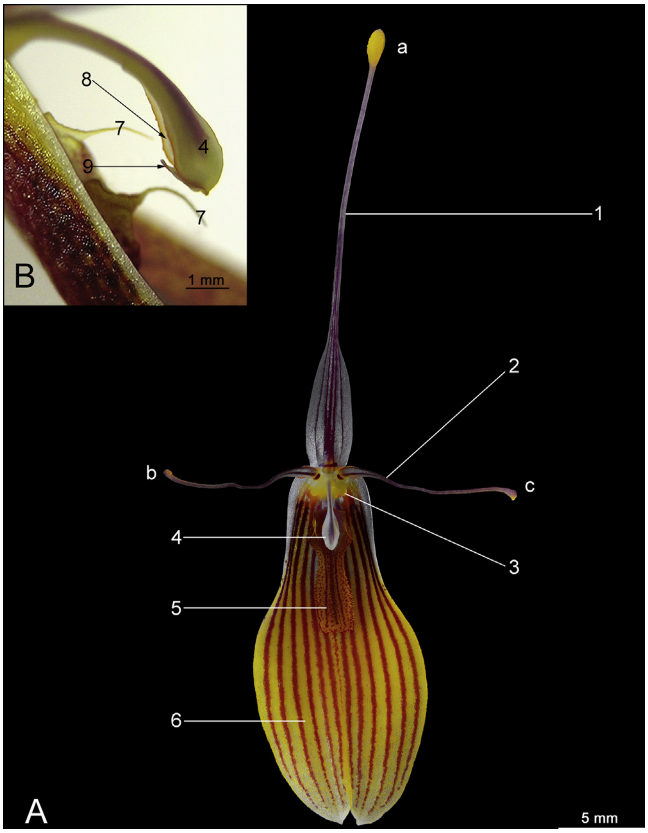

Fig. 1. Floral structures of *Restrepia*.
A: main photograph, *R. brachypus*. Flowers are resupinate and pedunculate or sessile in a minority of species. The dorsal sepal (1) and the lateral petals (2) are elongated and filamentous with clavate apices (a, b, and c) containing osmophores (Pridgeon and Stern, 1983) which resemble thorns. The column foot bears two calli (3), one either side of the base. The column (4) is slender, clavate with a ventral anther and stigma. The third, ventral petal is modified to form a smaller labellum (5), with two uncinate processes (Luer, 1996) or cirrhi (Pridgeon and Stern, 1983) which is the preferred term in this manuscript (inset, 7). The large, colourful synsepal (6) is formed by the joining of the lateral sepals.
B: inset, detail of the column. Detail of the column (4), the cirrhi (7), position of the anther cap (9), covering four equal sized ovoid pollinia and the stigmatic surface (8) positioned on the ventral surface of the column.

later described in more detail by Luer (1996). All species within the genus are similar in respect to their floral structure (Luer, 1996) and a typical exemplar of the genus, *R. brachypus*, Rchb.f., 1886, (WCSP, 2015) is shown in Fig. 1. With regard to the floral micromorphology, Pridgeon and Stern (1983) investigated the function of the apical osmophores of the dorsal sepal and lateral petals, and performed both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of these structures. However, the function(s) of the calli and the cirrhi have never been established, indeed Luer (1996) wondered 'what the function of these strange features (calli) could be'. Since this time, no further studies of the morphology or function of the floral organs in this genus have been published.

As described above, Pridgeon and Stern (1983) performed their investigation of osmophore structure in the early 1980s, prior to the commercial development of Environmental Scanning Electron Microscopy. As such, *Restrepia* floral micromorphology has not been studied using current ESEM/Cryo-SEM technology, capable of producing high-resolution images. In particular, the micromorphology of the calli and the labellar regions have never been recorded in detail. Three distinct areas of the labellum had been recorded by Luer (1996), but he did not study their micromorphology.

The primary objective of the current study therefore, was to perform an in-depth investigation of the morphology/micromorphology of the floral structures of *Restrepia* using SEM and macro-photography techniques, in order to examine the consequences for the pollination in the genus. From which any functional link between the floral morphology and the previously established gametophytic self-incompatibility breeding system of this genus (Millner et al., 2015) could be determined.

2. Materials and methods

2.1. Plant material

The *Restrepia* plants used in the current study came from the personal collection of H. Millner. *R. brachypus* was selected as the main subject for this study as it is easily obtained and is morphologically typical of the genus. All the plants were greenhouse grown under the same conditions. (Minimum night temperature = 58 °F/15 °C; day length = 14 h).

2.2. Scanning electron microscopy

A detailed study of the osmophores, labellum and calli of *Restrepia* was performed using ESEM techniques. In total, the floral organs from 16 flowers from six individual plants of *R. brachypus* were examined and the features confirmed by observations in other species i.e. *R. dodsonii, R. muscifera* and *R. guttulata.* Two flowers from one individual plant of each of these species were examined. This work was performed at the Centre for Electron Microscopy, University of Birmingham, United Kingdom.

Specimens were mounted onto a Cryo Stage (Quorum PolarPrep S2000 Cryo Transfer System, Quorum Technologies, Lewes, East Sussex, UK), and were then rapidly frozen using liquid nitrogen to a temperature of −180 °C and sputter coated with platinum. The Cryo Stage allows rapid freezing which results in improved sample integrity with fewer ice crystals. This produces images which are more 'true to life'. The specimens were examined under a FEI XL30 FEG ESEM, FEI UK Limited, Cambridge, UK, and the images processed in Photoshop.

2.3. Macro-photography

'Focus' or 'image stacking' techniques were used to produce the increased depth of field and detail in the macro photographic images. Multiple images, each with a slightly different plane of focus were taken and then combined, using computer software, into a final composite image. The programmes used were cameraRC, J-ProSoftware LLC, Saint Paul, Minnesota, to produce the image 'stacks' and Zerene Stacker, Zerene Systems LLC, Richland, Washington, to combine the images into a composite. The size of the image stacks produced ranged from 45 to 160 images. A Nikon d7100 DSLR camera and a 60 mm macro lens with combinations of 36 mm, 20 mm and 12 mm extension tubes were used. In some of the composite images the backgrounds were extracted and replaced with a solid black colour in Photoshop. This removed extraneous and irrelevant detail that distracted from the main subject in the image and improved the clarity of the final photographs in Figs. 1A, 2A, 3A, 4A, B and 5A.

2.4. Photographic measurements

Other photographs, not focus stacked, had been recorded of 18 species over the course of this and other research (Millner, 2013; Millner et al., 2008, 2015). A series of measurements was taken from these photographs in Photoshop in order to establish whether a precise size or 'functional' fit between flower and pollinator might