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Article Type: Original Research

Keywords: calli; cirrhi; myophily; osmophores; pollination mechanism; self-incompatibility.

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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 statistical 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.

Response to Reviewers:
15th September, 2016.

Dear Dr Dotterl,
Ms. Ref. No.: FLORA-D-16-00218R1

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Thank you for your favourable reply regarding our manuscript. Since which time, we have been addressing the minor revisions from your review. The main problem concerned entering species authorities into Table 1, about which I contacted you. We tried your suggestion to create a new table, but after due consideration found that this didn't work very well. Therefore, we decided to reformat Table I to include species authorities and have included the reference to the WCSP, (Kew) which is from where this information was obtained. Anyone wishing to check these data, should now be able to do so.
All the minor text, spelling, formatting changes that you suggested were accepted via track changes. Our reponse to some of the other points you suggested for amendment are highlighted below in red.
Pg 11 Line 207 eventually after consideration we decided on the following wording –
We consider them to be……
This expressed our hypothesis better, and still implied it was our idea and not proven fact
Pg 12 lines 212-213
As this sentence was unclear, it has been omitted. The previous few lines, really had said everything that was required.
Pg 12 lines 224-255
Comment 'thus, pollination may no longer occur during thus stage? and animals entering at this stage may not be pollinators?'

The sentence has been rephrased. This seems to be an adaptation to bring about pollination if it has not occurred when senescence begins. The wording should now make this clearer.
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This sentence has been omitted for clarity

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Section 3.1.5 Fly pollination in the Pleurothallidinae
This has now been moved after the description of the calli as suggested

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Many thanks.

Yours sincerely,

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Cover Letter

15th September, 2016.

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We hope that our revised manuscript is now acceptable for publication in *Flora* and we look forward to your response.

Many thanks.

Yours sincerely,

Dr Helen Millner and Dr Timothy Baldwin

HIGHLIGHTS

- *Restrepia* is a small Pleurothallid genus facing habitat loss
- Floral organs were imaged using SEM and macro-photographic techniques.
- *Restrepia* exhibits non-nectar rewarding myophily.

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.

Key words*:* calli; cirrhi; myophily; osmophores; pollination mechanism; self-incompatibility.

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, 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 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, (WSCP, 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 and Baldwin, 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 = 58ºF/15ºC; day length = 14 hours).

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 ºC 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 – 160 images. A Nikon d7100 DSLR camera and a 60mm macro lens with combinations of 36mm, 20mm and 12mm 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 Figures 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 exist, or if *Restrepia* species are pollinated by species of similar body proportions. All the images obtained were corrected to 300 d.p.i. Pixel measurements of the column, labellum and width between the cirrhi (Fig. 3, w) were taken. From these the ratio of labellar to column length and the ratio of the column length to width between the cirrhi (w) were calculated. As ratios were calculated from dimensions within a single image this method rendered different photographic magnification between images irrelevant.

2.5 Different illumination

The calli and surrounding areas were photographed under different illumination i.e. ambient daylight, torchlight and UV 380 nm in a dark room. This was considered a suitable UV

wavelength to use as it was in accordance with the model of fly colour vision (Troje, 1993) and the manner in which flies 'discriminate' spectral stimuli (Arnold *et al.*,2009).

2.6 Tissue staining for lipids

Flowers were stained for the presence of lipids with Sudan B using standard techniques after Howes and Satiat-Jeunemaitre (2001).

3. 1 RESULTS AND DISCUSSION

3.1.1 Osmosphores (Figure 2)

Osmophores are defined as floral tissues specialised for fragrance biosynthesis and secretion (Vogel, 1990; Dressler, 1993) and their structure in orchids was studied using light microscopy by Vogel (1990). The only SEM study to date of *Restrepia* osmophores was published by Pridgeon and Stern (1983). Subsequently, Vogel (1990) and Vogel and Renner (1992) discovered the functional layering of osmophore structures into storage, production and accumulation of lipid rich substances which were found to be precursors of the fragrance itself. The fragrance compounds were shown to accumulate beneath the cuticle and diffuse through it, thereby causing various indentations, shrinkage and rupturing of the osmophore cuticle. These features were not recorded by Pridgeon and Stern (1983).

Very similar structures to the papillate structures found on the adaxial petal and sepal apices of *Restrepia* have been found on the abaxial side of the labellum in *Cyclopogon elatus* (Orchidaceae) (Wiemer *et al.,* 2009). In Wiemer's study, similar features to those found on the cuticle layer in the current study were reported. Pridgeon and Stern's study had previously identified the substance produced by the osmophores in *Restrepia* as a 'fatty oil' or 'aminoid fragrance' (Pridgeon and Stern, 1983) which was further confirmed in *Cyclopogon elatus* (Orchidaceae) (Sazima *et al.*,1993).

The micrographs obtained in the current study confirm the structures described by Pridgeon and Stern (1983), and provide additional information related to the development and senescence of the osmophore papillae. Figure 2 (B, C) shows the papillae at 24 hours pre-anthesis, turgid, with the integrity of their structure uncompromised. By one day post-anthesis (Fig. 2D, E), vesicles or 'blisters' are visible on the surface of the papillae and by two days post- anthesis (Fig. 2F, G) characteristic indentations, shrinking and collapse of the papillae are observed. These images are almost identical to those reported by Wiemer *et al.* (2009). The shrinking and collapse of the papillae apices recorded in the current study agree with the literature – *Cyphomandra* (Solanaceae), (Sazima *et al.*,1993), *Cyclopogon elatus* (Orchidaceae), (Wiemer *et al.*, 2009) and *Diplopterys pubipetala* (Malpighiaceae), (Possobom *et al.*,2015).These authors suggest that the shrinking and collapse of the papillae occurs because the fragrance compounds have been released and diffused through the cuticle, leaving the cells empty and depleted (Possobom *et al.*, 2015) as explained by the functional layering of osmophore structures (Vogel and Renner, 1992).

In the current investigation, no pores were observed on the osmophore papillae as reported by Pridgeon and Stern (1983), which may have been an artefact caused by the SEM technique used at that time (Pridgeon and Stern, 1983). However, the presence of vesicles on the osmophore surface in our study is consistent with a secretory function and similar vesicles or 'blisters' were found on many of the floral papillae of *Restrepia* (Fig. 2H-J and Fig.3).

Pridgeon and Stern (1983) concluded that the location and arrangement of the osmophores did not have an assignable role in the pollination mechanism, but that the fragrances would act over long distances to 'advertise' the flower. We consider that the osmophores may enable the pollinating insect to locate the flower by the strength of their 'scent trails' which would increase as the insect approached the flower.

3.1.2 The synsepal (Figure 2).

The synsepal (Fig. 2H-J) was found to be covered with papillae arranged either longitudinally following the coloured stripes of the flower (Fig. 2H) as in *R. brachypus*, or, in patches, following the coloured spots of the synsepal (Fig. 2I) as in *R. fritillina.* The synsepal was shown to contain secretory papillae (Fig. 2J) with vesicles observed on the cuticular surface of the papillae (Fig. 2J). The scent emitted by the synsepal papillae may act as an olfactory clue or lure, guiding the insect, once it has landed on the flower towards the labellum and column where pollination occurs. The raised papillae may also serve as tactile guides for the pollinator. The synsepal papillae together with the raised papillae of the epichile (*see labellum and epichile*) may be postulated to operate in a similar manner to conical cells present on floral structures to enhance pollinator grip and to generate 'structural' colour, often in distinct patterns on the flower (Fig. 2H-J) (Whitney *et al*., 2009a; Rands *et al.*, 2011). Thus, the spotting and lines present on both the synsepal and labellum may serve important roles both as tactile and olfactory guides for the pollinator. The proportion of conical cells/papillae to other surface morphologies could depend upon the complex selective biotic and abiotic pressures occurring in each habitat (Whitney *et al.*,2011). Staining with Sudan B confirmed the presence of lipids along the stripes of the synsepal in *R. brachypus*, coinciding with the position of the secretory papillae.

3.1.3 The labellum (Figure 4).

The micromorphology of the labellum is similar for all *Restrepia* species with the exception of *R. aberrans* (Luer, 1996). The three regions of the labellum are angled differently, with the hypochile being the steepest region of the flower presenting itself to a visiting insect. The concave nature of the hypochile labellar region is shown in Fig. 3 A, B. The absence of papillae and cuticular vesicles in this region (B, C) suggest that it is non-secretory. This area provides a different surface texture to the visiting pollinator which may be an example of the flower

185 ‘manipulating’ the behaviour of their pollinator through tactile signals from different surfaces as
186 previously reported (Glover and Martin, 1998; Whitney *et al.*, 2009a; Whitney *et al.*, 2009b).
187 The cellular morphology changes noticeably in the isthmus region of the labellum. Individual
188 papillae are absent and the labellar groove or sulcus, (Fig. 3D) runs through this region. The
189 cells of the labellar groove bear numerous vesicles (Fig. 3C, E) suggesting a secretory function.
190 Any secretions so formed would thus be channeled towards the lower epichile by the sulcus.

191 In *R. brachypus* the margins of the epichile are coarsely denticulate, with a heavily papillose
192 surface (Fig. 3A). The surface papillae are in a linear arrangement (Fig. 3A), following the
193 stripes of the labellum. When these papillae are examined at high magnification the surface of
194 individual cells may be seen (Fig. 3G). There are numerous vesicles, together with evidence of
195 some cells having ruptured (Fig. 3I, J) indicating the presence of fragrance substances collecting
196 in the cuticles of these cells, which later diffuse through the cuticle causing the cells to rupture
197 (Sazima *et al.*, 1993; Wiemer *et al.*, 2009). These data provide supporting evidence that the
198 cuticular vesicles observed in various floral structures are secretory in nature. The epichile
199 stained the darkest with Sudan B, confirming the presence of lipids and suggesting that this is
200 the most active secretory region. These features correspond with the general description from
201 Luer (1996); and are in agreement with the features described by Sazima *et al.* (1993) and
202 Wiemer *et al.* (2009).

203 **3.1.4 The cirrhi (Figures 3 and 4).**
204 The position of the cirrhi in the flower is illustrated in Fig. 1, inset B7; Fig. 3A a, b and in detail,
205 Fig. 4B. While these structures have been recorded previously (Luer, 1996; Pridgeon and Stern,
206 1983), their function has never been established. They are distinctive structures unique to
207 *Restrepia* and we consider them to be structural adaptions that facilitate pollination in this genus.

208 The existence of different sized pollinators within the genus is suggested by the different ratios
209 of column length to cirrhi width between different species, e.g. 2.8 in *R. citrina*, compared to 1.8
210 in *R. purpurea* (Table 1). However, the ratio of column length to labellum length (approximately
211 2:1) is similar between species. From these ratios, only pollinators of the correct
212 width/proportions would be able to fit between the cirrhi and under the column (Fig. 4E). This is
213 in agreement with the hypothesis that orchid floral morphology is highly adapted to its
214 pollinators and characterized by a 'functional fit' between flower and pollinator (Benitez-Vieyra
215 *et al.,* 2006), but does not answer the question as to whether each *Restrepia* species may be
216 pollinator specific.

217 An oblique view of the position of the cirrhi on either side of the column is shown in Fig. 4B.
218 These are located in such a way that they protect the anther cap and pollinia and thus prevent the
219 pollinia from being 'robbed'. A pollinating insect would have to pass between the cirrhi and
220 under the column to bring about pollination. Once there, it would effectively be 'trapped' and
221 could only exit the flower by progressing along the labellum. One further intriguing feature of
222 these structures, is that the tips of the cirrhi 'splay out' as the flower senesces (Fig. 3A a, b) so
223 making entry under the column easier for the pollinator. This appears to happen after the vesicles
224 of the osmophores and labellar regions have begun to senesce, and the stigmatic surface has
225 become less receptive. This may be an adaptation of the flower in a final attempt for pollination.

226 One distinguishing feature of Diptera is the presence of *halteres*, the vestigial remains of a
227 second pair of wings. The loss of these has resulted in the development of strong muscles to
228 operate the forewings which enable the flies to be extremely agile in flight (Marshall, 2012).
229 The proposed action of the cirrhi is therefore vital for the flower, in order to 'trap' and slow-
230 down these pollinators, as they might otherwise exit the flower before pollination has occurred.
231 The distance between the cirrhi may also prove to be of importance in determining the type of

Dipteran pollinator.

Elaborate ‘trapping’ mechanisms have also been found in other Pleurothallid genera. In *Dracula,* the pollinator’s thorax is trapped by the incurved flaps of the rostellum (Endara *et al.,* 2010) which creates an angle between the scutellum and the abdomen for the removal and deposition of the pollinia. In *Specklinia pfavii,* species of *Drosophila* are trapped between the lip and column (Karremans *et al.,* 2015). In both these examples, a precise fit between the flower and pollinator is required (Benitez-Vieyra *et al.,* 2006) which suggests pollinator specificity and/or the operation of oligophily. The role of the rostellum is important in preventing self-pollination; in the case of *Dracula,* it remains partially attached to the fly being pulled forward to cover the stigmatic cavity.

3.1.5 The calli (Figure 4).

Macro photographs of the position of the calli within the flower and possible ‘false nectar guides’ are presented in Fig. 5A, B, and detailed micromorphology of the calli in Fig. 4C, D.

Although the presence of a labellar callus in orchids is well known (Arditti, 1992), e.g. in *Maxillaria* (Davies *et al.,* 2004), the structure and function of calli in *Restrepia* have yet to be established. While the callus is usually situated centrally on the labellum and on either the hypo- or mesochile (Arditti, 1992), *Restrepia* calli are uniquely positioned at either side of the column base where they are attached to the labellum (Luer, 1996) (Figs. 1A 3; 4A). Orchid nectaries are typically positioned in spurs located at the base of the labellum, as in *Angraecum* and *Aerangis* (Arditti, 1992), or form a depression at the base of the labellum, from where nectar collects on the labellum callus (Arditti, 1992). The papillate nature of the labellum and accompanying nectar secretion in *Maxillaria* were established (Davies and Turner, 2004; Davies *et al.,* 2003; Stpiczynska *et al.,* 2003). The labellar callus in *Bulbophyllum* species was shown to exhibit a

papillate form that collected nectar (Teixeira *et al.*, 2004).

In contrast, while in *Restrepia* the calli were shown to be papillate in nature (Fig. 4C, D), none of the images obtained showed evidence of secretions or vesicles as observed elsewhere on the labellum and osmophores. Therefore, we conclude that the *Restrepia* calli are not concerned with nectar secretion or collection. This was observed in *R. brachypus, R. dodsonii* and *R. sanguinea* (*Restrepia* subgenus *Restrepia*), and also in *R. muscifera* (*Restrepia* subgenus *Pleurothallopsis*), thus confirming the same morphology in both subgenera. One explanation for the observed lack of nectar is that *Restrepia* is a non-nectar rewarding genus. Many orchids do not produce nectar or any reward at all (van der Pijl and Dodson, 1966; Ackerman, 1985). Therefore, while it is not unusual for orchids to be non-nectar rewarding, the current study represents the first report of this phenomenon in the genus *Restrepia.*

The cuticle of the calli was observed to be variously folded and striated, radiating from the apex of the papillae (Fig. 4C, D). Cuticular folds in epidermal plant cells are often associated with iridescence, in which the image observed alters with the viewing angle. This has been attributed to cuticular folds acting as diffraction gratings (Whitney *et al.*, 2009c; Glover *et al.*, 2012), but for this effect to function, the cuticular layer should be flat and striated. Moreover, the generation of iridescence will only occur if the ridges are separated by specific distances (Glover, 2009). Rounded or conical cells do not allow directional reflection since they scatter light (Glover *et al.*,2012) and hence would not be associated with iridescence. Similar cuticular 'folding' to those observed in the current study have been reported previously in studies of orchidaceous labellar spurs (Bell *et al.*, 2009) and on non-orchidaceous petal surfaces (Glover, 2009). Bell *et al.* (2009) argued that the cuticular striations of the papillae acted as a tactile guide to the pollinating insect and so improved pollination, or were associated with nectar production by the spur. Glover (2009) concluded that these structures influenced the behaviour

279 of light, acting as a scattering mechanism to evenly distribute all wavelengths leaving the petal
280 surface.

281 Therefore, it is likely that since the calli and surrounding areas exhibit different optical
282 properties when illuminated under different conditions (Fig. 5) the features observed on the calli
283 are associated with the scattering of electromagnetic radiation (visible spectrum and near UV or
284 UVA), while the flatter areas below the calli (Fig. 5, arrowed in A, B, C and shown in D, C) may
285 exhibit a small degree of iridescence in the near UV region of the electromagnetic spectrum
286 (Whitney *et al.,* 2009c). These structures could therefore present a different appearance to the
287 insect depending on the viewing angle. Since flies are visually sensitive to radiation in the near
288 UV or UVA region (wavelengths 320-400 nm), it is possible that they perceive these areas
289 which reflect UV light as visual signals acting as 'landing lights' or guides. These areas only
290 appear 'bright' and attractive to the insect when it is in the correct position, or on the correct
291 'flight path' to enter the flower ventrally, beneath the column, where pollination can occur.

292 In such non-food rewarding flowers, the areas of contrasting colour, which usually guide the
293 insect towards nectar (Waser and Ollerton, 2006), serve to attract and deceive the insect. There
294 are many examples of such 'false nectar guides' in the Orchidaceae. Pollinators may be attracted
295 by the colouration of the flower, especially the labellum and spot patterns (Sugiura *et al*., 2002).
296 Bees are attracted by the purplish spots on the labellum of *Cymbidium lancifolium* (Cheng *et al*.,
297 2007). Nectar-seeking insects are guided to the central, reproductive area of the *Dendrobium*
298 *speciosum* flower by colour gradation, including an area of high UV reflection near the centre
299 and a bright yellow ridge along the labellum (Dyer, 1996; Slater and Calder, 1988). It is of
300 interest to note that many of these features can also be found in *Restrepia* flowers e.g. a spotted
301 or striped labellum (Figs. 1 and 3), yellow crests at the base of the two lateral petals (Fig. 5A, B),
302 dark spots on the synsepal and at the base of the petals and sepals (Fig. 5A, B), and bright yellow

calli and UV reflective areas in the flower (Fig. 5). As such, *Restrepia* may also be a non-nectar rewarding and 'deceptive' orchid genus.

3.1.6 Fly pollination in the Pleurothallidinae

Flies are often considered to be inefficient and unreliable pollinators, but their sheer numbers and presence throughout the year make them important pollinators for some plants (Gullan and Cranston, 2005; Tan, 2006; Woodstock *et al.*, 2014). They are of great significance at high altitudes where other insect groups may be lacking (Larson *et al.*, 2001). As *Restrepia* are typically found in montane rain forests (altitude = 1500-3500m) this would support the hypothesis for myophily by Dipteran species (Pridgeon and Stern, 1983; Luer, 1996). While there is much indirect evidence to support this, it has never been confirmed in the wild or in cultivation. Indeed, spontaneous capsule set was practically unknown in the collections studied by Luer (1996) and is also rare in UK collections (H, Millner, University of Wolverhampton, personal observations 2004-2014) suggesting pollinator absence in both instances.

In one form of myophily, visiting adult flies feed on nectar and are regular visitors who will leave the flower quickly if they obtain no reward (Jersáková and Johnson, 2006). Such plants tend not to emit/produce a strong scent and are often yellow or white in colour, with exposed stamens and stigma and may have complex traps to retain the insect on the flower for longer. While there are many examples of predominantly yellow *Restrepia* species e.g. *R. brachypus, R. trichoglossa, R. chrysoglossa, R. mendozae, R. falkenbergii* and R. *wagneri*, many others are dark red almost brown, e.g. *R. sanguinea, R. tabeae, R. peteersii* and R. *guttulata.* However, in all species the calli are bright yellow accompanied by yellow 'crests' at the base of the two lateral petals (Fig. 1 and Fig. 4A). These may act as guides or lures while the cirrhi provide a 'trapping' mechanism for the pollinator(s) (Fig. 4E).

In the second form of myophily, pollinators are attracted by deception variously through scents, colours and surfaces, which imitate flies' natural food sources or their brood site (Kowalkowska *et al*., 2015). Certain male Diptera (*Tephritidae*) are attracted by a specific floral attractant which acts as the male fly's sex pheromone precursor by flowers which do not produce nectar (Woodcock *et al*., 2014). This has been studied more fully in the genus *Bulbophyllum*, which is regarded as a 'vicariant of the Pleurothallidinae' (Kowalkowska, 2015). These two orchid groups are not closely related, being in different tribes of the subfamily Epidendroideae (Azevedo *et al*., 2007), but represent an example of floral convergence caused by similar pollination systems (Dressler 1993). In *Bulbophyllum*, these floral attractants were identified as either methyl eugenol (Tan *et al*., 2002), zingerone (Tan and Nishida, 2007) or raspberry ketone (Tan and Nishida, 1995). Given the secretory nature of the labellum in *Restrepia*, the intriguing question arises as to whether the exudate observed on the micrographs might contain any of these substances and is the subject of ongoing research in our laboratory.

3.2 CONCLUSIONS

3.2 .1 Pollination Hypothesis

Based upon the data presented, we propose a pollination hypothesis for the genus *Restrepia*: The fly (a small species of Diptera,) is attracted to the flower from a distance by scent produced by the osmophores (Pridgeon and Stern, 1983); and locates the flower by a combination of sight and the 'scent trails' produced by the osmophores. After landing on the synsepal/labellum, the conical papillae present provide grip/purchase for the fly (Whitney *et al.*, 2009; Rands *et al.*, 2011) and also provide tactile and olfactory 'clues' guiding it along the labellum. The cells of the epichile (lower labellum) produce waxes and oils (Fig. 3 I, J) which the fly can sense via its'

proboscis or other organs. The fly then progresses along the isthmus onto the hypochile (upper labellum), guided/lured by the structural optical effects of the calli, and their surrounding area. As the fly progresses along the labellum, the surface of the hypochile become smoother and steeper. This makes further progress more difficult, and it is at this point where the fly is positioned/trapped between the cirrhi and beneath the column. Pollination is then brought about by pollinia being deposited onto the stigmatic surface, or pollinia from the column becoming attached to the fly. The fly is then able to leave the flower having performed its role in the pollination of the flower, albeit unrewarded.

3.2.2 Pollination and breeding systems

Non-nectar rewarding myophily is considered to help prevent self-pollination, as the pollinator is discouraged from returning to the same flower (Jersáková and Johnson, 2006), thus reducing the likelihood of self-pollination and promoting out-breeding (Millner, 2013). Myophily has been previously linked to self-incompatibility by Barbosa *et al.* (2009) who considered self-incompatibility and myophily to be biological synapomorphies within the Pleurothallidinae (Barbosa *et al.,* 2009). To ensure the survival of any plant the pollination and breeding systems must work in conjunction with each other as complimentary mechanisms.

Restrepia has previously been reported to exhibit a gametophytically controlled self-incompatibility breeding system (Millner *et al.,* 2015) in which self-pollination results in capsule set, together with the formation of empty testae. It is therefore important for these species to avoid self-pollination, which agrees with the proposed existence of myophily and deceit pollination within the genus. However, in dwindling populations of obligate out breeders, such as the majority of *Restrepia* species, pollination rates may decrease. Such populations may no longer be self-sustaining through seed production (Borba *et al.,* 2002; Millner, 2013). This may be the case

in the remaining wild populations of *Restrepia,* which makes the understanding of both their pollination mechanism and breeding system of crucial importance.

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Table 1: Comparative mean length (pixels) of the column, labellum and width between the cirrhi

Species	Authors (WCSP, 2016)	L[1]	cv[2]	C[1]	cv[2]	L/C[3]	w[1]	cv[2]	C/w[4]
R. antennifera 1	Kunth, 1816	709.3	*2.3*	356.4	*1.9*	**2.0**	175.2	*0.6*	**2.0**
R. antennifera 2		702.0	*2.5*	361.6	*2.4*	**1.9**	157.7	*1.1*	**2.3**
R. brachypus	Rchb.f., 1886	841.4	*1.3*	438.5	*2.5*	**1.9**	180.1	*1.7*	**2.4**
R. citrina	Luer and Escobar, 1983	650.6	*0.7*	333.7	*0.8*	**1.9**	117.3	*2.0*	**2.8**
R. contorta 1	Ruiz and Pavon,1996	488.5	*0.8*	239.0	*1.8*	**2.0**	104.1	*3.8*	**2.3**
R. contorta 2		467.1	*3.2*	241.8	*2.8*	**1.9**	106.0	*1.2*	**2.3**
R. cuprea	Luer and Escobar, 1996	609.9	*0.9*	309.4	*2.3*	**2.0**	137.0	*4.3*	**2.3**
R. dodsonii	Luer, 1980	476.9	*1.2*	226.6	*2.2*	**2.1**	97.6	*4.8*	**2.4**
R. echinata	Luer and Escobar, 1996	568.5	*2.7*	271.6	*2.9*	**2.1**	107.2	*0.7*	**2.5**
R. elegans 1	Karst, 1847	672.2	*0.8*	328.0	*3.5*	**2.0**	150.2	*1.6*	**2.2**
R. elegans 2		466.0	*2.3*	238.4	*2.0*	**2.0**	114.7	*3.1*	**2.1**
R. guttulata 1	Lindl., 1837	548.3	*2.5*	283.8	*0.5*	**1.9**	103.8	*0.5*	**2.7**
R. guttulata 2		527.8	*2.1*	263.7	*2.5*	**2.0**	103.5	*2.2*	**2.5**
R. mendozae	Luer, 1996	719.3	*1.1*	367.5	*1.4*	**2.0**	189.8	*1.2*	**1.9**
R. purpurea	Luer and Escobar, 1996	489.4	*3.2*	254.2	*2.2*	**1.9**	138.1	*1.2*	**1.8**
R. schizosepala	Luer and Hirtz, 1996	642.4	*1.3*	312.5	*1.1*	**2.1**	167.8	*1.5*	**1.9**
R. seketii	Luer and Escobar, 1996	536.8	*1.0*	260.0	*2.6*	**2.1**	112.2	*1.4*	**2.3**
R. vasquezii	Luer, 1996	505.9	*3.8*	261.7	*3.3*	**1.9**	99.6	*0.5*	**2.6**
Mean						**2.0**			**2.3**
n						**18**			**18**
se						**0.02**			**0.07**

Values given (L^1, C^1 and w^1) are the pixel values from photographs of the species and do not represent a formal measurement i.e. mm. Pixel values are used for comparative analysis within each flower and not for size comparisons. Repeated measurements from one flower were used to ensure consistency.

L^1 Mean values from ten measurements, labellum length in pixels, C^1 Mean values from ten

measurements, column length in pixels, w[1] Mean values from ten measurements, width between the cirrhi in pixels (Fig. 3A, w).

[2]Coefficient of variation (cv) <5% for all values indicating good precision.

[3]Ratio, labellum length to column length, approximately 2:1, se = 0.02

[4]Ratio, column length to width across the cirrhi, se = 0.07.

FIGURE LEGENDS

Figure 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, 1996a) 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.

Figure 2: Dorsal sepal and lateral petal osmophores of *R. brachypus* together with synsepal papillae

A: *R. brachypus* flower. The clavate apices and the triangular arrangement of the dorsal sepal (a) and the lateral petals (b and c) are shown.

B and C: Dorsal sepal osmophores at one day pre-anthesis. The adaxial surface of the dorsal sepal and its osmophores are shown (B), and at higher magnification (C) in which the osmophore

papillae can be observed to be turgid and rounded, with a smooth cuticular surface on which no obvious vesicles are observed.

D and E: Dorsal sepal osmophores one day post-anthesis (D) and a higher magnification (E) in which raised vesicles on the cuticular layer of the cells are observed.

F and G: Dorsal sepal osmophores are shown one week post-anthesis (F) collapse of some of the osmophore papillae is observed. A higher magnification of the papillae is shown (G) in which ruptured vesicles and cell collapse are observed. This process of senescence was recorded to start between one and two days post-anthesis.

H and I: The arrangement of the synsepal papillae. A linear arrangement (H) that coincides with coloured stripes of the synsepal, as in *R. brachypus.* The synsepal papillae arranged in patches (I) that coincides with coloured synsepal spots in species without synsepal stripes, here, as in *R. fritillina*

J: Synsepal papillae, one day post-anthesis, *R. brachypus.* Raised vesicles on the cuticular surface can be seen.

Figure 3: *R. brachypus*, micromorphology of the labellum one day post-anthesis

A, B and C: *the hypochile region of the labellum.* A: Two cirrhi (a, b) are situated either side of the hypochile region; the width between the cirrhi is shown (w). The concave, glabrous, non-papillate hypochile with its rounded cells, lacking cuticular vesicles is shown (B); in which the origin of the labellar groove, or sulcus is arrowed. A higher magnification of region c (C) shows numerous small vesicles on the surface of the cells comprising the sulcus and some sculpting of the cuticular layer of other cells.

A, D and E: *the isthmus region of the labellum.* The region between the epichile and hypochile

(the mesochile) is narrowed to form an isthmus (A) distinguished by a labellar groove, running its length having originated in the hypochile region (D). In this region the labellar surface has become more uneven (D) and at higher magnification (E) vesicles are evident on the cuticular layer of the cells.

A and F-J: ***the epichile region of the labellum.*** The denticulate epichile margin and the end of the labellar groove are shown (F). At higher magnification (G) the cuticular layers of the cells have many vesicles which appear similar to those observed in (Fig. 2E) and to those on the cuticular surface of the osmophores (Figure 2E).

H-J: ***multicellular papillae of the epichile.*** One day post-anthesis vesicles are evident on the cellular cuticles (H) and the magnified view (I) shows that some of these have ruptured and exudate is visible between the cells. At two days post-anthesis (J) some rupturing of the cells has begun, the vesicles have shrunk and remains of the exudate are present on the cuticular surface between the cells.

Figure 4: Putative 'false' nectar guides, structure and proposed function of the cirrhi

A and B: ***Macro-photographs, R. brachypus, one day post-anthesis*****.** (A) ventral view of the base of the lateral petals and calli and (B) oblique view of the column illustrating the position of the cirrhi, each side of the column and anther cap. In older flowers, the cirrhi may splay sideways (Fig. 3A). Possible false nectar guides are indicated in (A and B): (i) bright yellow 'crests' to the lateral petals, (ii) and (iii) dark spots, (iv) concave reflective areas below the calli (cf. fig. 5), (v) bright yellow calli and (vi) bright edge to the stigmatic surface/ventral edge of the column.

C and D: ***Papillae of the calli.*** The papillae apices consisting of various cuticular folds radiating from the apices. These striations were observed laterally on the papillae and continued across from

one cell to another (C), further details are shown (D). These were found to be unique to cells forming the calli with no observed exudate on or between them. Presence of conical papillae was confirmed from calli in *R. brachypus, dodsonii, muscifera* and *sanguinea.*

E: *Function of the cirrhi.* Stylised diagram of the proposed function of the cirrhi in which the fly is positioned between the cirrhi. The only way it can progress is by going forwards, direction shown by arrow.

(i) stigmatic surface and (ii) tip of anther cap, both on the ventral side of the column.

Figure 5: Calli of *R. brachypus* photographed under different forms of illumination

A: *R. brachypus* flower in daylight, no reflection visible from the calli, or the area beneath them (arrow).

B: *R. brachypus* flower illuminated by torchlight in a dark room, the areas under the calli are highly reflective (arrow).

C: *R. brachypus* flower illuminated by UV 380nm in a dark room. The area below the calli fluoresced, appearing as two bright blue dots (arrow).

D and E: The arrowed reflective areas visible in (B) and (C) shown as a macro photograph (D) and a micrograph (E). Both confirm the papillate nature of the calli and the absence of papillae in the reflective area beneath them.

Graphical Abstract (for review)

Figure 1

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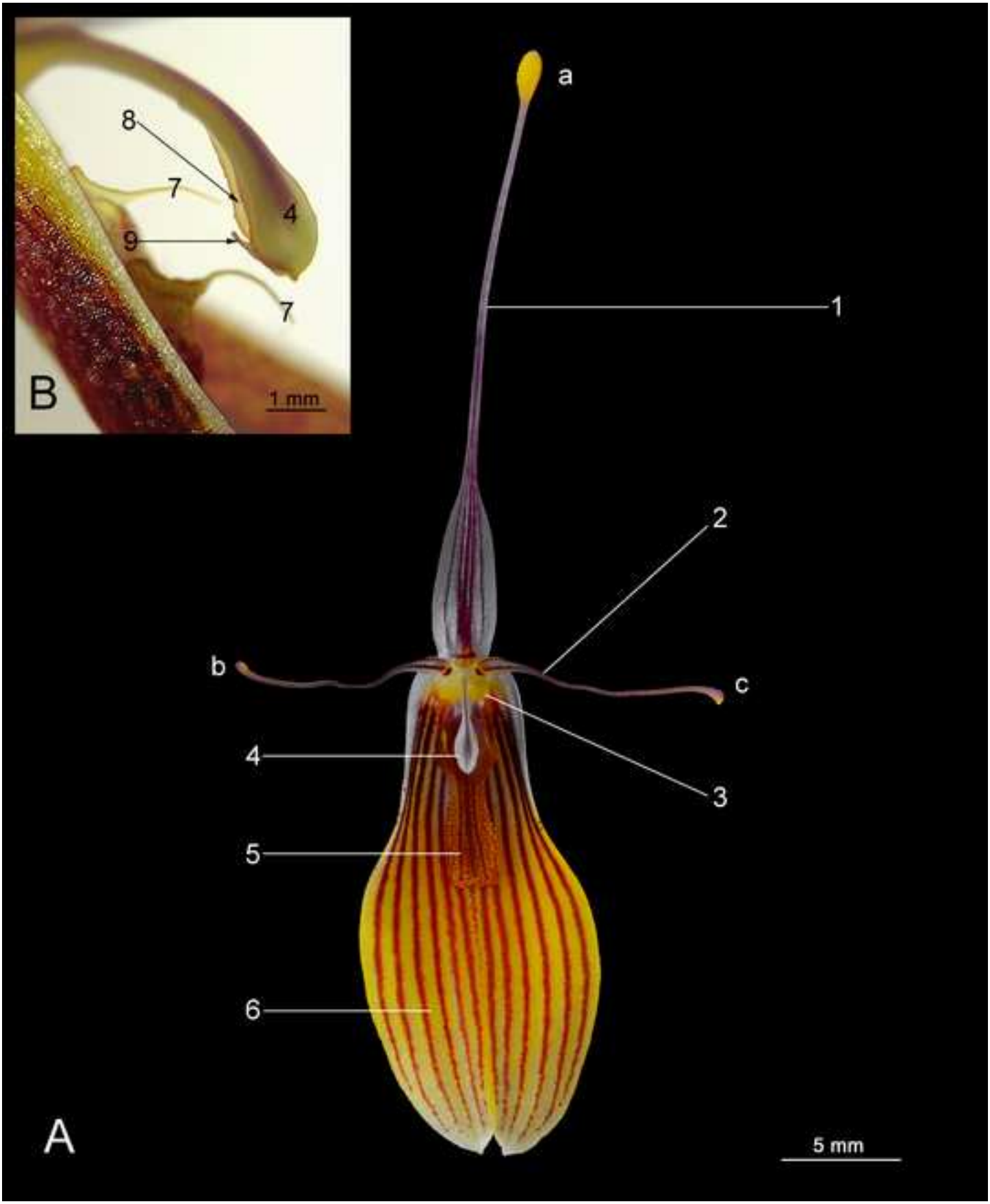

Figure 2

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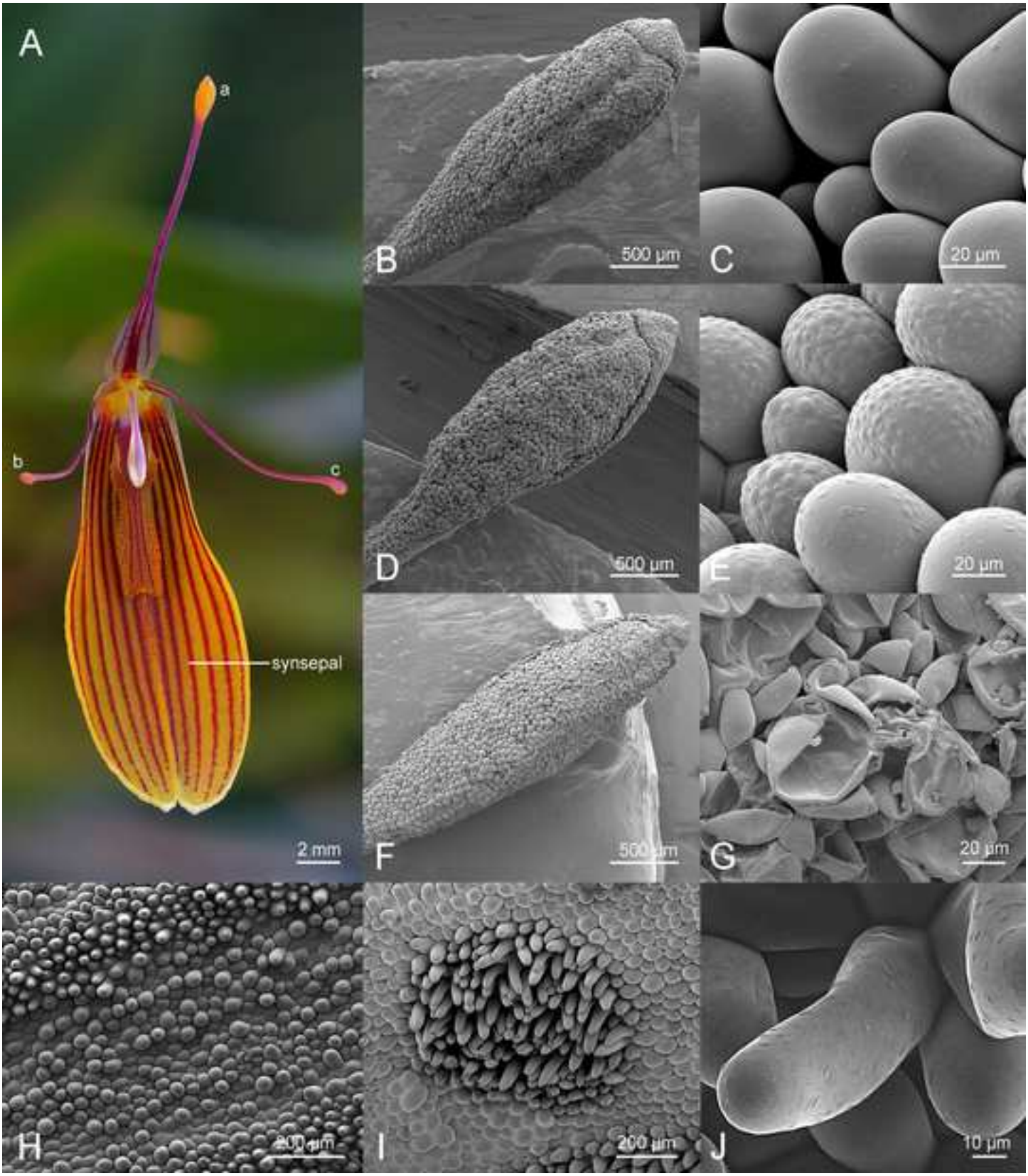

Figure 3

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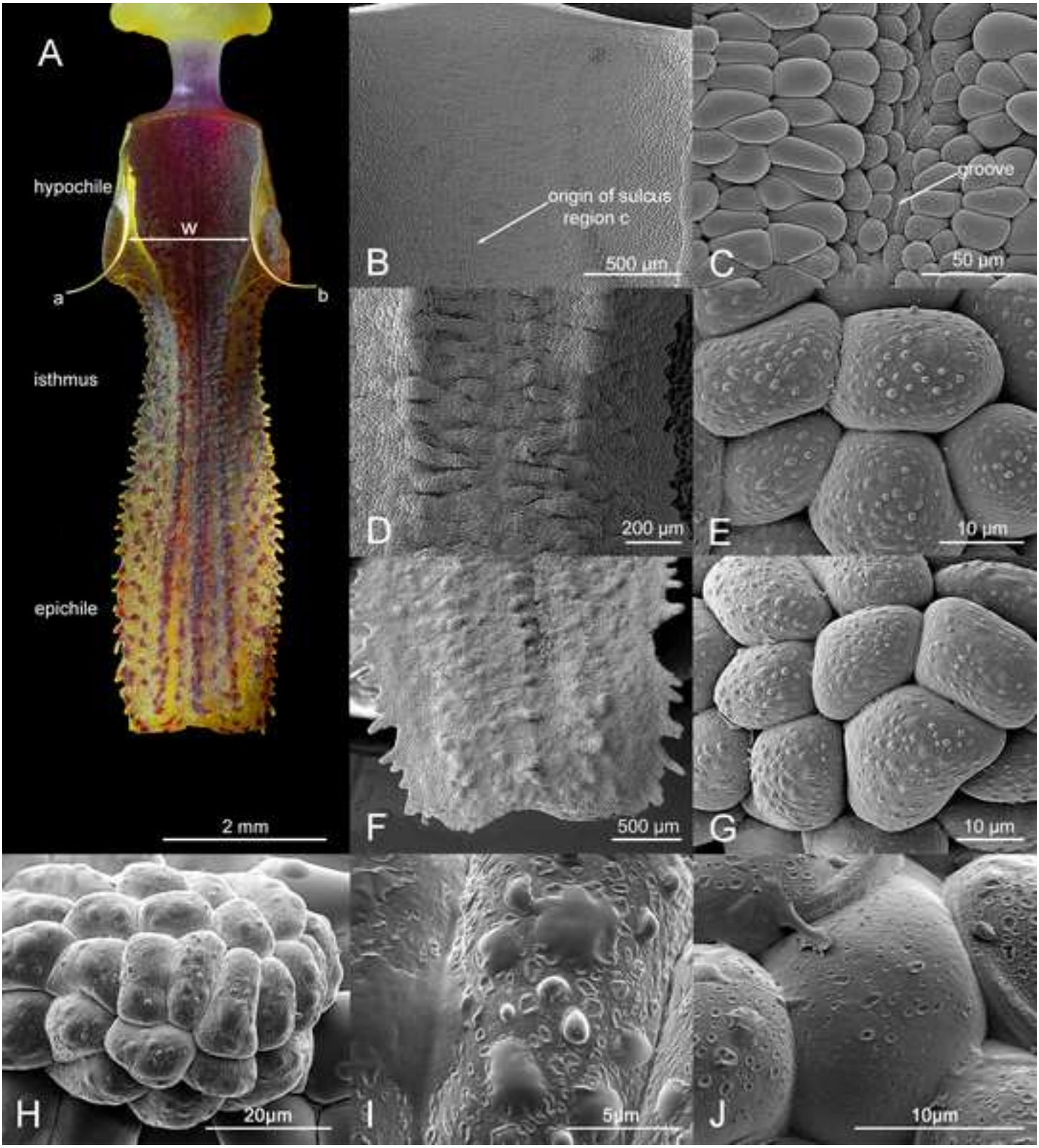

Figure 4

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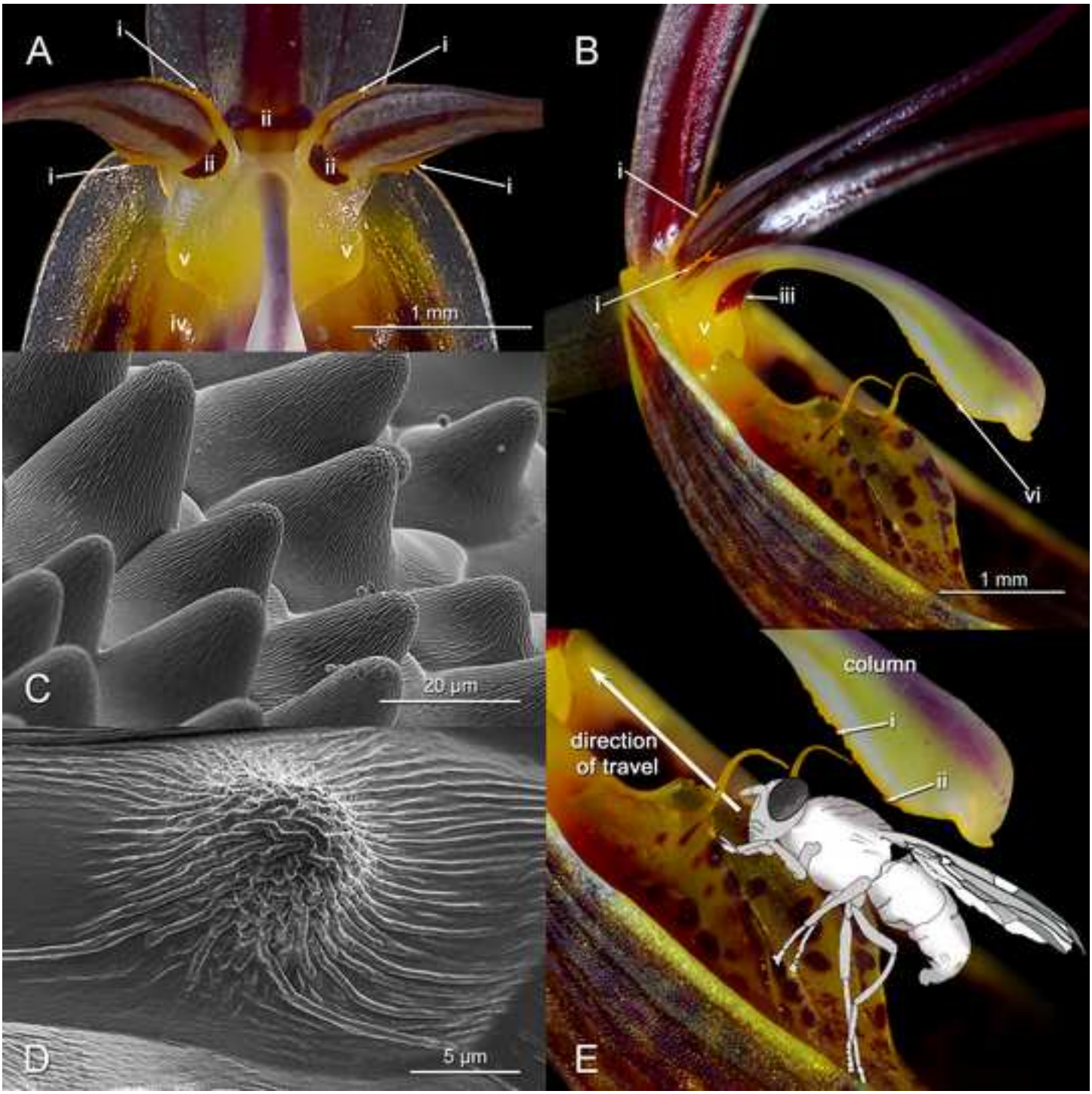

Figure 5

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