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Reproductive biology and *ex situ* conservation of the genus *Restrepia* (Orchidaceae)

A thesis presented for the degree of

Doctor of Philosophy

by

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Abstract

The genus *Restrepia* is well known to orchid enthusiasts but its micromorphology has not been described, and its pollination and breeding systems have not been investigated. The aim of this investigation was, therefore, to add to existing knowledge so that the resultant data could be used to facilitate *ex situ* conservation initiatives.

A detailed electron microscopy study (SEM) of the floral organs was performed. This confirmed the structure of the dorsal sepal and lateral petal osmophores, their secretory nature together with that of the synsepal and the labellum. It was postulated how, by manipulating different labellar surface textures, the flower might use these 'tactile guides' to steer the insect (fly) through the flower. The cirrhi were postulated to help by destabilising the pollinator in flight, trapping it and bringing about pollination. The papillate structure of the calli was established and their optical properties investigated.

Media comparison investigations established that Western medium supported the highest germination rates and, with the addition of banana supplement, the highest rates for seedling growth and development. This represented the first protocol for axenic germination of *Restrepia* in the literature (Millner *et al.*, 2008) and provided a tested methodology for investigating breeding systems and producing *Restrepia* plant material for both scientific and horticultural purposes.

Self-pollinations were found to produce fewer embryos compared to cross-pollinations. The operation of self-incompatibility (SI) was confirmed by the study of pollen tube growth which further confirmed the time interval between pollination and fertilisation. A time line from pollination/fertilisation to flowering was established. The type of SI in operation was best explained by gametophytic incompatibility. This demonstrated that it was possible to raise *Restrepia* hybrids and species from seed, by performing intra-specific crosses so helping to preserve them for posterity and relieve pressure on wild populations.

Narrow endemic *Restrepia* species face combined threats from habitat loss, habitat degradation and problems of viable seed production due to the effects of SI and inbreeding depression (ID). Recently developed online resources, such as GeoCAT, were used to perform a Red List assessment in order to identify the degree of threat individual species faced, both globally and nationally. All species were classified as facing substantial levels of threat; although this was lessened for populations in protected habitats. Conservation is needed for cultivated collections as well as these wild populations by keeping alive existing knowledge and expertise in growing these species.

Contents

List of Tables

List of Figures

List of Plates

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H J Millner, August 2013

Chapter One:

The genus *Restrepia*, a general introduction

Pleurothallis ospinae R.E. Schultes [Veitch]. (Hawkes, 1989)

"...a genus most confusing to botanists for many years".
The Pleurothallid Alliance website

1.1 Introduction

Restrepia is a small orchid genus of 53 species belonging to the sub-tribe Pleurothallidinae. These species are found throughout Central America and in Venezuela, Colombia, Ecuador, Peru and Bolivia in South America. They have been well known to orchid growers for many years due to their distinctive floral morphology and the relative ease with which the more common species may be cultivated. Although described as '….a remarkable orchid', (Hawkes, 1989) and '…my favourite genus', (Pridgeon, 2004), they remain in many respects an understudied genus.

To date the only morphological study of *Restrepia* was performed by Pridgeon and Stern in 1985, on *Restrepia* osmophores, almost 30 years ago. This study used scanning electron microscopy techniques in which there have since been considerable advances. In addition, subsequent studies have established further details of osmophore structure. The only monograph on the genus in which all *Restrepia* species were fully described is now almost 20 years old. This was the monograph 'Systematics of Restrepia (Orchidaceae)' (Luer, 1996a) which was volume XIII in the series *Icones Pleurothallidinarum*. Much has changed since this monograph was published in regard to habitat loss and orchid population decline in both Central and South America. Major changes have also taken place with regards to orchid taxonomy with the introduction of molecular based taxonomy which was pioneered for the sub-tribe by Pridgeon *et al.* (2001) and Pridgeon and Chase (2001). Added to which are the huge technological changes in computing and internet based scientific resources, now freely available online, which have occurred since the publication of Luer's monograph.

As such, it would seem that now is an appropriate time to revisit this genus using current technologies. Luer wrote in 1996, 'I feel as if there is more to be discovered

about this genus than we know today.' In fact, nothing has been published regarding *Restrepia* breeding systems, and certain floral morphological features remain a mystery. Moreover, *Restrepia* represent a novel and unique Pleurothallid genus with which to carry out research, as they have not been hybridised, their breeding system is unknown, their labellar micromorphology is unstudied and their pollinators have never been identified.

The purpose of this introductory chapter is, therefore, to examine and review what is currently known of the genus: the historical aspects of its discovery, its current distribution, its habitat and conservation status and its taxonomy. A comparison is made between general orchid floral morphology and the floral plan of *Restrepia* and the species *R. brachypus* is introduced, (which was the subject species for many of the investigations described in Chapters 2, 3 and 4).

In order to avoid unnecessary repetition, a detailed discussion of the development of computer and internet resources used to investigate the conservation status of the genus in the current study, is left until the introduction to Chapter 5. The areas that this study will investigate are presented at the end of this chapter together with the aims of the study.

1.2 The discovery of *Restrepia*

During the 19th century, many explorers, plant hunters and botanists undertook scientific and commercial expeditions to the Americas in search of new species. Orchids were the most highly prized plants because they could be sold for huge profits in Europe due to their scientific rarity and the beauty of their flowers. Two such botanists were Humboldt and Bonpland whose expedition to South America (1799 to 1804) became famous due to the remarkable number of new plant species discovered.

Plate 1-1: *Restrepia antennifera*, the type species for the genus, (Humboldt *et al.*,1816).

Image courtesy Missouri Botanical Garden, http://www.botanicus.org.

One such species was discovered near Popáyan, Colombia, in 1801. Humboldt and Bonpland named this genus, *Restrepia* in honour of José Manuel Restrepo, a university student and enthusiastic botanist whom they had met earlier in their expedition (Manning, 2010). The species they discovered was *Restrepia antennifera* (Plate 1-1). Although *R. contorta* (Luer, 1996a; WCSP, 2013) (Plate 1-2(a)) had been described previously as *Humboltia contorta* by Ruiz and Pavon in 1798, the description based upon specimens of *R. antennifera* (Plate 1-2(b)) published in 1816 by Humboldt, Bonpland and Kunth, remains the type species for the genus. Carl Sigismund Kunth worked for over ten years (1815-1825) in collaboration with Humboldt and Bonpland to complete the seven volumes of *Nova Genera,* in which the genus *Restrepia* was first described in Volume 1. José Restrepo subsequently went on to become a noted Colombian botanist (Bechtel *et al*., 1992; Pridgeon, 1992) and was the first person to study the natural history of the Antioquian Andes in North West Colombia (Manning, 2010).

1.3 Distribution of *Restrepia* species

Since these early discoveries, many more species of *Restrepia* have been identified, the majority of which were discovered since 1980. The currently recognised species (WCSP, 2013), together with their countries of origin, date of discovery and first description are presented in Table 1-1.

The earlier discoveries (e.g. *R. contorta, R. antennifera* and *R. guttulata)* are widely distributed geographically, being found in several countries, and exhibiting a high degree of within-species variation. In comparison, many of the more recently discovered species have only been identified at one or two locations (Luer, 1996a). While these later species may persist in other localities in the wild, they are clearly

Plate 1-2: *R. contorta* and *R. antennifera*

(a) *R. contorta*, the first species to be discovered, 1798, Ruiz and Pavon. (Private collection of H. Millner)

(b) *R. antennifera* 'Roseola' 1816, Humboldt, Bonpland and Kunth. (Private collection of H. Millner)

Table 1-1: *Restrepia* species, their year of discovery and countries of origin.

Restrepia species	Country of origin	Year	Reference
R. contorta	P, E, C, V	1798	Riuz & Pav., Syst. Veg,235, 1798
R. antennifera	E, C, V	1816	Kunth in F.W.H.von Humboldt, A.J.A.Bonpland & C.S.Kunth, Nov. Gen. Sp. 1: 367 (1816).
R. guttulata	P, E, C, V	1837	Lindl., Companion Bot. Mag. 2: 357 (1837).
R. elegans	V	1847	H.Karst., Allg. Gartenzeitung 15: 202 (1847).
R. lansbergii	V, E, P	1854	Rchb.f. & H.Wagener, Bonplandia (Hannover) 2: 23 (1854).
R. nittiorhyncha	C	1854	Rchb.f., Bonplandia (Hannover) 2: 23 (1854).
R. wageneri	V	1854	Rchb.f., Bonplandia (Hannover) 2: 23 (1854).
R. aspasicensis	C	1855	Rchb.f., Bonplandia (Hannover) 3: 70 (1855)
R. muscifera	Mx, CA, C	1859	(Lindl.) Rchb.f. ex Lindl., Fol. Orchid. 8: 7 (1859).
R. falkenbergii	C	1880	Rchb.f., Gard. Chron., n.s., 13: 232 (1880).
R. brachypus	E, C, V, B	1886	Rchb.f., Flora 69: 554 (1886)
R. pandurate	C	1888	Rchb.f., Gard. Chron. 1888(1): 244 (1888).
R. sanguinea	C	1894	Rolfe, Bull. Misc. Inform. Kew 1896: 44 (1894).
R. trichoglossa	Mx to P	1901	F.Lehm. ex Sander, Sander's Orch. Guide: 215 (1901).
R. aristulifera	C, V	1972	Garay & Dunst., Venez. Orchids Ill. 5: 258 (1972).
R. chocoënsis	C	1973	Garay, Orquideologia 8: 181 (1973).
R. dodsonii	E	1980	Luer, Phytologia 46: 382 (1980).
R. iris	E	1980	Luer, Phytologia 46: 383 (1980).
R. teaguei	E	1980	Luer, Phytologia 46: 384 (1980).
R. flosculata	E, C	1982	Luer, Selbyana 7: 127 (1982).
R. limbata	C	1982	Luer & R.Escobar, Selbyana 7: 76 (1982).
R. pelyx	C	1982	Luer & R.Escobar, Selbyana 7: 76 (1982).
R. citrina	C	1983	Luer & R.Escobar, Orquideologia 16: 40 (1983).
R. mohrii	P	1993	Braem, Schlechteriana 4: 44 (1993)
R. aberrans	Panama	1996	Luer, Orquideologia 20: 117 (1996).
R. chameleon	C	1996	Luer & R.Escobar, Orquideologia 20: 120 (1996).
R. chrysoglossa	C	1996	Luer & R.Escobar, Orquideologia 20: 123 (1996).
R. cloesii	P	1996	Luer, Orquideologia 20: 125 (1996).
R. condorensis	E	1996	Luer & R.Escobar, Orquideologia 20: 128 (1996)
R. cuprea	C	1996	Luer & R.Escobar, Orquideologia 20: 130 (1996).
R. cymbula	E	1996	Luer & R.Escobar, Orquideologia 20: 133 (1996).
R. echinata	C, P	1996	Luer & R.Escobar, Orquideologia 20: 135 (1996).

Table 1-1 continued: Restrepia species, their year of discovery and countries of origin.

Restrepia species	Country of origin	Year	Reference
R. echo	C	1996	Luer & R.Escobar, Orquideologia 20: 138 (1996).
R. ephippium	E	1996	Luer & Hirtz, Orquideologia 20: 141 (1996).
R. escobariana	C	1996	Luer, Orquideologia 20: 144 (1996).
R. jesupiana	V	1996	Luer, Orquideologia 20: 146 (1996).
R. mendozae	E	1996	Luer, Orquideologia 20: 157 (1996).
R. metae	C	1996	Luer, Orquideologia 20: 159 (1996)
R. purpurea	C	1996	Luer & R.Escobar, Orquideologia 20: 162 (1996)
R. radulifera	V	1996	Luer & R.Escobar, Orquideologia 20: 165 (1996).
R. renzii	V	1996	Luer, Orquideologia 20: 167 (1996).
R. roseola	V	1996	Luer & R.Escobar, Orquideologia 20: 170 (1996).
R. schizosepala	E	1996	Luer & Hirtz, Orquideologia 20: 172 (1996).
R. seketii	C	1996	Luer & R.Escobar, Orquideologia 20: 165 (1996).
R. tabeae	C	1996	H.Mohr, Leafl. Schlechter Inst. 2: 10 (1996).
R. tsubotae	C	1996	Luer & R.Escobar, Orquideologia 20: 178 (1996).
R. vasquezii	B	1996	Luer, Orquideologia 20: 180 (1996).
*R. piperitosa**	P	1998	Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 65: 119 (1998).
*R. portillae**	E	2002	Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 88: 109 (2002).
*R. howei**	E	2005	Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 103: 279 (2005).
*R. persicina**	E	2006	Luer & Hirtz, Monogr. Syst. Bot. Missouri Bot. Gard. 105: 255 (2006).
*R. fritillina**	C	2007	Luer & V.N.M.Rao, Monogr. Syst. Bot. Missouri Bot. Gard. 112: 110 (2007).

Notes:

The species distribution and references follow Luer, (1996a). The species epithets are those currently recognised on the World Checklist of Selected Plant Families (WCSP, 2013) and include five species (marked with *) not included in Systems of *Restrepia* (Luer, 1996a). Currently, *R. lankesteri* (Ames and Schweinfurth, 1930) although given specific status by Luer (1996a) is regarded as synonymous with *R. trichoglossa* (WSCP, 2013). This brings the current total number of species to 53.

Key: E Ecuador, V Venezuela, C Colombia, P Peru, B Bolivia, Mx Mexico, CA Central America, CR Costa Rica

much rarer and have a narrower geographical distribution compared to the earlier discovered species. Included in Table 1-1 are the two species discovered since 1996 for which formal descriptions have been published - *R. piperitosa* (Luer, 1998) and *R. portillae* (Luer, 2002) and the most recently discovered *R. fritillina* (Luer, 2007).

The majority of species described before 1901 have wide geographical distribution, e.g. *R. contorta, R. trichoglossa* and *R. brachypus* (Table 1-1). However, *R. falkenbergii* and *R. pandurata,* first described in 1880 and 1887 respectively, both remain uncommon species in both public and private collections. All species described since 1972, have been recorded from very few locations with two exceptions (*R. echinata* and *R. echo*) and those discovered from 1996 onwards from only one (as detailed in Luer, 1996a). This suggests that species which have only been recorded once or twice in the wild may occur as narrow endemics. This would also include *R. howei,* (Luer, 2005) which is unique in having no recorded locations. A single plant of *R. howei* was discovered in a collection of imported plants from Ecuador (Howe, 2006) and nothing further is known of its distribution or occurrence in the wild. This one plant has only been vegetatively propagated by leaf offsets, or 'keikis', thereby producing genetically identical offspring (Howe, 2006).

In Central America and Mexico, only two widespread species are known. Both of which were discovered prior to 1930, namely, *R. muscifera* (Lindley, 1859) and *R. trichoglossa* (Lehman, 1901). *R. trichoglossa* is also found throughout Venezuela, Colombia, Ecuador and Peru (thus making it the most widespread and common *Restrepia* species) and *R. muscifera* is also indigenous to Colombia. The third species native to Central America is *R. aberrans*. This was first identified in Panama in 1996 (Luer, 1996b) and has not been recorded in any other locations since. It is rare in the wild (Luer, 1996a) and in public and private collections.

1.4 *Restrepia* habitat – montane forests

Restrepia have their centre of distribution in the high Andes of Colombia and Ecuador and are to be found growing epiphytically in cool, wet conditions in the Andean montane forests (Luer, 1996a). They are often found in association with other Pleurothallids, (members of the orchid sub-tribe Pleurothallidinae) but are less common (Luer, 1996a). These montane forests are considered to be among the least known ecosystems in the tropics (Armenteras *et al.,* 2003) and the number and distribution of plant species, both epiphytic and terrestrial, have yet to be recorded for these habitats in many regions of South America. Many species are threatened by habitat destruction, and their long term survival, through self-sustaining populations, may be in question.

Tropical montane cloud forests occur in mountainous areas where local climatic conditions cause cloud and mist to be regularly in contact with the forest vegetation (Whitmore, 2001). These ecosystems contain an abundance of mosses, ferns, orchids and other epiphytic species on tree and rock surfaces (Whitmore, 2001). The definition of a cloud forest is not straightforward (Bubb *et al.,* 2004), as it occurs on a global scale within a wide range of annual and seasonal rainfall patterns (500-1000 mm/year) and at different altitudes. In the Andes of South America, it is typically found between 2000-3500m (Philips, 1997). In South and Central America the term 'cloud forest' is most commonly used in connection with montane forests. These forests support ecosystems of distinctive floral and structural form and contain a disproportionately large number of the world's endemic and threatened species, especially epiphytes (Whitmore, 2001).

Montane cloud forests are uniquely threatened both by human pressures and by climate change; impacting on temperature, rainfall and the formation of clouds in mountainous areas (Bubb *et al.,* 2004). *Restrepia* and other epiphytes are distributed throughout the

canopy on the basis of available moisture - either as rainfall or atmospheric humidity, which affects their diversity, abundance and distribution. Epiphytes (both vascular and non-vascular) are among the first species to be affected by phenomena such as global warming and changes in their populations may provide early indications of climate change, (Johansson, 1974; Benzing, 1990, 1998).

Cloud forests have also come under threat from changes in land use; such as the felling of trees for timber, farming or mining, all of which lead to deforestation. For example, in the Eastern Colombian Andes the most altered and fragmented ecosystems have been found to correspond to montane and sub-montane forests (Armenteras *et al.,* 2003). The cleared land is principally used for subsistence agriculture by resource-poor farmers (Bubb *et al*., 2004). The result, in a country such as Costa Rica, where rain forest was widespread until 50 years ago, is that the forest cover has now been reduced to isolated regions unevenly distributed throughout the country. This habitat fragmentation is happening throughout South America, but exactly how this is affecting *Restrepia* is unknown. Information has, however, been published on other genera within the Pleurothallidinae such as *Masdevallia* and *Dracula*. In common with *Restrepia,* the majority of *Masdevallia* and *Dracula* species have been found in single localities (Koopowitz *et al*., 1993). Most genera in the sub-tribe have similar distribution patterns. Using published deforestation rates and species distribution profiles, Koopowitz *et al.,* (1994) calculated that 402 of the total 3405 species within the Pleurothallidinae may already be extinct as a result of random deforestation. They later demonstrated these to be overestimates since they relied on the premises of total deforestation, even distribution of orchids and random felling (Koopowitz *et al*., 2003). Nevertheless, this represents one of the first attempts to predict extinction rates in orchids.

As early as the 1980s, it was realised that there was an urgent need to identify montane

forest areas with high concentrations of endemic species facing significant environmental threats. So the 'hotspots' analysis of tropical rain forests (Myers, 1988) was extended (Myers, 1990; Mittermeier *et al.,* 1999); thus identifying further high priority areas in temperate regions. By definition, a 'hotspot' has to contain at least 1,500 species of vascular plants (> 0.5 percent of the world's total) as endemics, and must have lost at least 70% of its original habitat (Myers, 1988). Total intact 'hotspot' habitats have been reduced from 12% to 1.4% of the land's surface (Brooks *et al*., 2002). These 'hotspots' contain 45% of known plant biodiversity (Myers *et al.,* 2000). In the Ecuadorian Andes, epiphytes constitute 30% of the vascular plant species in such biodiversity hotspots (Kuper *et al.,* 2004). Orchid 'hotspots' coincide with the centres of plant diversity, e.g. the Northern Andes, which contain proportionately high numbers of endemic orchid species (Cribb and Govaerts, 2005). The centres of diversity for *Restrepia* species coincide with the centres of orchid diversity. Of the 53 currently known *Restrepia* species, 28 are native to Columbia and 18 to Ecuador, many of which are narrow endemics (Luer, 1996a; Cribb and Govaerts, 2005).

Maps A, B and C (Plate 1-3) illustrate that the geographical distribution of *Restrepia*, montane forest distribution and Andean deforestation 'hotspots' coincide; thus highlighting the endangered nature of *Restrepia* habitats throughout South America. Although the genus as a whole may not be threatened with extinction, individual species in the most threatened locations most probably are. Furthermore, there may well be more undiscovered species; but habitat destruction may lead to their extinction before they can be identified.

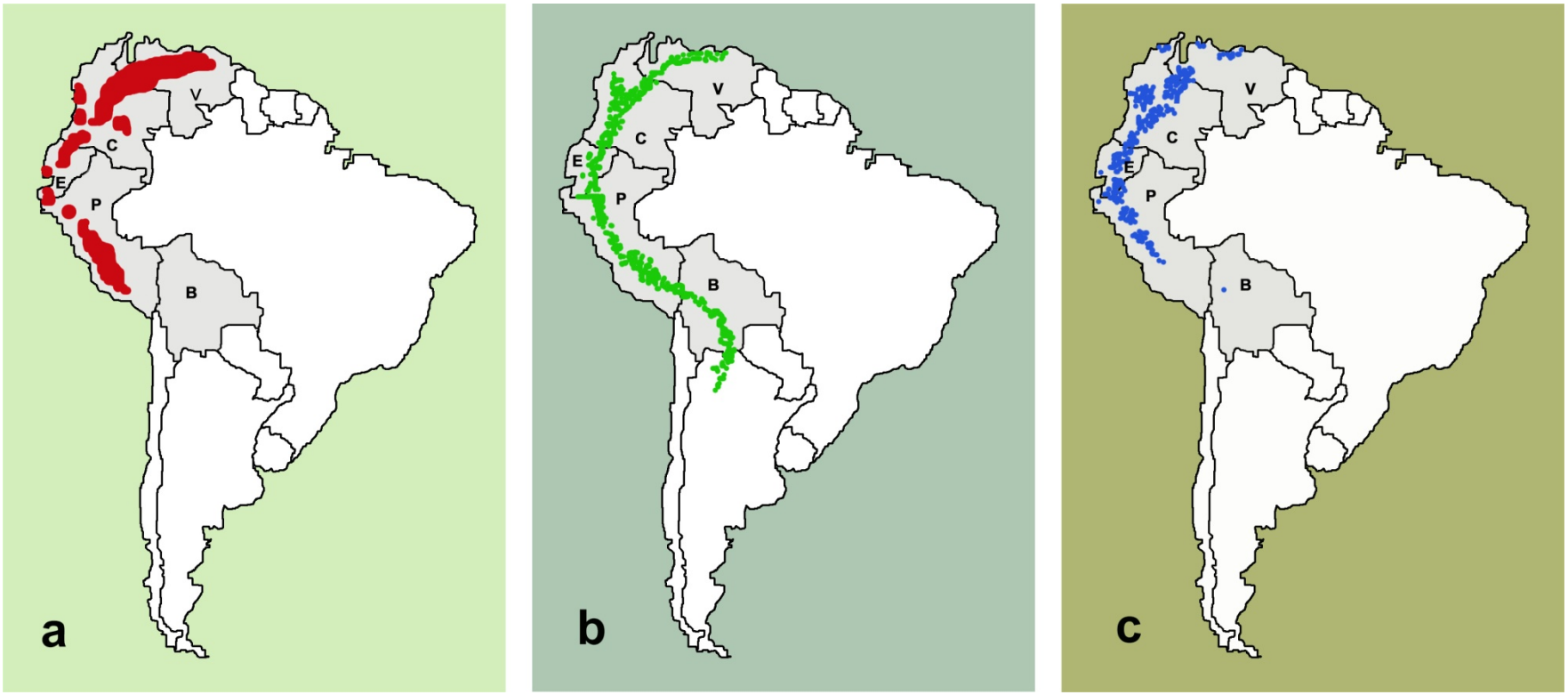


Plate 1-3: Maps of South America showing the distributions of montane forest, deforestation 'hot spots' and *Restrepia* species.

(a) 'Hot spots' of deforestation. Adapted from the TREE's project map, Global Land Cover Facility, University of Maryland. (Global Land Cover Facility, 2013).

(b) Distribution of montane forest vegetation. Adapted from the Tropical Montane Forest map (UNEP-WCMC, 2013).

(c) Distribution of *Restrepia* species. Adapted from information in – Systematics of *Restrepia* (Orchidaceae) (Luer,1996a).

Key: V Venezuela, C Colombia, E Ecuador, P Peru, B Bolivia

1.5 *Restrepia* taxonomy

Since first described, over 100 epithets have been attributed to members of this genus (Luer, 1996a). Over time many specific epithets have been reduced to synonymy and some species have been removed to form new genera e.g. *Barbosella,* (Schlechter, 1918), *Barbrodria* Luer and *Dresslerella* Luer (Luer, 1996a). The genus itself was reduced to synonymy with the related genus *Pleurothallis* by Williams in 1940, and in some literature *Pleurothallis* was still being used as the generic term for *Restrepia* as recently as 1989. For example, *Pleurothallis ospinae* (Hawkes, 1989) is synonymous with *R. antennifera* (WCSP, 2013). Currently, The World Checklist of Selected Plant Families (WCSP, 2013) lists over 135 synonyms for *Restrepia,* including one homotypic and seven heterotypic synonyms for *R. antennifera.*

1.5.2 Morphological taxonomy

In his series of monographs (*Icones Pleurothallidinarum),* Luer attempted to clarify classification and identification within the sub-tribe Pleurothallidinae using a traditional morphological approach. As such, *Icones Pleurothallidinarum XIII, Systematics of Restrepia* (Luer, 1996a) is the most comprehensive and important work regarding the taxonomy of *Restrepia.*

Prior to the publication of *Systematics of Restrepia,* many species could not be identified with any degree of certainty, (e.g. *R. purpurea,* and *R. mohrii*) and confusion still remains over many of the superficially similar forms of the more widely distributed species e.g. *R. antennifera*, *R. trichoglossa* and *R. brachypus*. The World Checklist of Selected Plant Families (WCSP, 2013) is the most important web based resource which provides information on the accepted scientific names and synonyms of monocotyledonous species. This resource has provided the *Restrepia* nomenclature used in the current study and follows that suggested by Luer, (1996a) with the exception of

R. lankesteri (Ames and Schweinfurth, 1930), which it reduced to synonymy with R. *trichoglossa*. Luer (1996a) recognised *R. lankesteri* as a separate species although stating that 'it was little more than a variation of a spotted form of *R. trichoglossa*'. The author himself, however, did not regard his monograph as definitive, and recognised that it was, "Only the best that could be done at the time using primitive, gross morphology," (Luer, 1996a).

The subtribe contains many plants which are small in size and have insignificant flowers which have been poorly described in the literature. This has made the identification of many species difficult and confusing. Pleurothallids were considered unimportant horticulturally compared to other brightly coloured, large flowered species as a consequence of which until the 1980s relatively little research had been performed on the subtribe (including *Restrepia*).

However, since the 1980s Pleurothallids have been the subject of various morphological and anatomical studies (Luer, 1986 – 2007; Pridgeon and Williams, 1979; Pridgeon, 1981, 1982a; Pridgeon and Stern, 1983, 1985; Stern *et al.,*1985; Neyland and Urbatsch, 1993; Stenzel, 2000, 2004). In 1986, Luer published his first *Icones Pleurothallidinarum* beginning with a generic survey. All of his classification then, and subsequently, was morphologically based. Pridgeon (1982b) and later, Neyland *et al.* (1995) tried to improve this taxonomic approach using numerical analysis, but morphological characteristics still provided the basis for their analyses.

1.5.2 Molecular taxonomy

In 2001, Pridgeon and co-workers presented the first molecular taxonomic data for the subtribe (Pridgeon *et al.,* 2001) which radically changed the Pleurothallid taxonomic system and changes in nomenclature were suggested in a subsequent publication,

(Pridgeon and Chase, 2001). Initially, this molecular taxonomic approach was rejected by Luer, but in later revisions of his earlier work he began to 'split' genera such as *Masdevallia*, which has resulted in a marked increase in the number of Pleurothallid genera from 31 to 129 in light of the molecular data (Luer, 2003, 2004, 2006, 2007; Tropicos, 2013).

Morphological traits that had traditionally been used in systematic studies of orchids, including the number of pollinia, presence or absence of an annulus, shape of the ramicaul and the transition area with the leaf base, degree of sepal connation, floral appendages and osmophores, shape and adnation of the labellum and other specialised floral structures exhibited a high level of parallel evolution within the subtribe. The subsequent homoplasy (Pridgeon *et al.,* 2001) made the distinction between homologous and analogous characteristics very difficult. Pridgeon and his colleagues took the view that due to the homoplasy common in vegetative and floral features (Pridgeon, 1982) within the sub tribe, that there was an absence of reliable homologous morphological and anatomical characters to interpret as synapomorphies.

In order to establish phylogenetic relationships within the sub-tribe, their studies combined evidence from nuclear and plastid DNA sequences (Pridgeon *et al.,* 2001). Nomenclature changes were suggested in a subsequent paper (Pridgeon and Chase, 2001). These works, together with the *Icones Pleurothallidinarum* monographs by Luer, are the most significant studies affecting nomenclature and taxonomy within the sub-tribe to date. So far, there have been no changes made in the genus *Restrepia* due to these two contrasting taxonomic treatments. However, this may change in regard to *R. aberrans* and *R. chocoënsis* as evidence from further DNA sequencing projects becomes available. Luer recognised three subgenera (Table 1-2) within the genus, with most species closely allied in the subgenus *Restrepia*. He isolated the only two species known to differ significantly (*R. aberrans* and *R. chocoënsis*) in two monotypic subgenera.

These two species are atypical of the majority of *Restrepia* species (Table 1-2), and neither were included in the 2001 study by Pridgeon *et al*. It is feasible that they may not be genetically closely related to the remainder of species in the genus. Not only do they differ morphologically, they also differ significantly in their pollination syndromes (as is discussed in Chapter 4).

In the study (Pridgeon *et al.*, 2001), there was strong support for the monophyly of *Restrepia* in contrast to other Pleurothallid genera, such as *Pleurothallis,* which were found to be polyphyletic (Pridgeon *et al.,* 2010). A monophyletic group is a taxon or group of organisms which form a clade consisting of a species and all its descendants. These are typically characterized by shared derived characteristics or synapomorphies (Pridgeon *et al.,* 2001). The close morphological similarities between *Restrepia* species would support this and would explain why there is little difference between the morphological taxonomy (Luer, 1996a) and the molecular taxonomy (Pridgeon and Chase, 2001; Pridgeon *et al.,* 2001) for this genus. In the study (Pridgeon *et al.,* 2001) there was moderate support in the combined analysis for a sister relationship with the monospecific genus *Pleurothallopsis* (Pridgeon *et al.,* 2010), which was previously considered to be unrelated. (This nomenclature should not to be confused with genus *Restrepia,* sub genus *Restrepia,* and section *Pleurothallopsis,* Table 1-2). It was the opinion of Pridgeon and colleagues that further sampling of DNA sequences of taxa in both genera is needed (Pridgeon *et al.,* 2010).

Table 1-2: A comparison of the morphological features within the subgenera of the genus *Restrepia* (Luer, 1996a).

Genus	Subgenus	Section	Number of species	Peduncle	Dorsal sepals	Lateral petals	notes
Restrepia	***Restrepia***	***Restrepia***	**42** Type species: *R. antennifera*	Elongated, flexible, bearing the flowers above the middle, near, or beyond the apex of the leaf.	Osmophores present at apex	Osmophores present at the apices	Habit and flowers of sections *Restrepia* and *Pleurothallopsis* are too similar to be segregated at the subgeneric level.
		Pleurothallopsis	**9** Type species: *R. muscifera*	Short, erect, usually less than half the length of the leaf bearing the flower against the back of the leaf	Osmophores present at apex	Osmophores present at the apices	
	Echmeles		**Monotypic:** *R. aberrans*	Elongate	Non-clavate, without osmophores	Free sepals without osmosphores	Labellar hypochile with obtuse lobes
	Pachymeles		**Monotypic:** *R. chocoënsis*	Elongate	No osmophores	Elongated, free apices, no osmosphores	Narrow, thickly coriaceous sessile leaves.

1.5.4 Problems of taxonomy

The taxonomy of orchids at the species level will always be problematic, due to the fact that they do not conform to any of the species concepts in the literature, (Pridgeon, 2003). This is due to their inter-specific and inter-generic hybridisation, often with no clear-cut demarcations between individuals, populations and species (Luer, 1996a). Therefore, the term 'species complex' is often used to describe wild populations of a particular orchid species. In *Restrepia,* the *muscifera, trichoglossa, antennifera* and *contorta* species complexes are well known and all contain a confusing number of types, between which it is often difficult to distinguish. For example, no two populations of the *contorta* complex have identical flowers (Luer, 1996a). This can make identification of plants in cultivated collections very difficult, and makes it essential that photographic records are kept by researchers and placed in herbaria, and that the provenance of plants is established as far as is practically possible.

As worldwide orchid conservation efforts increase, accurate species identification becomes increasingly important for biodiversity assessments, monitoring of habitats and identifying future targets for conservation (Schuiteman and de Vogel, 2003). DNA based methodologies are increasingly used as reference tools (APG, 1998) which often results in changes to the specific and generic names of many orchids. What is important is not which specific epithet is applied, or which species concept is adopted, but that the taxonomic authority used and the species referred to, are unambiguous (Pridgeon, 2003) so that any experimental work carried out with these species can be repeated.

1.6 Floral morphology of *Restrepia*

All *Restrepia* species have similar vegetative phenotypes and, apart from differences in size between some species, it is impossible to differentiate or identify the species with any degree of accuracy when not in flower. The coriaceous leaf is usually elliptical or ovate, and only the rigid, conduplicate leaf of *R. limbata* and the thick, linear leaf of *R. chocoënsis* are different to the rest of the genus (Luer, 1996a).

It is *Restrepia* flowers which exhibit the unique features of this genus, and although they share the general floral structure of orchid flowers, they possess several adaptations specific to the genus. A comparison of the morphology of a generic orchid flower and a typical *Restrepia* flower is presented in Plate 1-4. Flower sizes range from 1-10cm in length and are constant in some species, but are variable in others (Luer, 1996a). Unusually large flowers are known to occur in some populations (e.g. *R. antennifera* 'Gigantea') and these forms are the most sought after horticulturally.

In common with other orchids, *Restrepia* flowers are zygomorphic (i.e. show bilateral symmetry) compared to the majority of angiosperms whose flowers are actinomorphic (i.e. show radial symmetry). In many orchid species, the pedicel twists through 180º while the flower bud is developing, so that the labellum is lowermost, (a process termed resupination. In *Restrepia* the flower attains the resupinate position by bending backwards on the peduncle, no twisting of the ovary or pedicel occurs (Luer, 1996a). Their single flowered inflorescences are borne at the apex of an axis called the peduncle continuous with an inferior ovary (Bechtel *et al.,* 1992; Luer, 1996a).

The flowers themselves consist of a calyx comprising three sepals, with the dorsal sepal attenuate and clavate at the apex. The lateral sepals are connate into an elliptical synsepal. The synsepal is usually brightly coloured and often mistaken for petals, it is

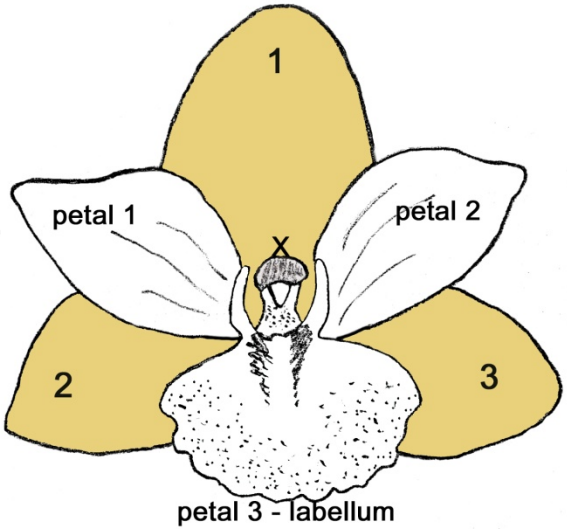
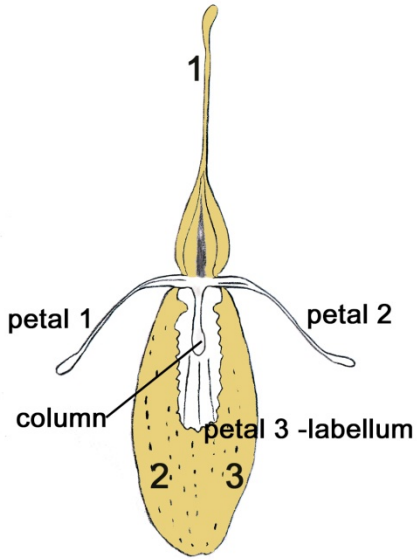
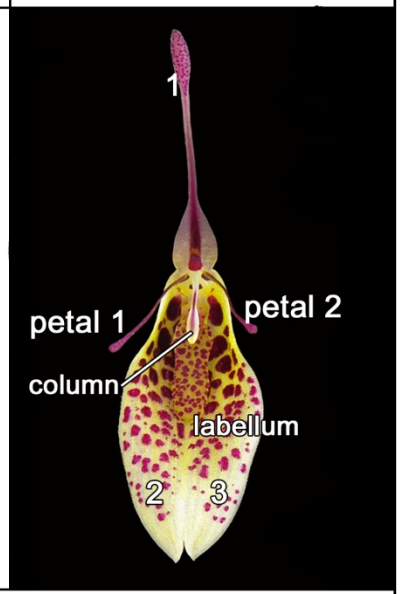
Diagram of a generic orchid flower	Diagram of a *Restrepia* flower	Photograph of *R. citrina*
		
Key: 1, 2 and 3 = sepals X = position of anther cap on head of the column The shape of the lip is often the distinguishing feature of a genus,in this case a generalised shape has been shown.	**Key to diagram and photograph:** 1, 2 and 3 = sepals 2 and 3 = joined synsepal Anther cap, shown at X on the first diagram is on the ventral surface of the column.	

Plate 1-4: A comparison of the morphology of a generic orchid flower and a typical *Restrepia* flower

the most conspicuous feature of all the species (Luer, 1996a). The basic colour of the synsepal varies from white, pink, purple, yellow, orange or tan with the addition of red, purple or brown pigmentation in the form of minute dots, spots or stripes. Some photographic examples of the species illustrating their colouration are shown in Chapter 2, Plates 23 and 24.

The two lateral petals are slender and clavate while the third petal is modified into an oblong lip (labellum). The detailed structure of the labellum together with the structure of its calli and cirrhi are discussed further in Chapter 2.

In *Restrepia,* the column, a structure unique to orchids, consists of a ventral stigma and anther with four pollinia. These are made up of four, free, equal-sized, ovoid pollinia in two pairs. A rostellar flap separates the pollinia from the stigma on the underside of the column. The pollinia taper into a stalk (caudicel) which attaches each one to a sticky gland termed the viscidium (Bechtel *et al.,* 1992). A comparison of the floral characteristics of the subgenera is shown in Table 1-2 and a more detailed description and discussion of the floral micromorphology of *Restrepia* follow in Chapter 2.

1.7.1 *Restrepia brachypus*

The species currently recognised as *Restrepia brachypus* (Plate 1-5) was discovered in 1859, and has since been known by various specific epithets -

Restrepia antennifera Lindl. 1859

Restrepia striata Rolfe 1891 (Plate 1-6)

Renathera striata Rolfe 1892

Pleurothallis hawkesii Flickinger 1963 Orchid Rev. 71:336, 1963, non *P. striata* Focke.

Restrepia hawkesii Flickinger 1963

Restrepia antennifera subsp. *striata* (Rolfe) Mohr, Leafl. Schlr. Instit 2:13, 1996

Restrepia brachypus Luer, 1996a, now the accepted specific epithet (WCSP, 2013).

This species was chosen as a model species with which to investigate self-incompatibility (SI) and inbreeding depression (ID) in the genus (Chapter 4) and floral micromorphology (Chapter 2). It was selected for the following reasons:

- it is a widely distributed species with two distinguishable geographical forms,
- it is common in cultivation and plants for study were readily available through the trade, through Plant Heritage and in the private collection of H. Millner,
- there were several recognisable, although unnamed, clones available for study,
- in cultivation it had never been hybridised nor propagated by seed, therefore genetically it may still resemble its wild counterparts.

The species was first known as *R. striata* from the drawing of a flower in the Kew Herbarium at the Royal Botanic Gardens, Kew, made from a specimen sent there in 1892 (Plate 1-6) and published in Curtis Botanical Magazine. The specific epithet was derived from the Latin *striatus,* meaning "striped," and referred to the striped synsepal; the number of stripes being considered a distinguishing feature of the species. The later epithet *brachypus* was derived from the Greek *brachypus,* "short-footed," referring to the short ramicaul (Luer, 1996a).

The specific epithets *striata* and *brachypus* are frequently confused today. However, the epithet *striatus* was reduced to synonymy with *brachypus* by Luer (1996a) and *brachypus* is the currently recognised epithet for this species (WCSP, 2013).

Plate 1-5: Typical *R. brachypus* flower

Internal scale bar represents 5mm

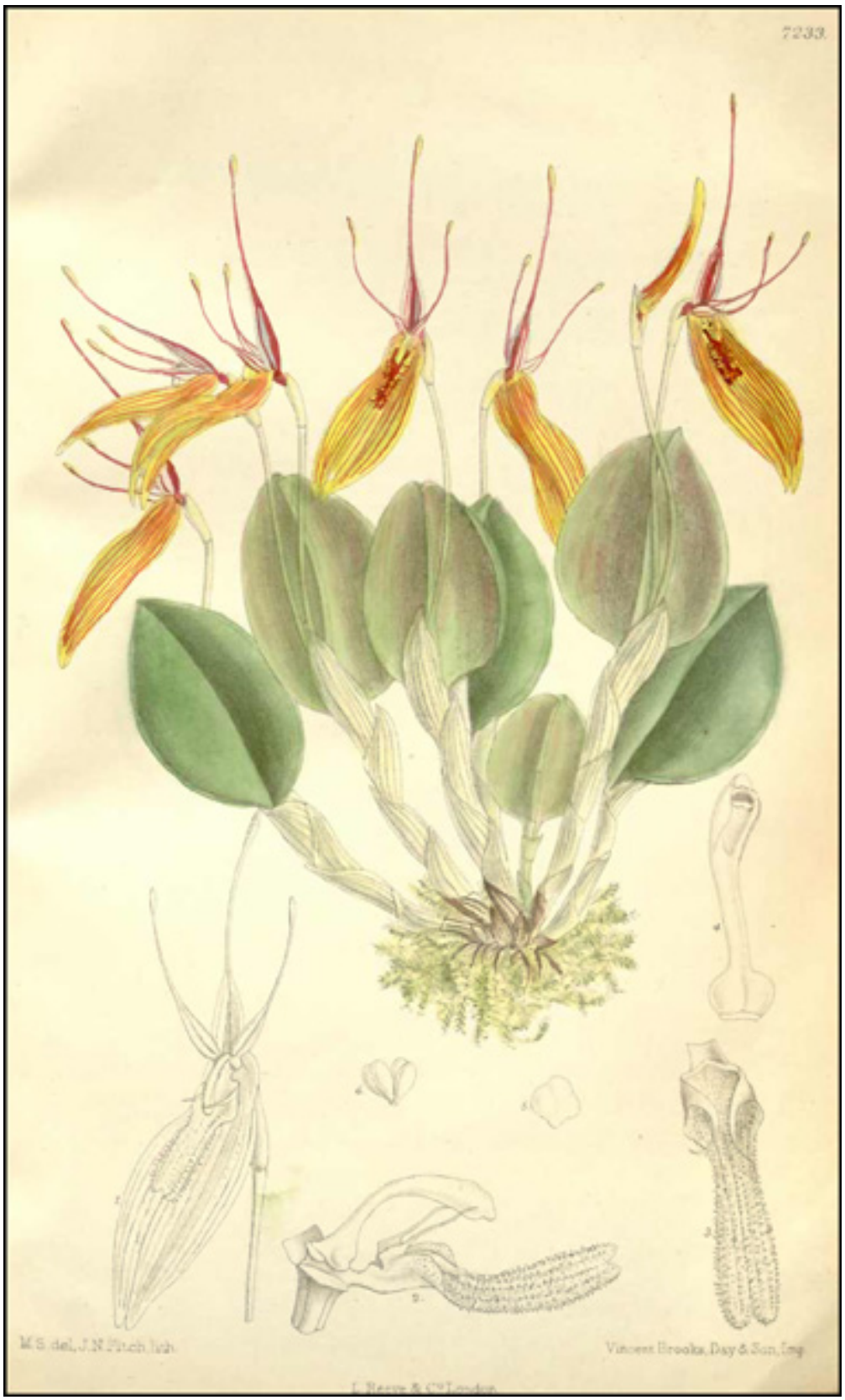


Plate 1-6: *R. striata* (syn. *R.brachypus)* Curtis Botanical Magazine (1892)
Image courtesy Missouri Botanical Garden, http://www.botanicus.org.

1.7.2 Formal description

The following formal description of *R. brachypus* is based on the description by Luer, (1996a) page 34.

Plant: medium in size to large, epiphytic, caespitose; roots slender. Ramicauls erect, 5-16 cm long, sometime prolific, enclosed by 5-10 thin, whitish, loose, compressed, more or less imbricating sheaths, the lowermost lightly dotted with black.

Leaf: erect, coriaceous, elliptical-ovate, subacute, 4-8 cm long.

Inflorescence: a solitary flower, produced successively in a fascicle up the back surface of the leaf; peduncle slender, 4-8 cm long; floral bract thin, tubular, 5-6 mm long; pedicel stout, 2-3 mm long, with a short filament; ovary purple, lightly sulcate, 3-4 mm long.

Sepals: membranous, the dorsal sepal free, erect, translucent, veined in red-brown, narrowly ovate below the middle, attenuated above the middle with the apex clavate-thickened, 22-37 mm long, 3-4mm wide above the base, 5-veined, the lateral sepals connate to near the apex into a shallowly concave, elliptical lamina, yellow, yellow-orange or tan, longitudinally striped in brown, sometimes with the stripes confluent towards the base, 21-37 mm long, 10-11 mm wide expanded, multiple-veined, the apex acute to subacute, minutely bifid.

Petals: membranous, translucent white, veined and more or less suffused in red-purple, narrowly linear-ovate, the margins minutely toothed near the base, attenuated above the middle with the apex clavate-thickened, 15-19mm long, 1.5mm wide at the base.

Labellum: yellowish, with 3 usually prominent, longitudinal stripes, marked with red-brown on both sides, narrowly oblong-subpandurate, 9-13 mm long, 2-2.75 mm wide, the epichile oblong, truncate, coarsely verrucose with fimbriate margins, the hypochile suborbicular, concave with thin, erect margins, each side with a capillary, uncinate process, the disc with a pair of low carinae extending forward from the base of each

process onto the epichile, the base subtruncate connected to the column-foot by a rigid, cylindrical neck.

Column: greenish white, slender, clavate, 5-6 mm long, the base pedestal-like with a pair of obtuse calli.

1.7.3 Distribution and provenance of *R. brachypus*

R. brachypus is widely distributed in Venezuela, Ecuador, Colombia and Peru and is one of the two *Restrepia* species indigenous to Bolivia. It inhabits wet, montane rain forests as a miniature, cool growing epiphyte, growing at elevations of 1180 to 3200 metres. Luer distinguished two geographical forms of the species, one, originating from Bolivia, having shorter pedicels and smaller flowers and the other, from Colombia, with larger, more slender flowers (Plate 1-7). Determining the exact provenance of cultivated plants and their country of origin is difficult, but many *R. brachypus* plants found in cultivation, closely resemble either the Colombian or Bolivian forms described by Luer (Plate 1-7). In this study (Chapter 4) two clones (designated Clone 2 and Clone 3) were used both of which were similar to the Colombian form described by Luer (Plate 1-7a) and a third (designated Clone 1) which was similar to the Bolivian form described by Luer (Plate 1-7b). It is possible to trace the importation of both of these clones. Clone 1 was imported by H. Millner (via J. and L. Orchids, Connecticut, USA) in 1995, country of origin unknown. Clones 2 and 3 were imported by H. Millner (via Ecuagenera, Ecuador) from Ecuador in 2004. Therefore, it is probable that Clone 1 originated from a different geographical location to Clones 2 and 3.

The floral dimensions of Clones 1, 2 and 3 all fall within the limits of natural variation for the species as identified by Luer (1996a), as shown in Figure 1-1. This confirms the suitability of these clones for use in the study as they are representative of the species.

The original accompanying text to the illustration (plate 1-6) from Curtis Botanical Magazine reveals some interesting details -

> '…*R. striata* (*sic*) was first known from the drawing of a flower in Kew Herbarium bearing the ticket, Schlim No. 68, and no doubt made from a new Grenadian specimen, from the Cauca range in which country Messrs. H. Low, of Clapton, sent specimen to Kew in 1892; but not till after the plant from which the accompanying drawing was made, which was sent in February, 1889, by Mr. Moore from the Glasnevin Gardens (still, as heretofore, in his father's time), so justly celebrated for its Orchid collection…'

Mr Moore was the son of David Moore who carried out much of the original work on orchid seed germination (Chapter 3). So, the first plants of *R. brachypus* or *R. striata,* as it was then known, came into this country via Glasnevin Gardens, Dublin. Thus, it is probable that the provenance of some currently cultivated plants is via vegetative offspring from these original ones.

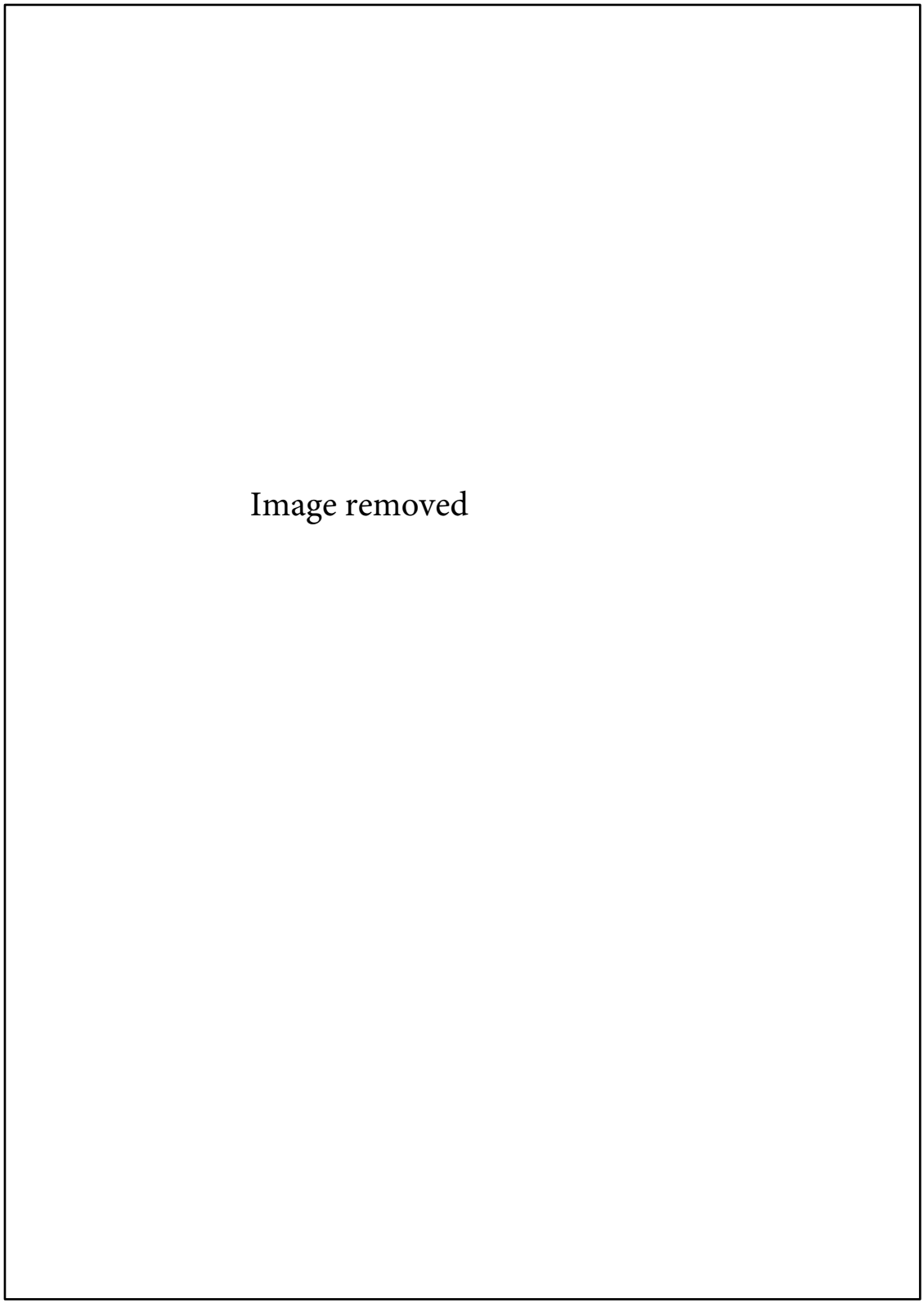


Plate 1-7: two forms of *Restrepia brachypus* as identified by Luer (1996a) The differences in size of plants and floral morphology is shown

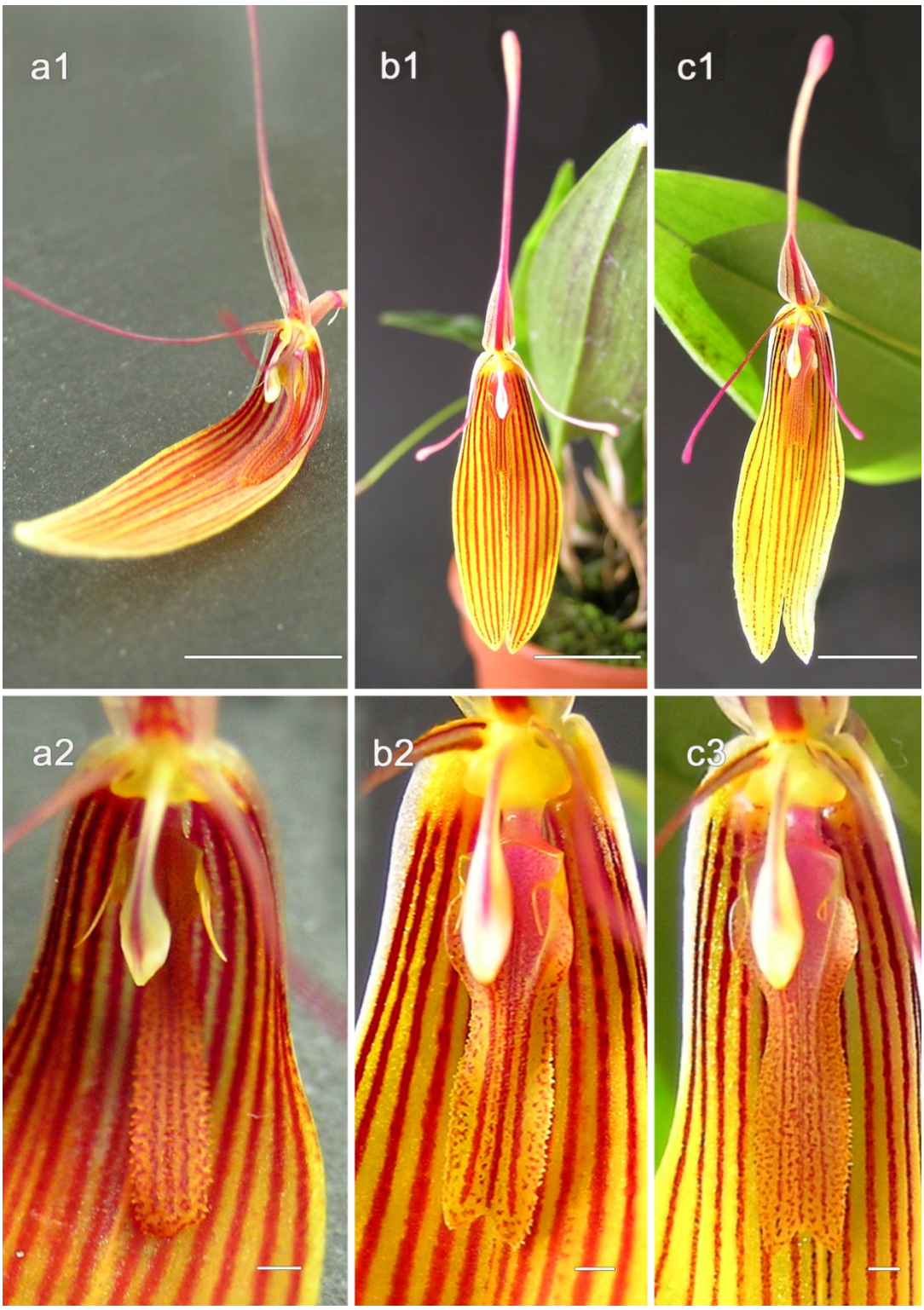


Plate 1-8: Comparison of the flowers and lips of the *R.brachypus* clones used in this study

Clone 1: (a1) flower (a2) lip detail; similar to the Bolivian form (Luer 1996a, Plate 1-7b)
Clone 2: (b1) flower (b2) lip detail; Clone 3: (c1) flower (c2) lip detail;
Clones 2 and 3 are similar to the Colombian form (Luer 1996a, Plate 1-7a).
Internal scale bars (a1, b1 and c1) represent 1cm, (a2, b2 and c2) represent 1mm

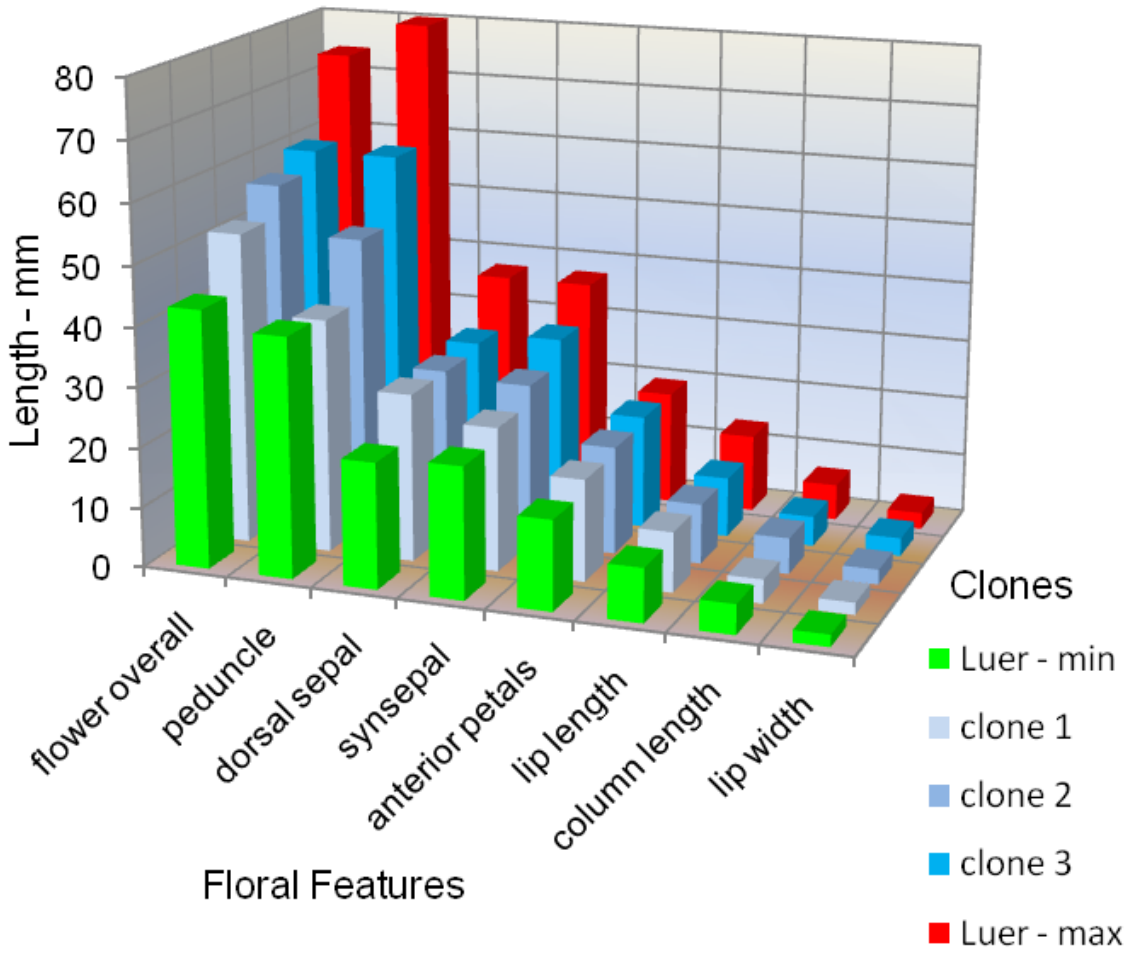


Figure 1-1: Floral feature measurements of the clones of *R. brachypus* used in the current study compared with the maximum and minimum values given by Luer (1996a) for *R. brachypus* flowers.

The maximum and minimum values for floral measurements found by Luer are shown by green (min.) and red (max.) bars. The corresponding average values for the clones used in the study are shown by the other variously blue coloured bars. These values fall within the limits of natural specific variation for *R. brachypus* flowers as identified by Luer (1996a), and confirm that these clones used are 'typical' of *R. brachypus* flowers.

1.8 Speciation and biodiversity

As mentioned previously, the centre of distribution for the genus is located in the high Andes of Colombia and Ecuador (Luer, 1996a). Species occur as epiphytes in rain and cloud forests at elevations between 350-3500 metres (Pridgeon *et al.,* 2010). There is one species-complex from which all other species seem to emanate - the *R. contorta* complex (Luer, 1996a). The results from the molecular taxonomic study (Pridgeon *et al.,* 2001) would appear to substantiate this, as *Restrepia* species (subgenus *Restrepia* and sections *Pleurothallopsis* and *Restrepia*, Table 1-2) are regarded as forming a separate clade i.e. a group of organisms derived from a common ancestor.

The explosive orchid speciation that has occurred recently in the Neotropics has been documented by various authors (Gentry, 1982; Gentry and Dodson, 1987; Haffer and Prance, 2001; Dodson, 2003; Kay *et al.,* 2005; Pinheiro and Cozzolino, 2013). *Restrepia* speciation would have occurred as part of the adaptive radiation of orchid species in these areas in response to dramatic changes in geology and climate (Gentry, 1982); the causative agents of allopatric speciation (Haffer and Prance, 2001). The following account of how rapid speciation may have occurred along the Eastern Andes in Ecuador and Colombia has been adapted from Dodson (2003).

Localities in the Andes are known to exhibit high species diversity. One locality may contain over 300 species of orchids. Similar habitats on the eastern side of the Andes in Ecuador, at the same elevation and with similar rainfall, 100 km apart, may have only 10% of species in common (Gentry & Dodson 1987). Orchids in these localities share a range of physical characteristics which predispose them to adapt to the changing nature of these habitats. These characteristics include epiphytism, wind borne seed dissemination, numerous seed production from a single pollination event, preadapted pollinators, pollinator specificity through deception and genetic flexibility.

Historically, the climate has gone through striking change with advancing and receding glacial periods and consequent changes in temperatures. Only ten thousand years ago the region came out of a glacial period in which the average temperatures were 5º C lower than those of the present day (Colinvaux, 1987). There is evidence to show that few orchid species would have been able to occupy their present range during the glacial periods (Dodson, 2003). It is estimated that orchid populations had to recede at least 1000 meters lower than today in order to survive. A lesser glacial period occurred in Ecuador as recently as 3000 years ago.

Volcanic activity has been constant in this region over past millennia. The extensive deposition of lava and volcanic ash quickly killed any existing plant cover, but provided new habitat for invasion by plants. Orchids were among the first pioneers being able to travel long distances. Given the physical characteristics of orchids, rapid change in the genetic nature of populations leading to explosive speciation was possible.

As the glaciers receded, vast areas became available for migration of the pioneering survivors. Due to the fractured and fragmented nature of the montane habitat these survivors would probably have constituted small populations with built-in pollinator specificity, and thus strong reproductive isolation. Any mutants with preadaptive selection for existing pollinators would have become quickly fixed and would have developed into new species very rapidly. Fixing of variants would have led to the explosive orchid speciation seen today. It is likely that many of the orchid species currently found in the Neotropics have developed since the last major glaciation period (Dodson, 2003).

Dodson (2003) explained the gene fixation of the small interbreeding populations by the Wright effect or 'Shifting Balance Theory of Evolution' (Wright, 1977), which attributed evolution to genetic drift or change from random events. In contrast

Tremblay *et al.* (2004) argued that gene flow was an essential component of evolutionary processes and an important component of diversification in orchids. The amount of gene flow among local populations would determine whether or not individual populations could evolve independently, be genetically distinct and plausibly lead to cladogenesis. Their current view of orchid evolution involves both genetic drift and natural selection working simultaneously. They regarded genetic drift as a common occurrence in local populations followed by natural selection, ultimately causing sufficient differentiation and the beginning of cladogenesis. Subsequently, this new taxon, (for example *Restrepia contorta*, the mother species-complex) would colonize new sites. Low reproductive success, leading to a small proportion of reproducing individuals makes genetic drift combined with episodic selection a more likely source of evolutionary change in orchids and explains much of the diversification of the family (Tremblay *et al.,* 2004) and *Restrepia* therein.

1.9 Biodiversity

Without genetic variation, populations cannot evolve in response to changing environmental pressures and may be at risk of extinction even if their population size has not decreased. Biodiversity at the genetic level may be seen as the driving force of evolution (Heywood, 1995; UNEP, 2001). A taxon with limited genetic variation such that it no longer represents a viable population was termed 'functionally extinct' by Koopowitz (2001). Subsequently, the question arises as to just how many narrow endemic orchid species, including *Restrepia* species, may now be in this category?

Studies of genetic variation within the Pleurothallid genera *Pleurothallis* (Borba *et al.,* 2001a) and *Lepanthes,* (Tremblay *et al.,* 2006), concluded that genetic variation is high, because these genera were out-breeding, and that this had worked to ensure high genetic variability within the studied populations. However, if environmental pressures

continue to increase, out-breeding may become less feasible and species numbers may decline.

For out-breeding populations, such as those of the Pleurothallidinae, habitat fragmentation and destruction pose much greater problems than dwindling population size alone; since, as natural habitats become fragmented, the chance of successful cross-pollination within any given species is reduced and self-pollination increases. Enforced self-pollination for these species can lead to in-breeding depression. This is characterised by a loss of 'productivity' or reduction in the rate of conversion of resources to biomass per unit area, per unit time (Waide *et al*., 1999). This may be expressed in fewer viable seeds, fewer offspring or less vigorous offspring. Data from this research suggests that this may be true for *Restrepia* also and this is discussed in detail in Chapter 4.

1.10 Pollination

The link between orchid diversity and pollination systems has been made by various authors (van der Cingel, 2001; van der Pijl and Dodson, 1996; Tremblay *et al.,* 2004). In the most recent of these publications (Tremblay *et al.,* 2004), the authors suggested that the predominance of pollination limitation had had a significant effect on the evolution of the Orchidaceae and explained both the intricate pollination mechanisms as well as the diversification of the family. Variation in fruit set was dependent on pollinator activity which varied between populations and between years which made orchids severely pollination limited (Tremblay *et al.,* 2004).

Darwin (1877) had linked the unusual pollination mechanism of orchids with evidence for both natural selection and the advantages of cross-pollination (Tremblay *et al.,* 2004); and had made the link between floral morphology and pollinator

anatomy/morphology. He accurately predicted the anatomical features of the moth required to pollinate the orchid *Angraecum sesquipedale*, now commonly known by various epithets such as 'Darwin's Orchid', 'Darwin's Comet Orchid' or 'Christmas Star Orchid'. When the pollinator, a moth species, was later identified it was named *Xanthopan morganii praedicta* (Rothschild and Jordan, 1903). Current literature still recognises the adaptation of flowers to pollinators and the concept of 'functional fit' between flower and pollinator in a variety of pollination syndromes (Benitez-Vieyra *et al.*, 2006).

The insect vector for pollination in *Restrepia* has yet to be confirmed (Luer, 1996a), although there is wide acceptance in the literature that this genus is most probably myophilous or fly pollinated (Pridgeon and Stern, 1983; Christensen, 1994; Blanco and Barbosa, 2005). Indirect evidence for this hypothesis comes from comparisons with related genera within the sub-tribe, such as *Pleurothallis,* for which there is substantial documented evidence of fly pollination (Borba and Semir, 2001; Borba *et al.,* 2001a, 2001b, 2002; Blanco and Barbosa, 2005). Other authors, (van der Cingel, 2001; Primack and Corlett, 2005) have stated that the Pleurothallidinae, as a whole, is a fly-pollinated group. Most of the observations by van der Cingel were of small, *Drosophila*-like flies. Vogel and Renner (1992) suggested that the fly pollinators of many Pleurothallidinae are guided by osmophores occurring on appendages, or inside the flower. In *Restrepia* and *Scaphosepalum,* osmophores have been shown to be present at the petal apices and the dorsal sepal apex (Pridgeon and Stern, 1983, 1985), thus providing further circumstantial evidence for fly pollination in these genera. The osmosphores were found to produce aminoid fragrances, which were thought to act as fly-attractants that could function over long distances (Pridgeon and Stern, 1983).

1.10.2 Mechanisms preventing self-pollination

Genetic SI, ID and mechanical barriers that prevent self-pollination have all been observed in the fly-pollinated genus *Pleurothallis* (Borba *et al.*, 2001b). The genetic similarity among conspecific populations is known to be high for species with very short-range flying pollinators (Borba *et al.*, 2001b) though much of the literature considers orchids to be usually self-compatible (van der Pijl and Dodson, 1966; Dressler, 1990, 1993; Borba *et al.*, 2001b), *Pleurothallis* species display weak self-incompatibility and inbreeding depression (Borba *et al.*, 2001b). These mechanisms in concert appear to be effective in promoting seed set by cross-pollination, as suggested by the high degree of genetic variability observed (Hamrick and Godt, 1990; Borba *et al.*, 2001a). Borba and colleagues (2001b) noted that these combined genetic attributes frequently occur in fly-pollinated orchids, or in species in which the pollinators visit for a long time in flowers of the same individual.

Many orchids are thought to deceive pollinators by offering no rewards (Dafni, 1984; Ackerman, 1985); and fly pollination has also been linked with a lack of floral nectar or pollinator reward (Jersáková and Johnson, 2006; Schiestl, 2005). Jersáková and Johnson (2006) hypothesised that this seeming contradiction worked to discourage self-pollination, by frequent visits from the same pollinating insect, thus reducing geitonogamous and autogamous self-pollination.

In conclusion, although there are general assumptions (as outlined) that *Restrepia*, in common with other Pleurothallid genera, is a myophilous genus, there are no recorded details of the ways in which pollination occurs. Nothing is known regarding whether *Restrepia* is a rewarding or non-rewarding genus or to what extent SI, ID, and mechanical barriers that prevent self-pollination operate within the genus.

1.11 Recording and identifying endangered species

Although the identification of the *Restrepia* species is now possible through the morphological details published by Luer (1996a; 1998; 2002; 2007) and their synonyms published by WCSP (2013), identification in itself, does not provide any indication of the extent to which these species may be endangered. They are known to occupy habitats in the Andes which have undergone deforestation and fragmentation (Plate 1-3). Therefore, while it would seem reasonable to assume that *Restrepia* populations have suffered and declined as a consequence of these changes to their habitat there is currently no study to establish the precise nature or extent of the threats to *Restrepia* species. Without such knowledge it is difficult to formulate any action plans for their conservation or even establish if these are required.

Plant species identified as endangered have been recorded in Red Data Books and Red Lists produced by the World Conservation Union, (IUCN), since 1963. These have been compiled in order to provide data on taxonomic and conservation status plus distribution information on the taxa that have been evaluated (Kerry and Gillett, 1998). This system was designed to determine the relative risk of extinction, catalogue and highlight those taxa facing a high risk of global extinction. Thus, Red Lists represented an important input to scientific conservation work.

In recent years, the Red List categories and criteria have been modified (Hilton-Taylor, 2000; IUCN, 2012a) in order to produce a more objective system. All new assessments and reassessments must follow this revised system (IUCN, 2012a). The Species Survival Commission (SSC) is a network of more than 7000 scientists who provide assessments with independent peer review (Lamoreux *et al.,* 2003), of these, approximately 200 members belong to the Orchid Specialist Group (OSG). The SSC is responsible for the current Red Lists; whereas the orchid Red Lists are the work of the OSG.

For some orchid genera, there is currently too little data to compile a comprehensive and accurate global Red Data Book, although many countries have produced their own national Red Data Books, listing threatened species within the country. Unfortunately, in biodiverse tropical countries, these Red Lists may range from 'highly subjective to erroneous' (Ibisch *et al.,* 2003). A common problem with national Red Data Books is that they do not distinguish between taxa of local and global importance (Sutherland, 2000). This is a crucial distinction with regard to the conservation status of *Restrepia*.

To date, no *Restrepia* species have been included in the global Red List of Endangered species (IUCN, 2013a). However, this may not be a true reflection of the Endangered status of *Restrepia* in the wild. For example, although *R. chocoënsis* and *R. guttulata* were both classified as being Locally Endangered by Calderon (1996) this is not an accurate reflection of their relative status. *R. chocoënsis,* as a narrow endemic in the Choco region of Colombia should be listed as Endangered locally, nationally and globally, but it has not been to date. In contrast, although *R. guttulata* is Endangered in the Cauca region of Colombia, it is a common species elsewhere and hence should not be considered as globally Endangered.

In Ecuador fieldwork has been undertaken to update the Red Lists (Valencia *et al.,* 2000; Léon-Yánez *et al.,* 2011), but the situation is still far from perfect. In 2000, more than a third of endemic plant species registered were known from a single population, fewer than 25% of the species recorded occurred within protected areas (such as national parks), a majority of the endemic species classified as Critically Endangered were not represented in Ecuadorian herbaria (Valencia *et al.,* 2000). The implication from this is that any narrow endemic *Restrepia* species in Ecuador would be unlikely to have been recorded either. This will most probably apply in their other countries of origin also, since Ecuador is considered to have documented more of their orchid

species than other South American countries due to their greater collecting efforts (Dodson, 2003).

In Bolivia, of the 380 Pleurothallid species, approximately a quarter are vulnerable due to their restricted distribution. This includes one endemic species, namely *R. vasquezii* (Ibisch *et al*., 2003). *R. aberrans* from Panama and *R. chocoënsis* from Colombia are also both known to be endangered, as they have only been found in two locations. Other narrow endemic species thought to be endangered, require their conservation status to be confirmed by field data, as emphasized by Koopowitz *et al*., (2003).

1.12 *In situ* and *ex situ* conservation

In light of the previously stated threats to *Restrepia* habitat the question arises as to which methodologies should be used to conserve the genus. Without doubt, the most effective means of conserving orchids is to protect their habitats (Cribb *et al.,* 2003). *In situ* conservation would study a habitat together with the role(s) of an organism within that ecosystem. For example, orchids might be studied in conjunction with their pollinators, their distribution and their environment. In the past, conservation has aimed at the preservation of an ecosystem, in its perceived original condition. More recently, however, conservation has increasingly become concerned with the active restoration of habitats and recovery of species in the areas of forest decimated by felling, farming and historical over-collection of orchids and other species. It has become recognised that such *in situ* approaches to conservation are no longer sufficient in themselves, and that there is a need for complementary methodologies (Fay and Krauss, 2003). The maintenance or restoration of genetic variation is vital for such populations to avoid genetic decline and maximise their evolutionary potential (Fay and Krauss, 2003). Research on a rare, endemic *Lepanthes* species (Tremblay *et al.,* 1998) demonstrated that low reproductive success had management implications for all endangered orchid species. Statistical

methodologies were then developed to identify orchid populations at risk (Tremblay and Hutchins, 2003). Other research has increasingly recognised inbreeding depression as having an important effect upon conservation biology (Hedrick and Kalinowski, 2000).

The second approach available for orchid conservation is via *ex situ* conservation. Until recently, this was often thought to consist only of public and private botanical collections. However, in recent years *ex situ* conservation has come to be used for a variety of purposes. For example, the production of material for conservation research, the supply of material to reduce pressure from wild collecting and the production of material for reintroduction, reinforcement, habitat restoration and management. Such *ex situ* collections including living plant collections, seed banks and tissue cultures all of which need to be managed according to strict scientific and horticultural standards to maximise their value for conservation purposes (BGCI, 2012). As wild populations of orchids have become depleted it is clear that cultivated orchid species and collections, together with *ex situ* methodologies are a vital but currently underutilised set of conservation resources (Maunder *et al*., 1997).

The Convention on Biological Diversity (CBD), (UNEP, 2001) recognised that *ex situ* conservation complemented *in situ* conservation by providing material for recovery programmes, and in developing research and education programmes. CBD supports the sustainable use of the habitat as a last line of defence against extinction in the wild in contrast to the previous conservation ideal of a return to a pristine 'original' state. The original, primary objective of the CBD was that 60% of threatened plant species should be held in accessible *ex situ* collections, preferably in the country of origin, with 10% of these included in recovery and restoration programmes by 2010, Target 8, Global Strategy for Plant Conservation (GSPC), (CBD, 2006). In addition, genetic studies are now seen as an important tool as part of an integrated approach to conservation studies (Fay and Krauss, 2003).

From a conservation perspective, orchids are in a unique situation. They are studied *in situ* by scientists and they are grown *ex situ* by many orchid enthusiasts worldwide. Some private orchid collections have provided source material and formed the basis of scientific studies, (Stpiczynska *et al.,* 2003; Oakeley, 2003, 2005; Ryan and Oakeley, 2003; Davies and Turner, 2004;) enabling species to be studied without endangering wild populations. In relation to *Restrepia* and its conservation, there are several organisations worldwide that are concerned with the subtribe Pleurothallidinae and its study e.g. The Pleurothallid Alliance, (USA branch) and Pleurothallid Alliance UK. Members of these groups have provided plant material for scientific study (Pridgeon *et al.,* 2001) and some are Plant Heritage national collection holders (Plant Heritage, 2013). These collections include some *Restrepia* species (Howe, 2005). The current study has only been possible with help from members of some of these organisations.

1.13 Summary

It has been the intention throughout this introduction to outline the current state of knowledge regarding the genus *Restrepia* and thus to identify areas where there are suitable avenues for further research. Its history and discovery has been presented, its habitat and distribution together with a consideration of the threats to its montane habitat. The effects of molecular taxonomy on its nomenclature have been discussed together with a brief consideration of theories on how the genus may have originated and subsequently diversified through speciation. An outline of *Restrepia* floral morphology as it pertains to general orchid floral morphology was presented and current views on fly pollination within the Pleurothallidinae discussed. Finally, the current Red List status of the genus species was described.

From this review of the literature the 'gaps' in the knowledge that this study will seek to address are as follows:

1. While the general floral structure of *Restrepia* is well documented the micromorphology of the labellum and its associated structures is unreported in the literature. Both the cirrhi and calli remain unstudied. Previous studies of the micromorphology of the sepal and petal osmophores are now nearly 30 years old and SEM technology has improved markedly since then.
2. *Restrepia* species in cultivation have not been raised from seed, making these plants still genetically similar to their wild counterparts. Nothing is known of seed, seedling development, developmental timescale or generation time as these have never been recorded. It has not been established whether this genus has specific requirements for axenic seed germination and seedling culture.
3. The precise pollinator has never been identified for any species in this genus or the mechanism by which pollination occurs. In particular the link between the pollination syndrome and breeding system has not been established.
4. The breeding system in *Restrepia* has never been studied. Nothing is known of pre- and post-pollination mechanisms in *Restrepia*. The effects of ID, SI and their effects on the post-pollination biology within the genus are unstudied.
5. The present conservation status of most species within the genus is unknown. It seems reasonable to assume that many may be endangered, but a Red List assessment of all the species so that their direct threats could be identified has not been carried out.

The main works regarding *Restrepia*, without doubt, have been those of Pridgeon and Stern (1983) and Luer (1996a). Since these works were published there has been an enormous change in the ways in which scientific research and data can be freely accessed via the internet. Many organisations now publish extensively, although not

exclusively, online (Tropicos, 2013; RGB Kew, 2013a; BGCI, 2013) whereas the Global Red lists have been published exclusively online since 2003 (IUCN, 2013). There are now resources available with which conservation assessments may be made, in particular the GeoCAT tool released by the GIS unit, based in the Herbarium at Kew which is intended to help with Red List assessments (Bachman *et al.,* 2011). So, not to pre-empt the introduction to Chapter 5, a more detailed discussion will be left until then, but it is necessary to mention the GeoCAT tool here as it is integral to the aims of the study.

In order to address some of these questions and provide answers, which may enable this and other Pleurothallid genera to survive long-term both in cultivated and wild populations the following aims of the study were formulated.

1.14 Aims of the study:

1. To use scanning electron microscopy (SEM) and photographic techniques to perform a study of the floral micromorphology of *Restrepia*, and a subsequently to observe and record the micromorphology of the labellum and associated structures and use these observations to formulate a putative pollination hypothesis.
2. To establish protocols for the axenic seed germination of *Restrepia* to enable species and hybrids to be propagated from seed, thus helping to maintain, or increase genetic diversity within cultivated populations. Subsequently to study the development of the seedlings into flowering plants thus providing horticultural protocols to facilitate the *ex situ* conservation of this and related genera.
3. To establish the presence or absence of breeding barriers within the genus such as SI and ID by using cultivated populations to elucidate breeding systems that may operate in their wild counterparts.

4. To carry out a Red List assessment of *Restrepia* species using recently developed internet resources in order to a) identify the direct threats to the species and b) assess the usefulness and effectiveness of such resources for this purpose.

"…flowers have evolved with distinct combinations of characteristics which make them recognizable and distinct".
C.A. Luer (1996)

2.1 Introduction

2.1.1 Floral reproductive structures of *Restrepia*

The main distinguishing features of the flowers of this genus were originally documented by Humboldt (Humboldt *et al.,* 1816) and were later described by Luer (Luer, 1996a). These features include a joined synsepal, an elongated dorsal sepal and lateral petals (Plate 1-4). While these features serve to distinguish *Restrepia* from other Pleurothallid genera, they do not include all the floral reproductive structures which occur in the genus.

An illustration of the reproductive structures that occur in *Restrepia* flowers is shown in Plate 2-1. The structures of the labellum (lip) and the column are shown together with the positions of the calli and uncinate processes. The term 'uncinate process' was used by Luer (1996a) and is shown in Plate 2-1. Pridgeon and Stern (1983) referred to these structures as 'cirrhi', which is the preferred term for the remainder of this thesis. In addition, the clavate apices of the dorsal sepal and lateral petals which contain osmophores are also shown.

The relative position of these reproductive structures within the flower is presented in Plate 2-2. Since all species in the genus are very similar in this respect (Luer, 1996a) the exemplar used is *R. guttulata*, *R. brachypus* having already been described and illustrated in Chapter One (Plates 1-5 and 1-6). Further illustrations which show the similarities between species may be found in Plates 2-23 and 2-24, which follow further on in this chapter. A further detailed description of the floral organs follows in which the numbers in parenthesis refer to Plate 2-2.

The flowers are resupinate and pedunculate or sessile in a minority of species. The lateral petals (1) and (2) and the dorsal sepal (3) are elongated and filamentous with clavate apices (a, b, and c) which contain osmophores (Pridgeon and Stern, 1983). The

most striking feature of the *Restrepia* flower is the large colourful synsepal (4), which is formed by the joining of the lateral sepals. The third, ventral, petal is modified to form a smaller labellum or lip (5), with two uncinate processes (Luer, 1996a) or cirrhi (Pridgeon and Stern, 1983) (6) which resemble thorns. The column (7) is slender, clavate with a ventral anther and stigma. The column foot has two calli (8), one either side of the base.

Plate 2-2 (inset) shows detail of the column (7), the cirrhi (6), the position of the anther cap (10), which covers four equal sized ovoid pollinia and the stigmatic surface (9) on the ventral surface of the column.

While all of these structures have been previously documented (Pridgeon and Stern, 1983; Luer, 1996a) the functions of the calli and the cirrhi have never been established and nor imaged using current microscopic or photographic technologies. Pridgeon and Stern investigated the function of the apical osmophores of the dorsal sepal and lateral petals, and carried out scanning electron microscopy (SEM) of their structure (Pridgeon and Stern, 1983). Since this investigation, no further studies of the morphology or function of the floral organs in the genus have been published. Luer (1996a) recorded the details of the floral structures including the calli but wondered 'what the function of these strange features could be'. While scanning electron microscopy is still the technique of choice for performing detailed morphological studies, there have been significant advances in SEM technology since work published by Pridgeon and Stern thirty years ago (Pridgeon and Stern, 1983).

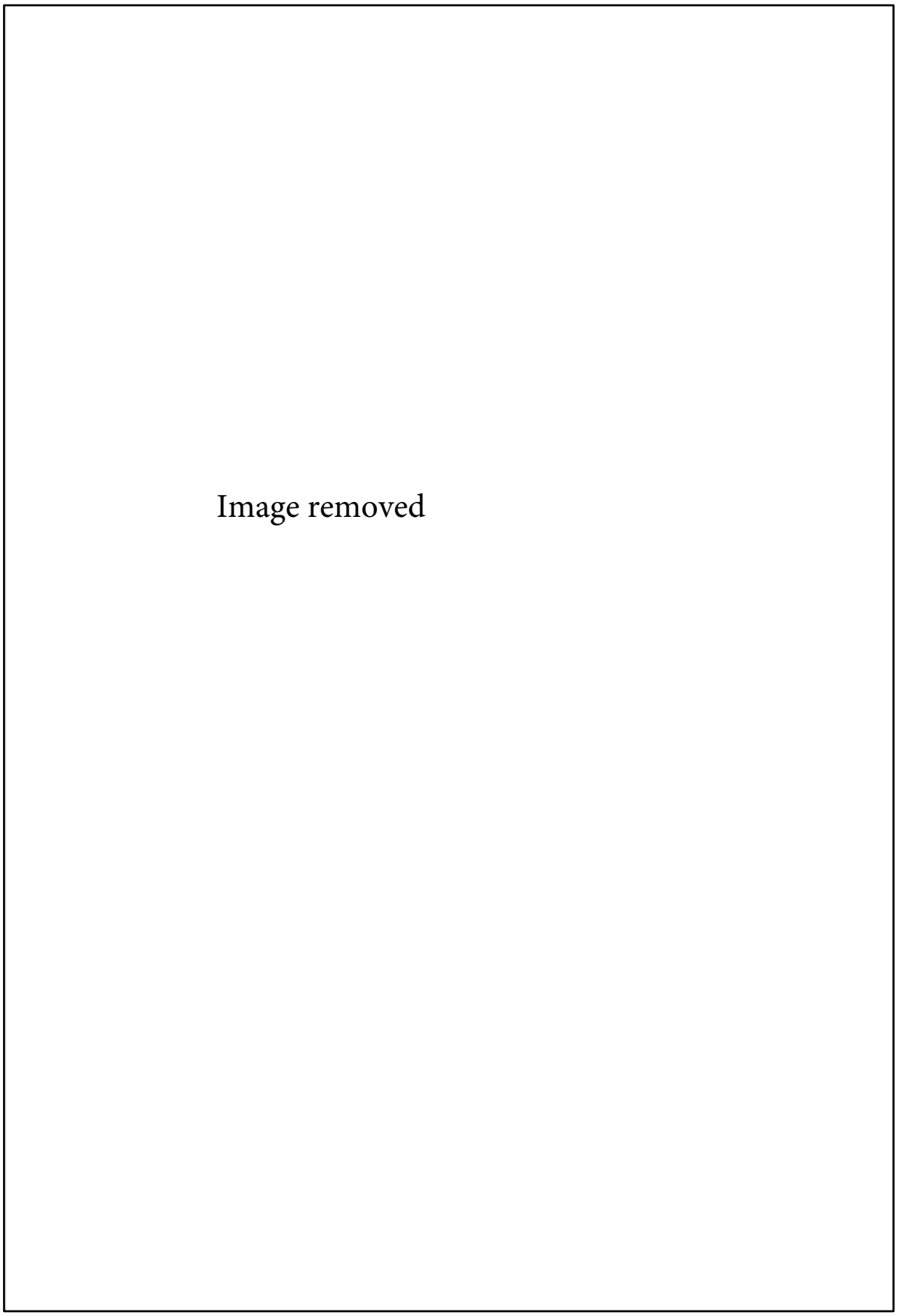


Plate 2-1: Morphology of the floral organs of *Restrepia* (Luer, 1996a)

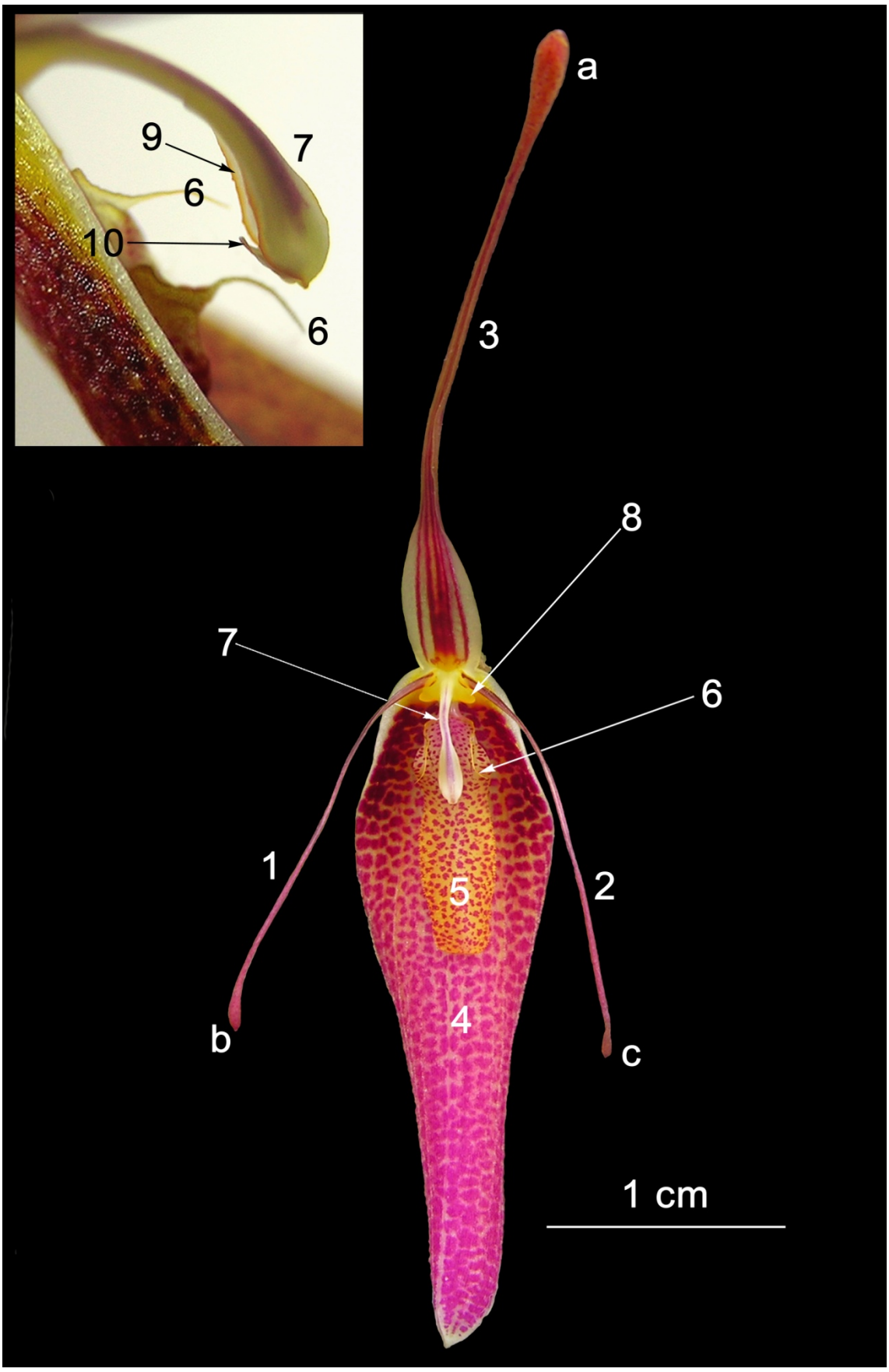


Plate 2-2: Floral morphology of a *Restrepia* flower. Exemplar *R. guttulata.*

2.1.2 Scanning electron microscopy

In scanning electron microscopy (SEM), a fine beam of electrons with energies typically up to 40 keV is focused on a specimen, and scanned along a pattern of parallel lines. Various signals are generated as a result of the impact of the incident electrons, which are collected to form an image. These are mainly secondary electrons, with energies of a few tens of eV, high-energy electrons backscattered from the primary beam and characteristic X-rays (Bogner *et al.,* 2007).

Electron microscopy was developed during the 1930s, as it was considered that electron microscopes would provide a better resolution than light instruments (Ruska, 1993). The first ‘‘scanning microscope’’ was built in 1935 by Knoll and the theoretical principles underlying scanning electron microscopy were established in 1938 (Haguenau et al., 2003). It was not until 1963, that a prototype for the first commercial SEM was produced by Pease and Nixon (Pease and Nixon, 1965; Hawkes, 2004; Oatley, 2004) and was later developed in 1965 (Breton, 1999).

The recent development of environmental SEM (Bogner *et al.,* 2007) was directly linked to the high vacuum required for the function of electron microscopes, which imposed restrictions on the way that biological specimens needed to be prepared. Research by Danilatos and Robinson in the 1970s led to the first SEM capable of maintaining a relatively high pressure, thus removing the need to dry and coat the specimens (Danilatos, 1991). This gave rise to the possibility of imaging biological specimens in a more ‘natural’ state (Danilatos, 1991). The term ‘‘environmental’’ SEM was introduced in 1980 and by the late 1980s the first commercial environmental scanning electron microscopes (ESEM) were being produced. This opened up new possibilities for observing untreated biological specimens and led to the ESEM gaining rapid acceptance. The main difference between ESEM and conventional SEM is the presence of a gas in the specimen chamber. Samples are thus not viewed under high

vacuum, but under a deteriorated or ‘‘low’’ vacuum. This was made possible by the special design of the electron optics column (Danilatos, 1993).

Pridgeon and Stern performed their investigations of osmophore structure in the early 1980s, before ESEM had been developed commercially and thus they were unable to view the structures in the ‘natural’ state that is now possible. Added to which were the limitations in the way that the images were recorded, i.e. as photographic plates or film rather than as digital images. The use of digital imaging for ESEM or SEM machines did not become commonplace until the early 1990s. Current ESEM machines can produce significantly higher resolution images, recorded digitally, thus enabling further enhancements of contrast and definition to be made, should this be necessary.

2.1.3 Developments in photography

The past thirty years has also seen a correspondingly rapid change in photographic technologies. Fairly modest digital cameras are now capable of producing detailed close-up or macro images. Thirty years ago these would have required a high-end SLR (single lens reflex) camera with special accessories. The images would have been recorded on film which required subsequent further developing and printing. Digital technology enables the image to be viewed immediately either through the camera or a computer so enabling further images to be taken if necessary. Alongside the growth of consumer digital cameras, computer software technologies for storing and editing the images produced have also rapidly advanced. Images can be digitally stored, replicated or enhanced in ways that were not possible thirty years ago. Adobe Photoshop, a graphics editing program developed and published by Adobe Systems, has become the industry standard for image manipulation. The most recent version of this programme, Adobe Photoshop CS6 extended, was used extensively to produce the final images presented in this thesis.

2.1.4 Rationale for the morphological study of *Restrepia*

These technological advances have opened up new ways with which to study the morphology and micromorphology of *Restrepia* floral structures. The following issues were identified regarding the floral morphology of *Restrepia* to which current technology could be applied in order to further present understanding and knowledge.

1. To confirm or refute the original findings of Pridgeon and Stern on *Restrepia* osmophore structure. Two contrasting ESEM technologies were available with which to perform this study, the technical details of which are provided in the Materials and Methods section (2.2).

2. (a) The reproductive organs of *Restrepia* have not been imaged using current ESEM technologies which should be capable of producing higher resolution images.

   (b) In particular the micromorphology of the labellar regions had never been studied, although three distinct areas of the labellum had been previously described by Luer (1996a). Any labellar secretions have never been identified, nor their role in attracting a pollinator explained.

3. Nothing was known concerning the mechanism of pollination in this genus, neither had there been any link established between pollination syndrome and breeding system. It was hypothesized that an in-depth investigation of the morphology/micromorphology of the floral structures of *Restrepia* flowers would provide a means to address these issues.

The intention in this section of the study was therefore to use current ESEM and photographic technologies in order to investigate the floral morphology and the labellar micromorphology of *R. brachypus* so as to address the gaps in the knowledge of the genus.

2.1.5 Chapter objectives:

1. To observe and record the floral structures, including the labellum, of *R. brachypus* flowers by performing an ESEM study.

2. To perform a comparative photographic study of the floral morphology of various *Restrepia* species including *R. brachypus.*

3. To postulate a hypothesis for pollination in this genus, based on the morphological data obtained.

2.2 Materials and methods

2.2.1 Plant material

The plant material used came from the personal collection of H. Millner; all the plants used in this part of the study had been green house grown under the same conditions. Night temperatures were maintained at a minimum of 58ºF/15ºC by electric fan heaters controlled by an independently wired thermostat. Supplementary lighting to extend day length during the winter months was provided by the use of T5 CFTs (compact fluorescent tubes). Humidity was maintained by enclosing the plants in large propagators with adequate ventilation to ensure that the temperatures inside did not rise above ambient greenhouse temperatures. Humidity and temperature inside the propagators were monitored via gauges.

Plants of *R. brachypus* which resembled Clone 1, the Bolivian form, (see Chapter 1 - Introduction) were used for the investigations. The original plant had come from J & L Orchids, Easton, Connecticut, USA. Plants of other species used had originated from Ecuagenera, Ecuador, via the trade in the UK.

A detailed study of the floral organs of *Restrepia* was performed using two different ESEM techniques (Cool Stage 2.2.2 and Cryo Stage 2.2.3). Floral specimens of *R. brachypus, R. dodsonii*, *R. muscifera* and *R. guttulata* were examined on the Cool Stage (see 2.2.2) but only *R. brachypus* specimens were examined on the Cryo Stage (see 2.2.3). In total, the floral organs from 16 flowers of *R. brachypus* were examined on the Cool Stage. Some of the features observed were confirmed in other species i.e. *R. dodsonii*, *R. muscifera* and *R. guttulata*, but only two flowers from each of these species were examined. An additional 20 flowers of *R. brachypus* were used for the Cryo Stage investigation. All of the images presented in the Results section are of *R. brachypus*, except where noted.

2.2.2 Variable Pressure Scanning Electron Microscopy (VPSEM) (Cool Stage)

The initial images for the current investigation were obtained using variable pressure scanning electron microscopy (VPSEM) using a Cool Stage (Deben UK Ltd., Suffolk). This method (Chen *et al.,* 2011) was used as it provides results close to the examination of fresh, untreated biological material and is a rapid technique that does not require a long time for the preparation of the samples. This work was carried out at the University of Wolverhampton.

Floral specimens were prepared by dissection under a low power dissecting microscope, and then were attached to the Cool Stage of the microscope using carbon sticky pads. Samples were then rapidly frozen in liquid nitrogen to -20°C and examined in a Carl Zeiss EVO LS 15 SEM with variable pressure capability at 20 kV, imaged using a variable pressure secondary electron (VPSE) detector. In order to reduce charging artefacts, a low pressure of about 30 Pa of air was used. This very low pressure is sufficient to compensate for specimen charging and provide a gas phase scintillation signal for the VPSE detector. The Cool Stage is a Peltier device that is able to rapidly reduce the temperature of the specimen to -20°C. Extended periods of SEM imaging are then possible, since hydration of the specimen is maintained.

The images produced were 1024 x 760 pixels for 70 dpi monitor display, but this resolution is too low for photographic printing which requires 300 dpi. The images were manipulated in Photoshop by having distracting details in the backgrounds removed, contrast and brightness adjusted and some sharpening. Where necessary, to enable the original images to be reproduced as shown, digital resampling (interpolation) techniques were used. In the VPSEM images, scale bars have been taken from the original images.

VPSEM images of the osmophores, synsepal, labellum (epichile, isthmus and hypochile), column, anther caps, pollinia, calli and cirrhi were obtained using this protocol.

2.2.3 VPSEM (Cryo Stage)

It was not possible to resolve certain morphological details on the micrographs using the initial VPSEM technique (utilising the Cool Stage). So a second, more advanced VPSEM technique using a Cryo Stage was employed to further investigate the surface features of the osmophores, synsepal, labellum and calli. This work was carried out at the Centre for Electron Microscopy Unit at the University of Birmingham.

Specimens of *R. brachypus* were prepared as described previously and were mounted onto a Cryo Stage (Quorum PolarPrep S2000 Cryo Transfer System). They were rapidly frozen using liquid nitrogen to a temperature of -180°C and sputter coated with platinum. The Cryo Stage allows more rapid freezing to a lower temperature than the Cool Stage which therefore results in better sample integrity with fewer ice crystals. This results in improved images which are more 'true to life'. The specimens were examined under a FEI XL30 FEG ESEM and the images later manipulated in Photoshop as described previously.

2.2.4 Photographic study

There is a particular difficulty when photographing *Restrepia* flowers, in that they are not flat. Consequently, close-up photographs that have limited depth of field often result in images in which not all the floral details are in focus. In particular, it is extremely difficult to obtain images in which both the dorsal sepal and the centre of the flower are in focus. Natural light was used for all the photographs presented (except where noted) and small apertures (f10 or less) were used to ensure a maximum depth of field.

The calli were photographed under three different types of illumination: daylight, torch light in a darkened room and long wavelength ultra violet light (UV) (450nm) in order to determine their optical properties.

A detailed photographic study of the relative positions of the cirrhi, column and labellum in different species was also performed and a Nikon Coolpix 800 digital camera was used to produce close-up digital images of these structures. Using the measurement tool in Photoshop CS6, accurate pixel measurements of the labellum and column were performed. This enabled the ratio of labellar length to column length to be accurately calculated for a range of species. Mean values of 10 measurements were used for the calculations.

2.2.5 Pollination Hypothesis

By using a combination of the data obtained from the ESEM study, in particular the labellar micromorphology, the photographic data and measurement data a pollination hypothesis for *Restrepia* was formulated.

2.3 Results

2.3.1 Osmosphores

2.3.1.1 Cool Stage

The adaxial surface of the dorsal sepal of *R. brachypus* showing its papillate structure, at anthesis, is presented in Plate 2-3A (a) and (b). In some images 'pale patches' were evident on the cuticular layer of the cells (c) and (d). These 'pale patches' were found on the cuticular surfaces of all the species observed - *R. brachypus, R. muscifera, R. dodsonii and R. sanguinea*.

The papillate structure of the dorsal sepal of *R. brachypus* two days post-anthesis is

presented in Plate 2-3B. The shrunken osmophore papillae are illustrated (a). Indentations and shrinking in the cuticular layer of the osmophores occurring since anthesis may be observed (b) and (c). This shrinkage was observed to occur between one and two days post-anthesis in all the species studied - *R. brachypus, R. muscifera, R. dodsonii and R. sanguinea*.

2.3.1.2 Cryo Stage

The adaxial surface of the dorsal sepal of *R. brachypus* at one day pre-anthesis is presented in Plate 2-4. The osmophores appear turgid and rounded, their cuticular surface smooth, with no 'pale patches' and without cuticular shrinkage (a), (b) and (c). In images (d), (e) and (f) rough patches are observed corresponding to the positions of vesicles (Plate 2-5). In comparison one day post-anthesis (Plate 2-5) the osmophores still appear relatively turgid but some possible shrinkage is observed at the sides (a) and (b). The cuticular surface was uneven and vesicles could be observed on the surface (c), (d) and (e), corresponding to the positions of the 'pale patches' found previously. No 'pores' in the cuticular surface were observed (Plates 2-4 and 2-5) which is in agreement with previous studies (Pridgeon and Stern, 1983).

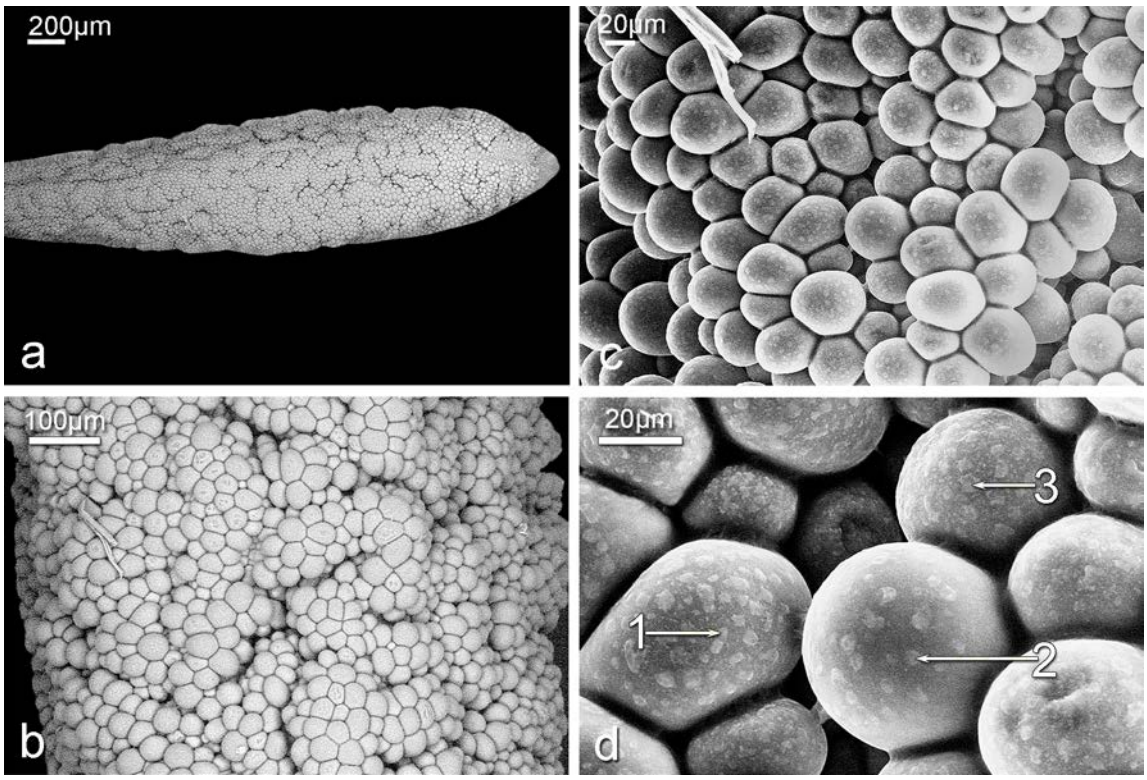


Plate 2-3A: Adaxial surface of the dorsal sepal of *R. brachypus* at anthesis (Cool Stage). Papillate structure and (d) 1, 2, 3 'pale patches' on cuticular layer of the cells

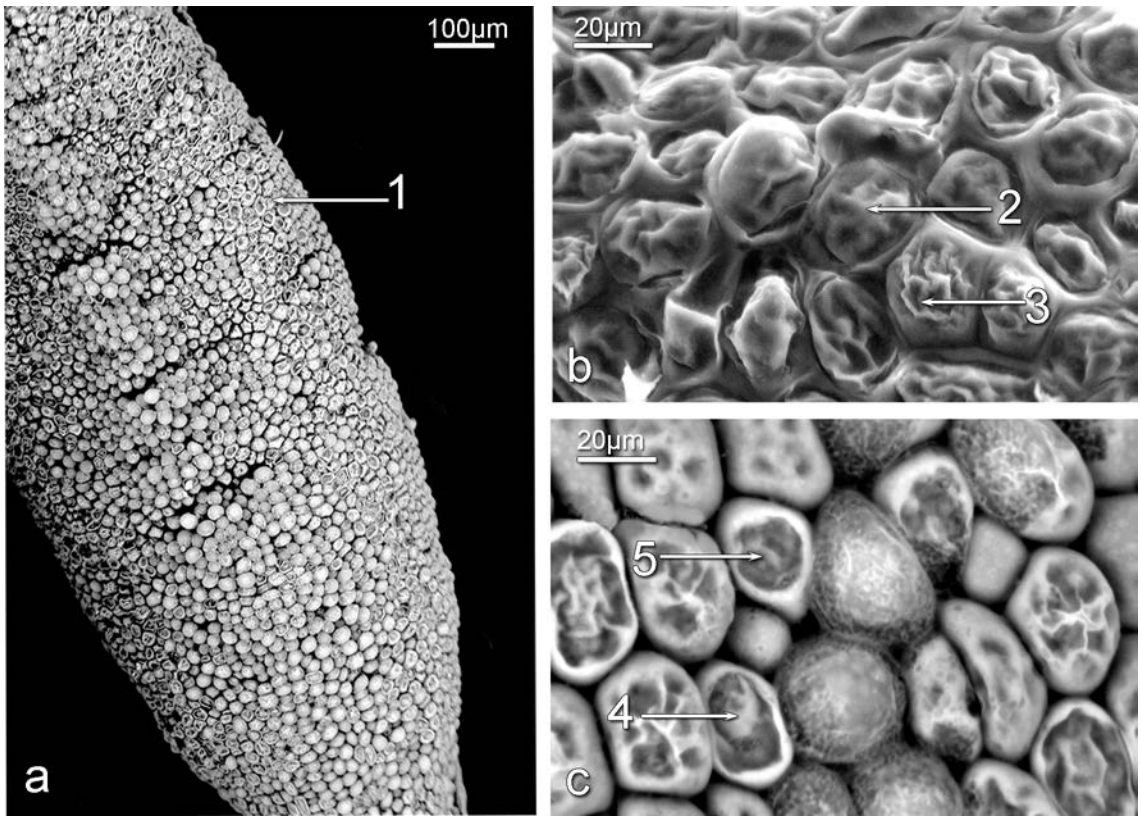


Plate 2-3B: Adaxial surface of the dorsal sepal of *R. brachypus,* two days post-anthesis (Cool Stage)

(a) Region 1, papillae appear shrunken; (b) and (c) 2, 3, 4 and 5 indentations and shrinking in the cuticular layer of the papillae.

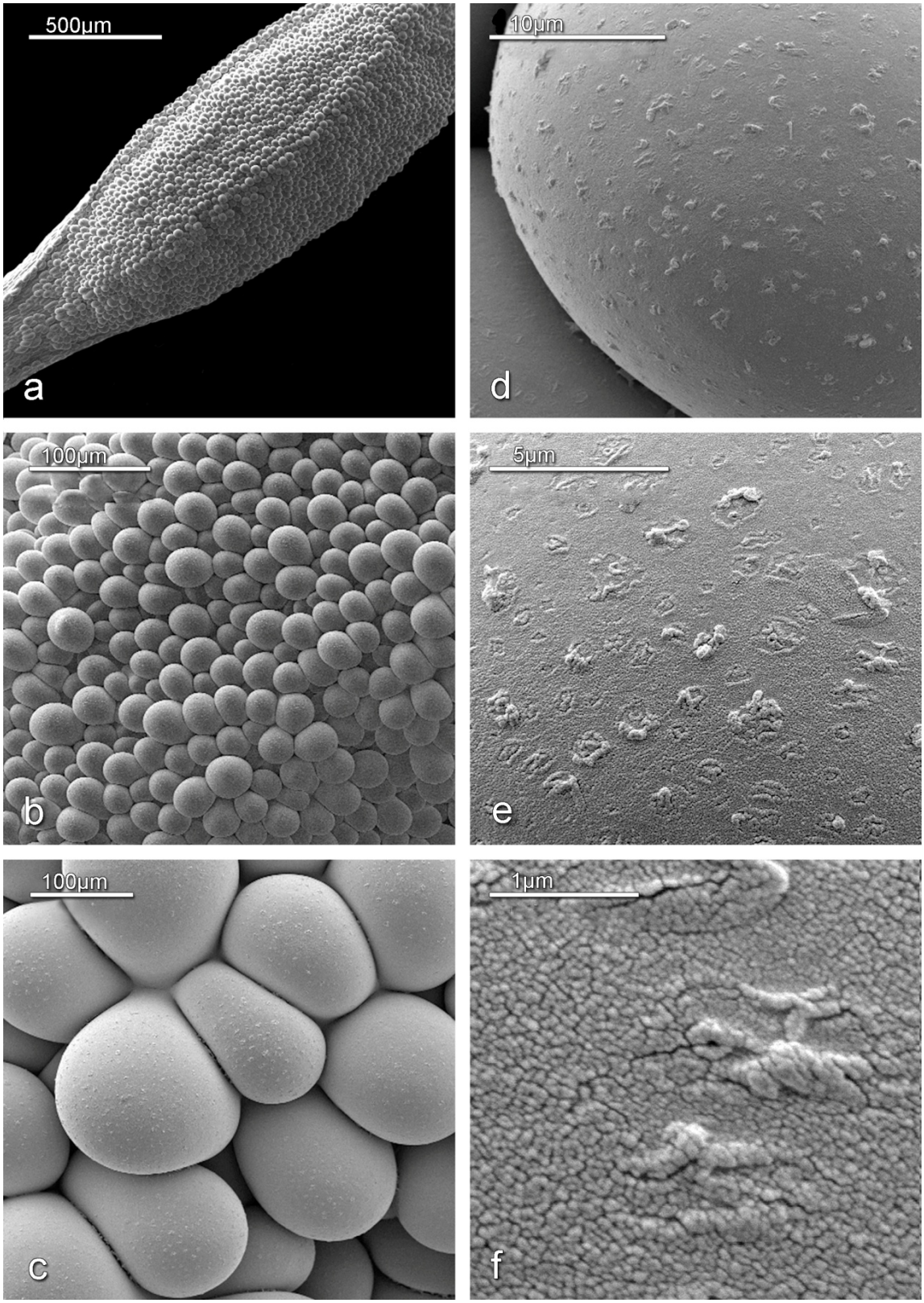


Plate 2-4: Adaxial surface of the dorsal sepal of *R. brachypus,* one day pre-anthesis (Cryo Stage)

(c) Papillae smooth, no 'pale patches' and no vesicles with no shrinking or indentations in the cuticular layers; (d), (e) and (f) rough patches on the cuticle corresponding to position of vesicles.

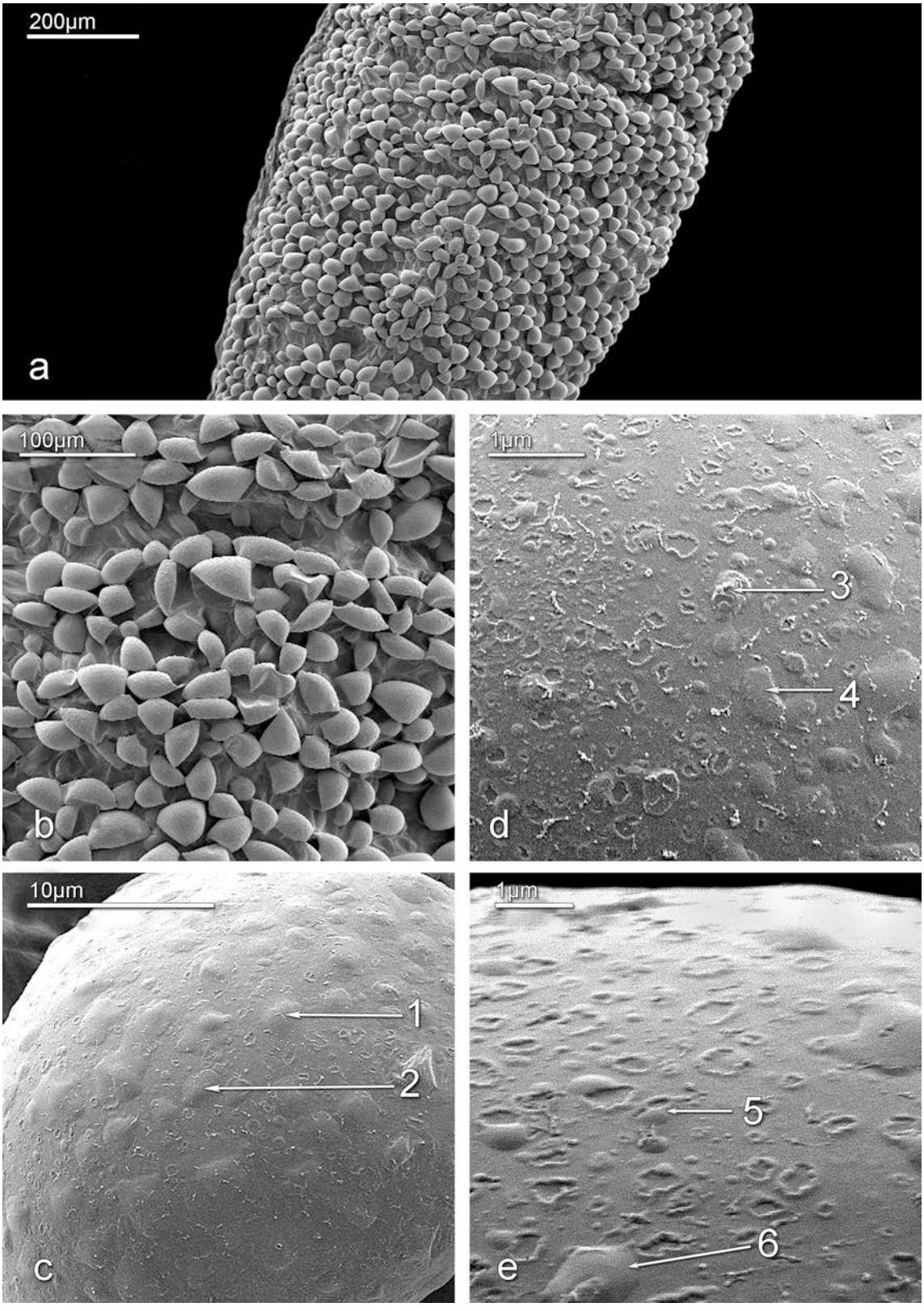


Plate 2-5: Adaxial surface of the dorsal sepal of *R. brachypus* one day post-anthesis (Cryo Stage)

(*cf.* Plate 2-3A) (c) 1 and 2, (d) 3 and 4 (e) 5 and 6, vesicles on osmophore surface;

2.3.2 The synsepal

2.3.2.1 Cool Stage

Images of the papillae present on the surface of the synsepal of *R. brachypus,* one-day post anthesis, are presented in Plate 2-6. The linear arrangement of the papillae (a) corresponds to the stripes observed on the striped synsepal of *R. brachypus* (photograph). Some of the papillae have already begun to shrivel (a). Higher magnification images of the papillae (b) and (c) illustrate the individual papillae and the non-papillate areas between them more clearly. Raised 'bumps' on the cuticles of the individual papillae (d) are observed. The cellular region between the papillae (e) 3 and 4 is non-papillate while the cellular cuticles in this region do not exhibit any raised 'bumps'.

2.3.2.2 Cryo Stage

In comparison, Plate 2-7 illustrates the synsepal of a flower of *R. brachypus* at anthesis, in which all the papillae appear turgid (a), (b) and (c). The younger papillae are also smaller in this example. Higher magnification reveals further details of the inter-papillae region (d) and of the papillae (e) and (f). The cuticular surface of the cells between the papillae is smooth, with no visible 'bumps' or vesicles (d) 1. The rough areas on the papillae surface (e) and (f) do not seem to correspond with the position of the 'bumps' observed on the papillae in Plate 2-6 (d) 1 and 2. These rough areas appear towards the apices of the papillae, while the 'bumps' are arranged along their length.

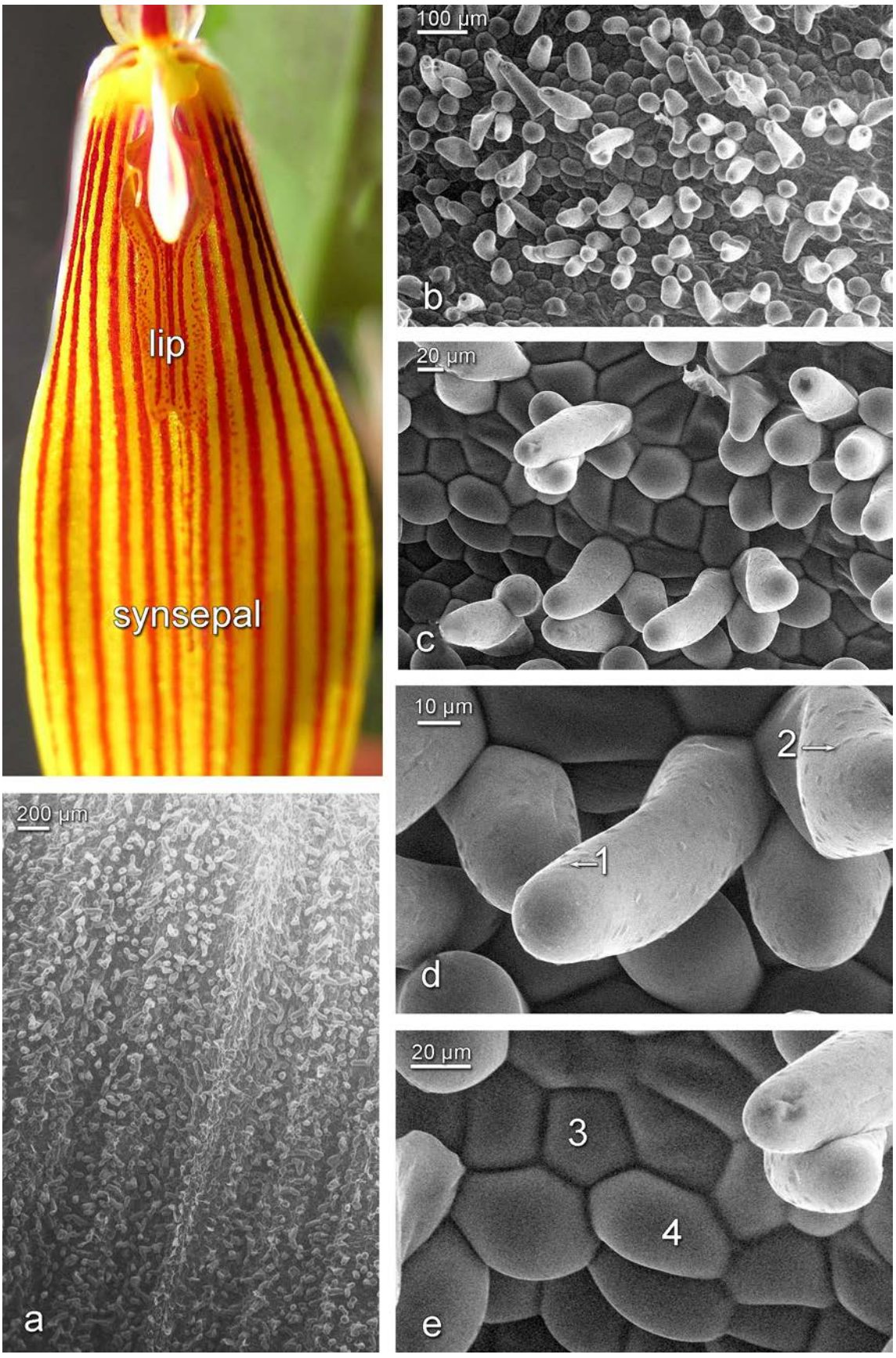


Plate 2-6: Synsepal papillae, one day post-anthesis (Cool Stage) Photograph- the striped synsepal of *R. brachypus*; (a), (b) and (c) linear arrangement of papillae corresponding to the synsepal stripes; (d) 1 and 2, raised 'bumps' on the cuticles; (e) 3 and 4 area between the papillae, cells have no 'pale patches' or raised areas in the cuticular layer.

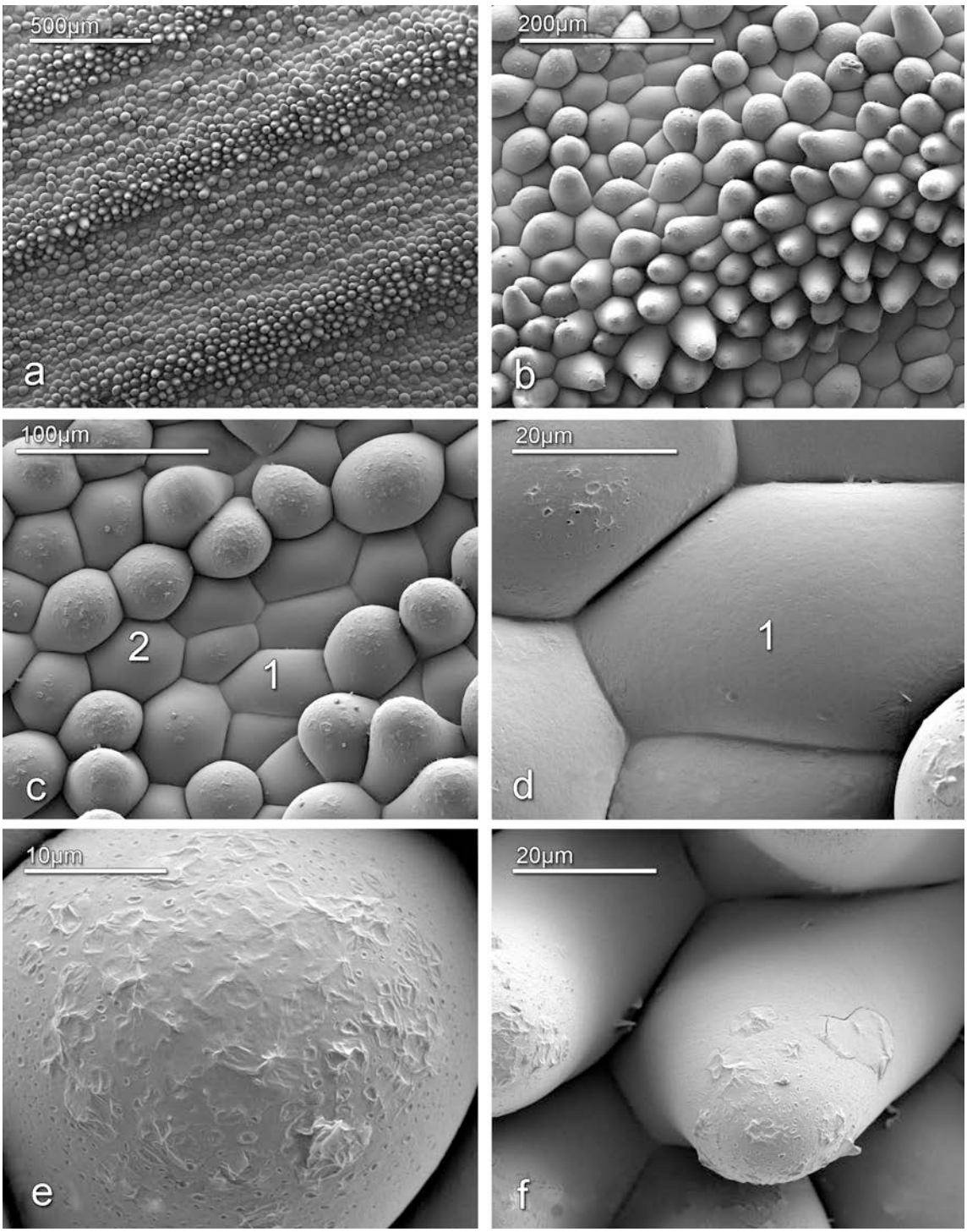


Plate 2-7: Synsepal papillae of *R. brachypus* at anthesis (Cryo stage)
(a) and (b) Linear arrangement of the papillae, corresponding to the synsepal stripes. These papillae are younger and have not elongated (*cf.* Plate 2-6);
(c) and (d) 1 and 2, cells in between the papillae, no vesicles can be seen on them;
(e) and (f) rough surface of individual papillae.

2.3.3 The labellum – general structure

2.3.3.1 Cool Stage

The labellum in all *Restrepia* species is divided into a hypochile and epichile, divided by a narrow isthmus (Luer, 1996a). Using *R. brachypus* as an exemplar the arrangement of the different labellar regions illustrating this general structure is presented in Plate 2-8. Contrasting views (a) photographic, (b) diagrammatic and (c) as a micrograph, are shown. The position of the column overhanging the hypochile area can be observed in the photograph (a) (and in Plate 2-25). The column was omitted in the diagram (b) and removed for the micrograph (c). The narrowing of the isthmus may be seen in (a) 2 and (c) 2 while the papillate nature of the epichile compared to glabrous nature of the hypochile is shown in (c) 1 and 2. The raised papillae of the epichile are depicted in (d) and its denticulate edges in (e).

The labellum is not flat, but rather is laterally curved (see Plate 2-25). This curvature is illustrated diagrammatically in Figure 2-1 below. The incline of the isthmus and hypochile depend on the angle at which the plant supports the flower. Some species hold their flowers more erect than others, which may depend, in part, on growing/environmental conditions.

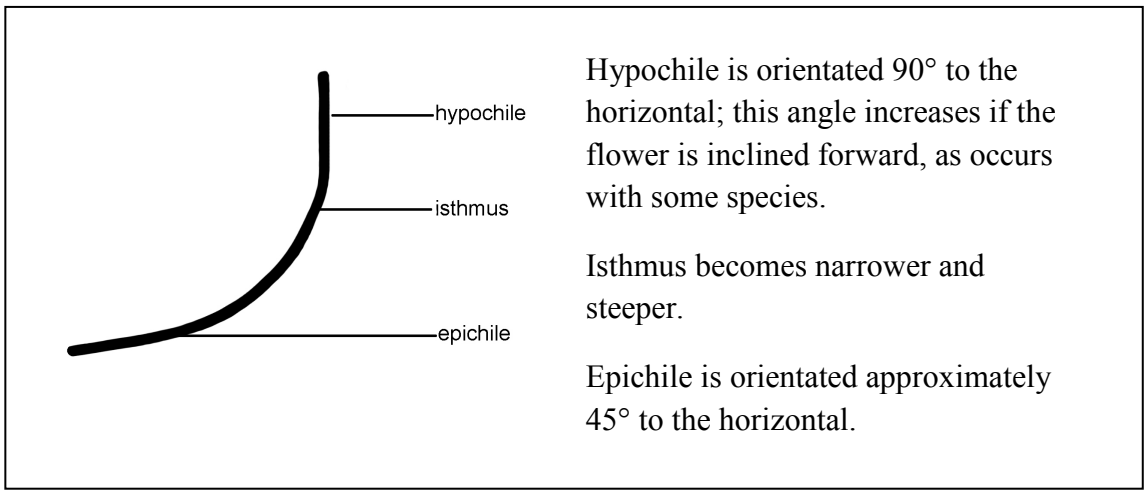


Figure 2-1: Longitudinal, lateral diagram of curved labellum

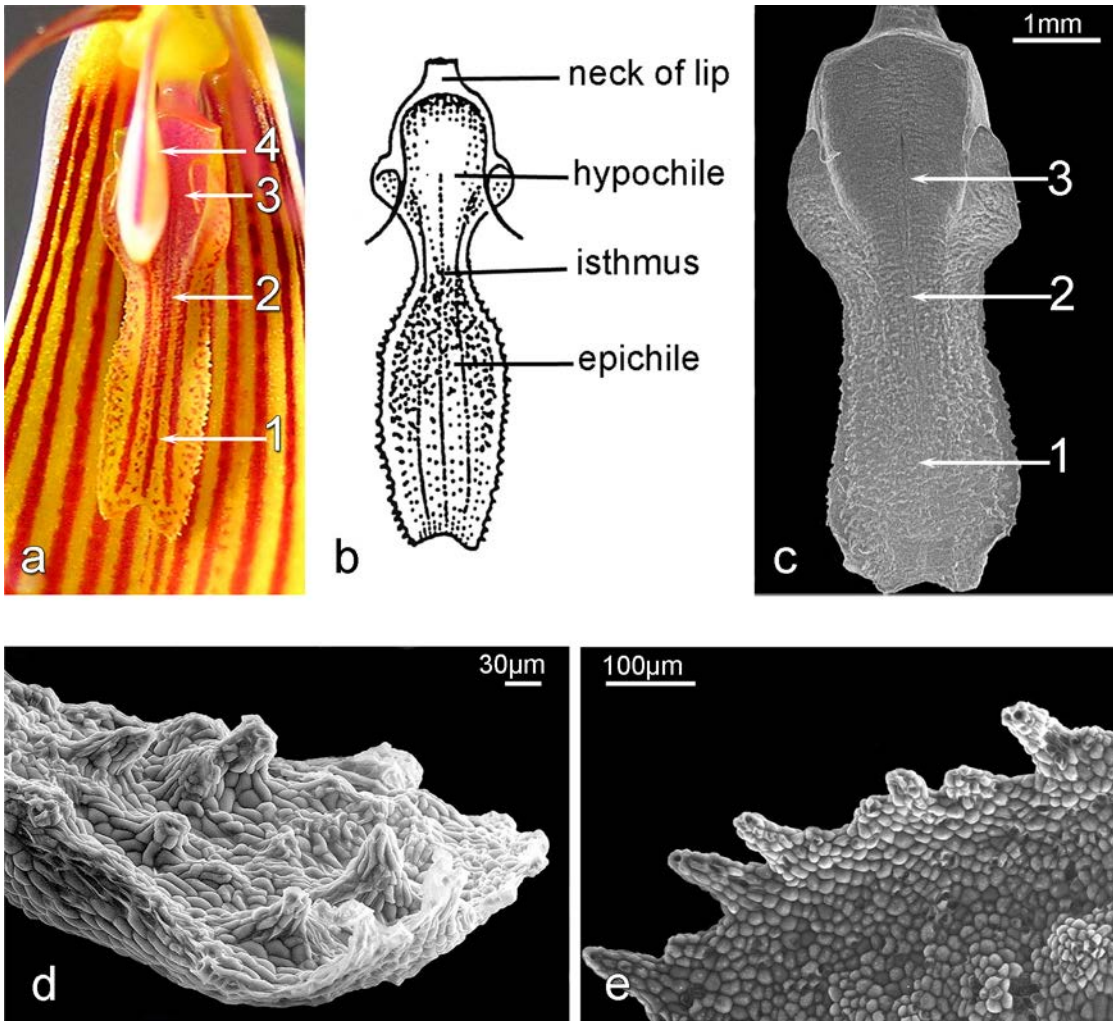


Plate 2-8: General structure of the labellum of *R. brachypus* (Cool Stage)
The different labellar regions: 1= epichile, 2= isthmus, 3= hypochile

(a), (b) and (c) present different views of the labellum;
(a) photograph, column (4) *in-situ*;
(b) diagram (Luer, 1996a), column not shown;
(c) VPSEM, column removed;

(d) and (e) lower edge of the labellum (epichile);
(d) labellar papillae, making the epichile uneven (*cf.* Plates 2-9 (d) and 2-10 (a));
(e) the irregular, denticulate labellar margin *cf.* Plate 2-11(c)

2.3.4 The epichile

2.3.4.1 Cool Stage

Cellular detail of the epichile region of the labellum of *R. brachypus*, two days post-anthesis, is presented in Plate 2-9. The linear arrangement of the papillae is shown (a) and (b) and their structural detail (c) and (d). The central 'labellar groove' may be observed towards the upper part of the epichile region in (a). The cuticular layer has many 'pale patches' (d) 2 and 3, which appear similar to those observed on the cuticular surface of the osmophores. In (c) 1 and (d) 1, some papillary cells have ruptured. The inter-papillary area has fewer 'pale patches' (e) 4 and 5, than observed in the inter-papillary region of the synsepal.

2.3.4.2 Cryo Stage

Higher magnification images of the epichile papillae of *R. brachypus*, one day post-anthesis, are presented in Plate 2-10 (a) and (b). Raised vesicles on the cuticular surface (a) 1 and 2, and (b) 3 may be seen which correspond to the 'pale patches' previously observed in Plate 2-9 (c) and (d). An exudate is present on the cuticular surface between the cells (b) 4, appearing to have come from one of the vesicles. No rupturing of the papillary cells was visible in these younger papillae.

The cells of the denticulate margin, presented in Plate 2-11, were observed to exhibit similar features. Vesicles were observed on cells adjacent to the denticulate edge (a) 1 and (b) 2; and the denticulate edge (c) 3. These vesicles correspond to the position of the 'pale patches' seen in Plate 2-9 (c) and (d). Exudate was observed on cells adjacent to the denticulate edge (a) 4 and (b) 5, and ruptured vesicles on the edge papillae (c) 6 and 7.

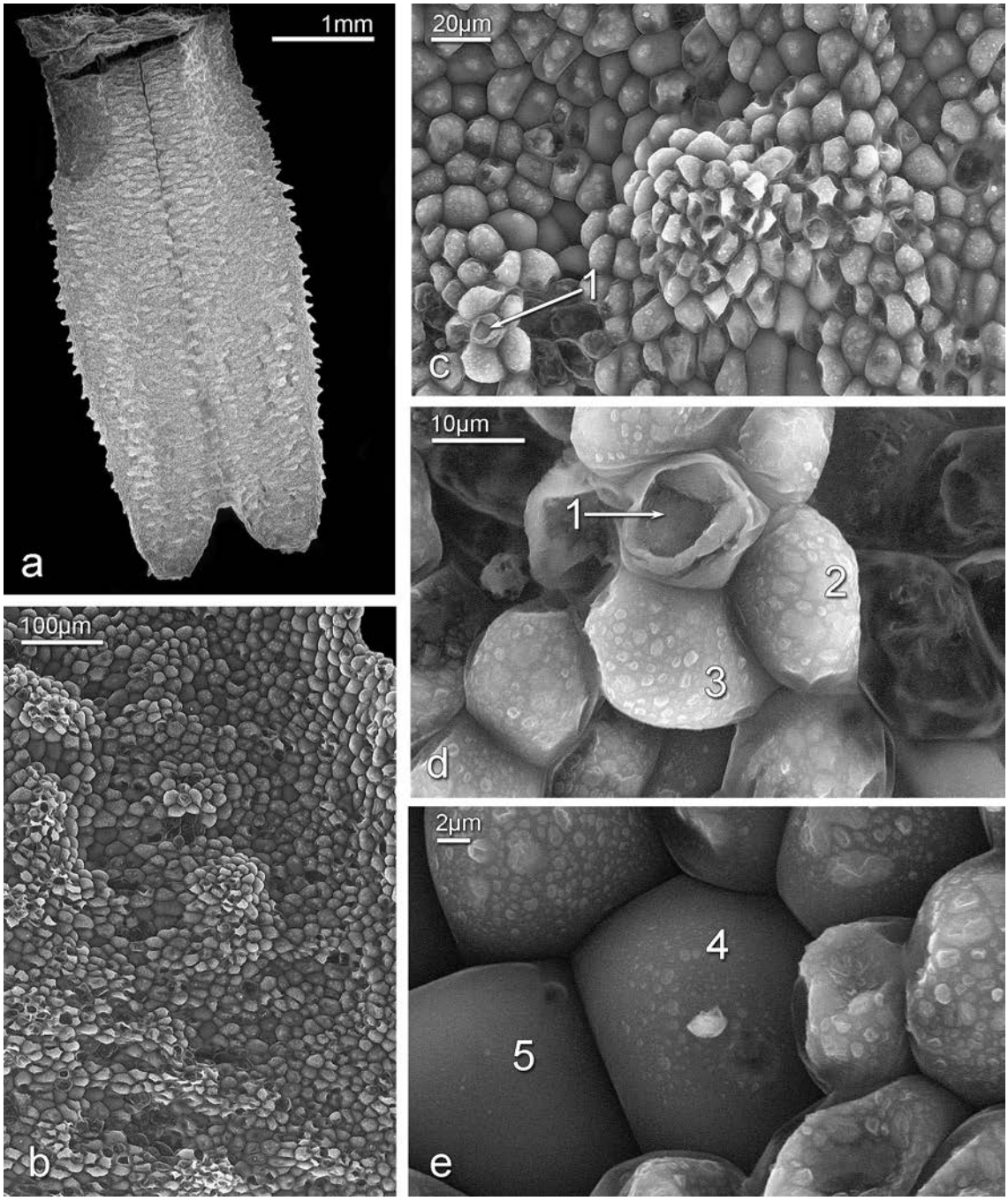


Plate 2-9: The epichile region of the labellum - *R. brachypus* (Cool Stage)

(a) The entire epichile, showing raised papillae;
(b) linear arrangement of papillae coinciding with the epichile stripes in this species;
(c) and (d) burst, ruptured cells, 1;
(d) papillae with ‘pale patches’ on the surface of the cells, 2 and 3;
(e) cellular region between the papillae, 4 and 5 with few ‘pale patches’ on the cuticular surfaces.

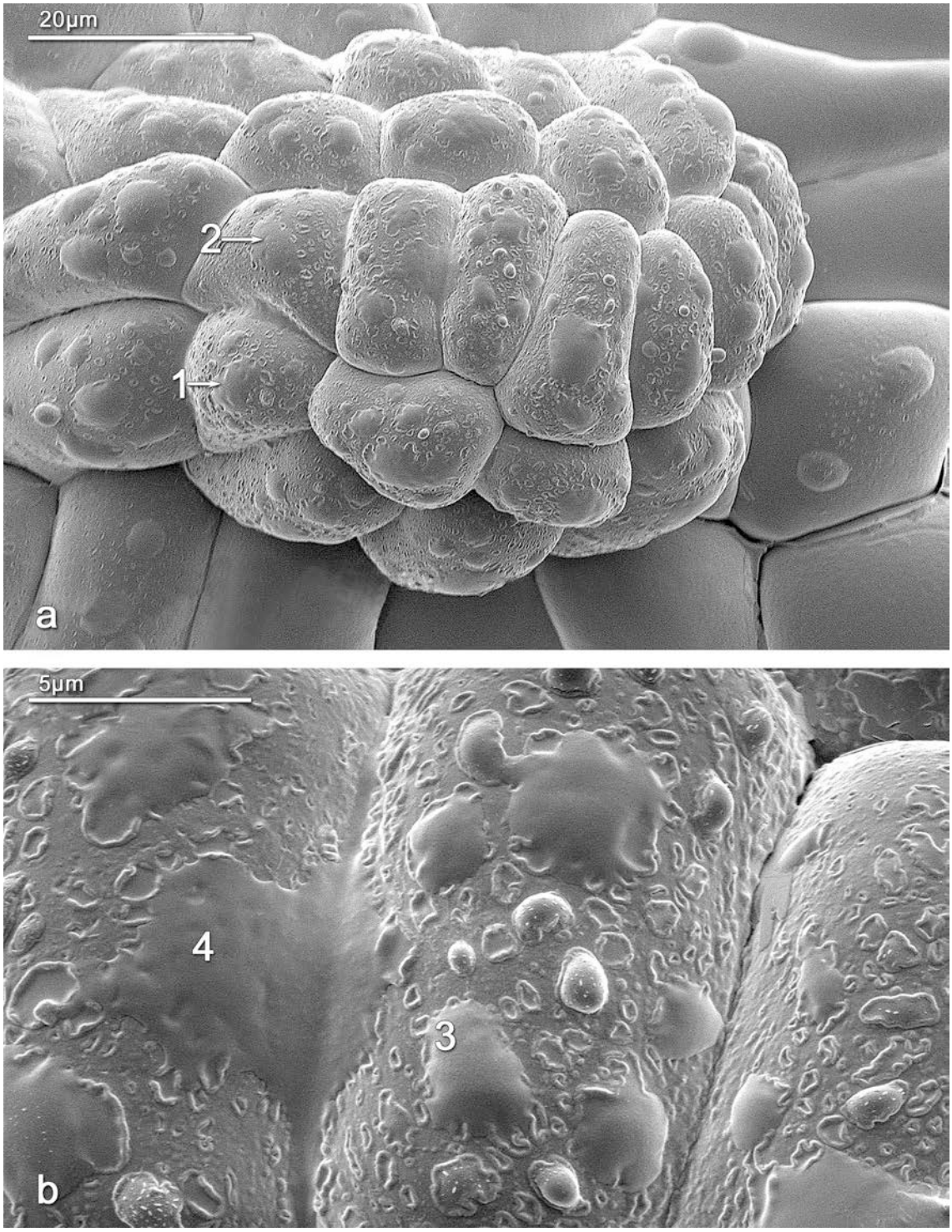


Plate 2-10: Details of the epichile papillae - *R. brachypus* (Cryo Stage)
(a) Detail of papilla, 1 and 2 fluid filled vesicles; (b) 3, vesicle and 4, exudate visible on cuticular surface between cells.

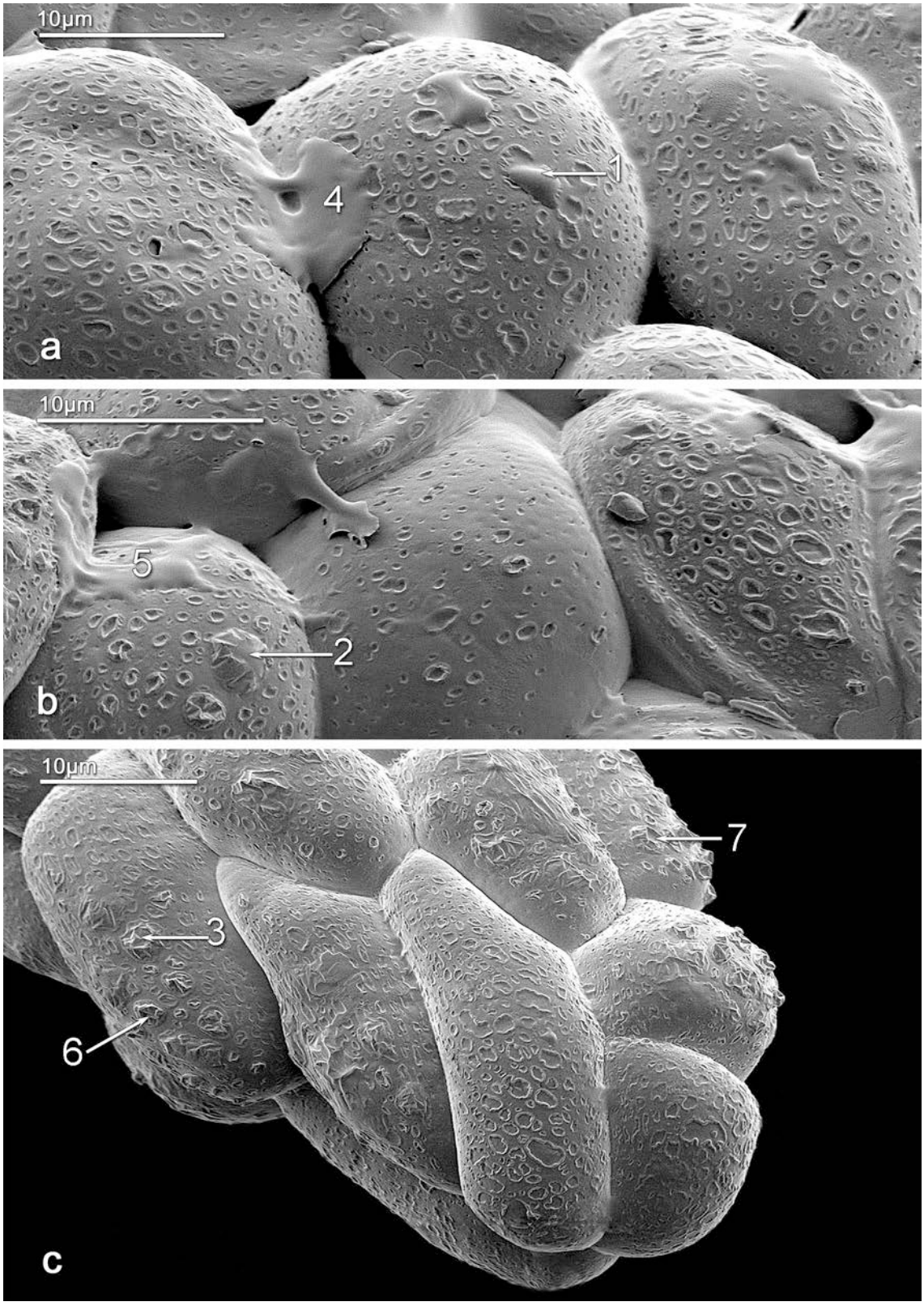


Plate 2-11: Detail of the epichile denticulate margin - *R. brachypus* (Cryo Stage)
(a) 1, (b) 2 and (c) 3 fluid filled vesicles; (a) 4 and (b) 5 exudate running down between the cells; (c) 6 and 7 ruptured vesicles.

2.3.5 The isthmus

2.3.5.1 Cool Stage

The labellar region between the epichile and hypochile, (the mesochile), in *Restrepia* species is narrowed to form an isthmus. Structural details of the isthmus region of the labellum of *R. brachypus,* two days post-anthesis, are presented in Plate 2-12A. The position of the isthmus region is indicated by X (a) and (b).

This region of the labellum is distinguished by the labellar groove (b) which runs down the centre of the isthmus, originating from the hypochile region. Changes in the cellular structure and arrangement from the epichile regions may be observed. The labellar surface has become more even (b) and (c) and individual papillae are not evident. There is some apparent shrinking of the cuticular surface of some cells (b) and (c). There are also fewer 'pale patches' on the cuticular layer of the cells.

The results shown in Plate 2-12B are described in section 2.3.6

2.3.5.2 Cryo Stage

Cellular details of the upper labellar groove of the isthmus of *R. brachypus,* one day post-anthesis are presented in Plate 2-13, and the lower labellar groove of the epichile in Plate 2-14. In the upper region, the cells adjacent to the groove show a 'raised' arrangement (a) 1 and (b), although they do not form obvious papillae. There are very few vesicles visible on the cuticular surfaces and no visible exudate (c).

The lower region of the labellar groove which runs into the epichile is shown in Plate 2-14. The cells in this region, adjacent to the labellar groove, exhibit different structural features from those cells adjacent to the labellar groove in the upper region. The cuticular surfaces of these cells exhibit both vesicles (a) 1 and 2 and (b) 3 and 4 and possible exudate (c) 5. No cuticular rupturing or shrinking may be seen.

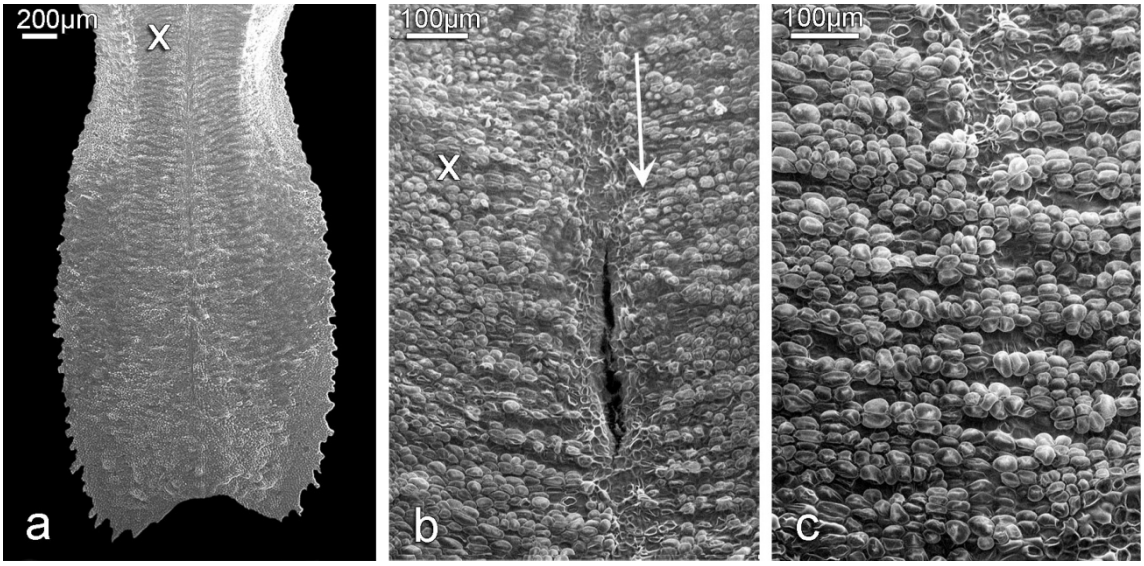


Plate 2-12A: Isthmus region of the labellum - *R. brachypus* (Cool Stage)
(a) Isthmus region, X; (b) and (c) cells raised, no distinct papillae and few 'pale patches' in the cuticular layer. Arrow = direction of labellar groove

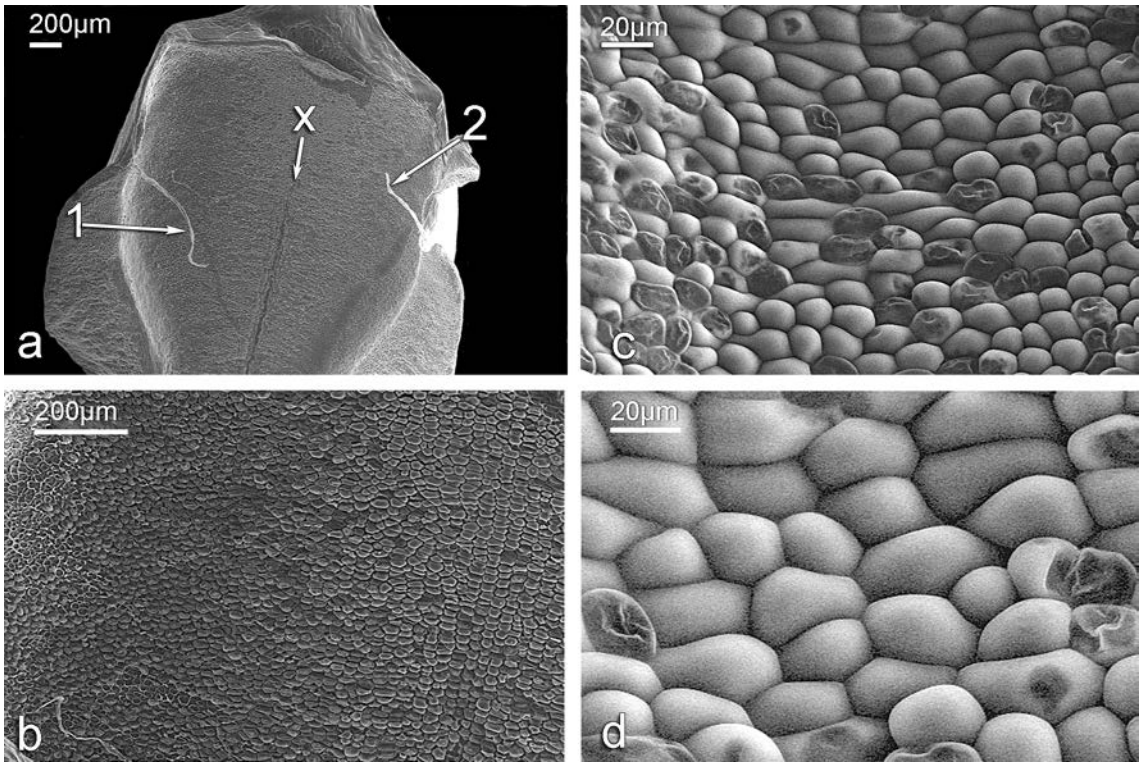


Plate 2-12B: Hypochile region of the labellum - *R. brachypus* (Cool Stage)
(a) Hypochile region, X, origin of the labellar groove Plate 2-12A (b), 1 and 2, remains of the cirrhi; (b) and (c) show the concave shape of the hypochile, papillae and 'pale patches' are absent on the cuticular layer of the cells.

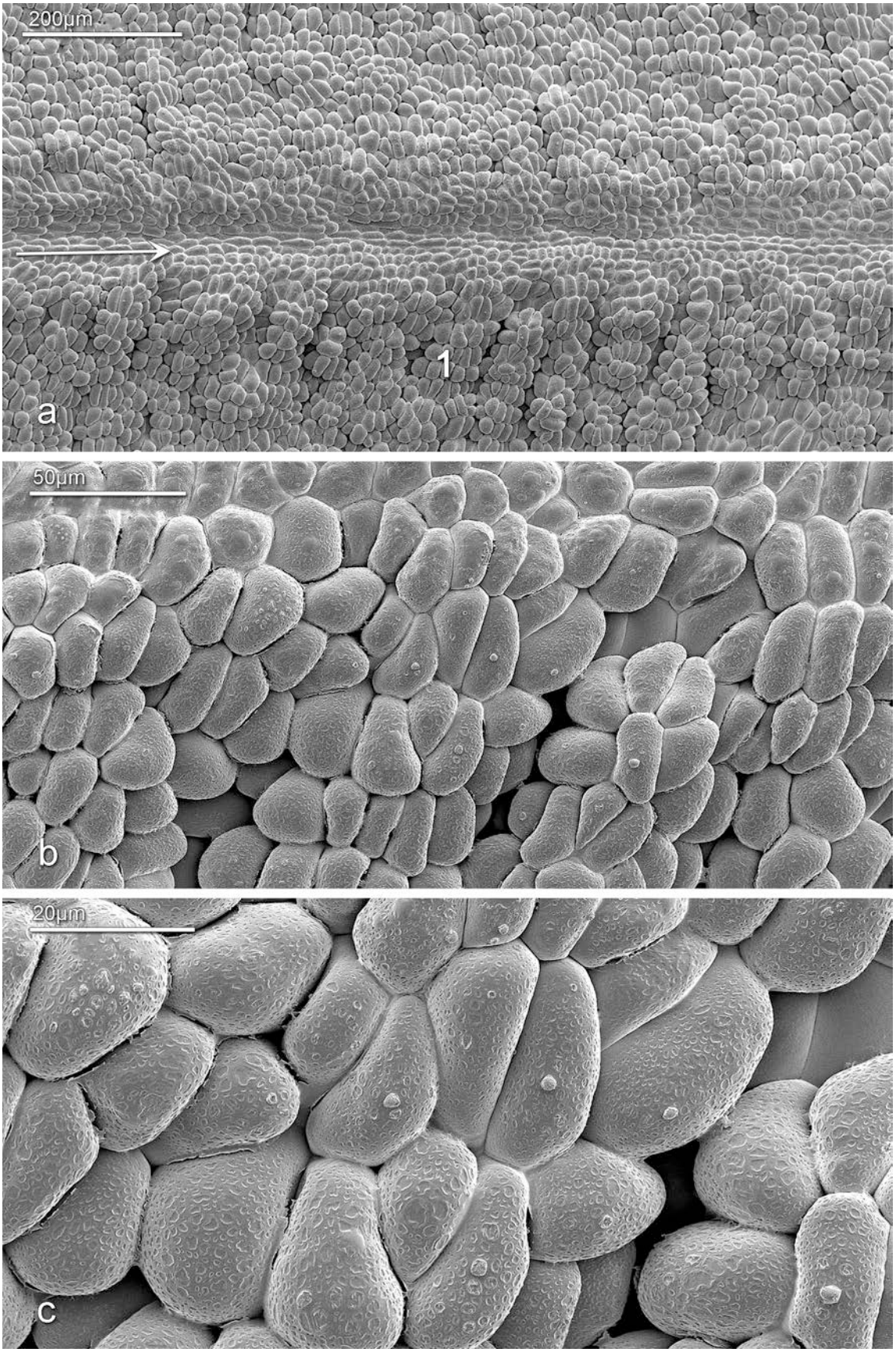


Plate 2-13: Detail of the isthmus and upper labellar groove - *R. brachypus* (Cryo Stage)

(a) Arrow = direction of labellar groove; (b) detail of (a)1, cells beginning to form papillae; (c) irregular cuticular surfaces, but no vesicles in this region.

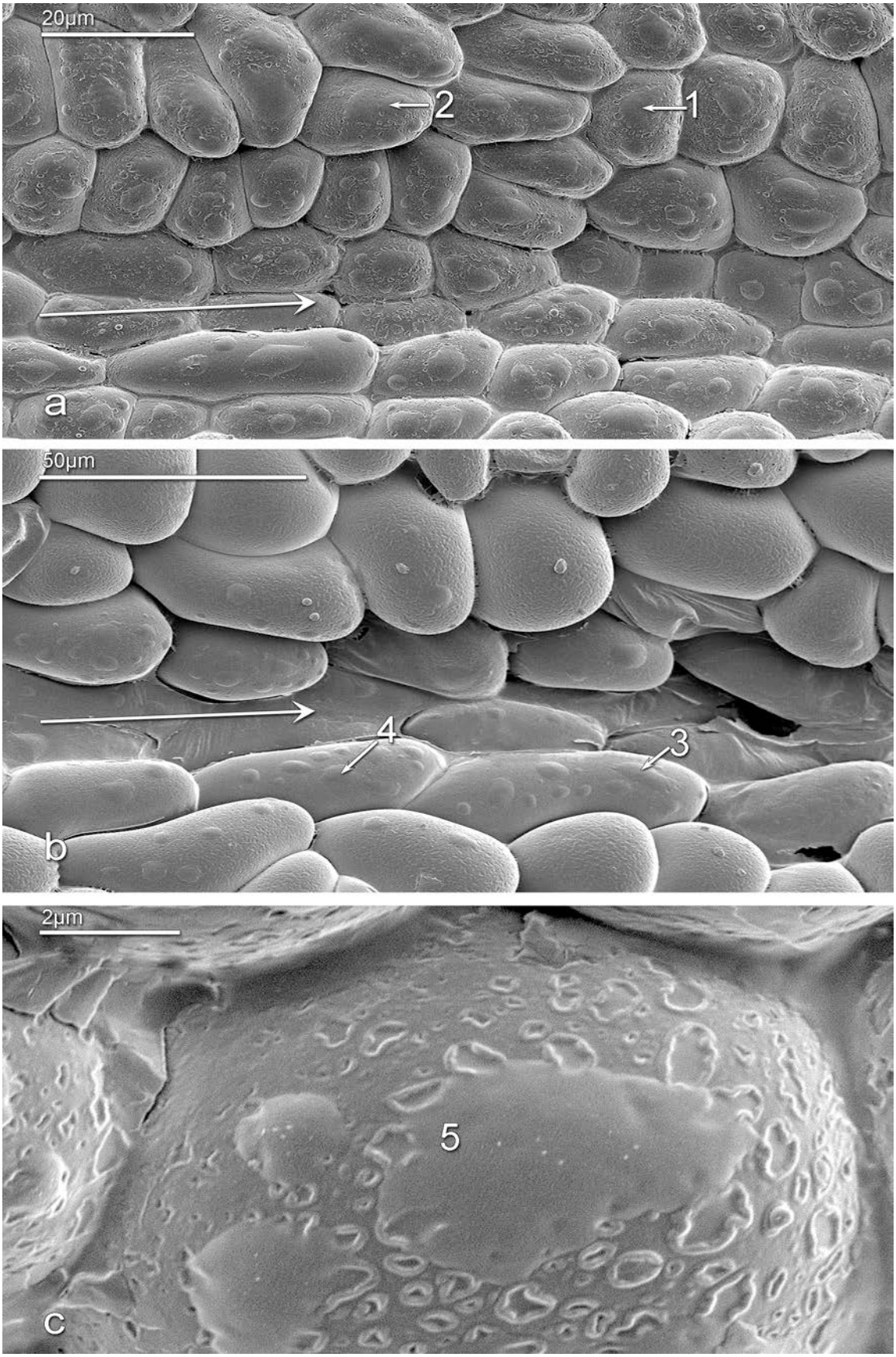


Plate 2-14: Details of the lower labellar groove - *R. brachypus* (Cryo Stage)
Arrow = direction of labellar groove; (a) 1 and 2, and (b) 3 and 4, vesicles on cells in this region; (c) detail of cell and vesicle, 5.

2.3.6 The hypochile

2.3.6.1 Cool Stage

Cellular detail of the hypochile is presented in Plate 2-12B. On either side of this area are the cirrhi (a) 1 and 2. These have shrivelled in this specimen of *R. brachypus*, but are still recognisable. The hypochile region is glabrous, non-papillate and the cells are rounded, having no 'pale patches' on their cuticular layer (b), (c) and (d). The labellar groove, X, originates in this region (a) see also Plate 2-15 (a) and (b).

The concave nature of this region may be observed in (c) and (d). It should be noted that the three regions of the labellum are angled differently (Figure 2-1) when the flower is attached to the plant.

2.3.6.2 Cryo Stage

Higher magnification images of the cells of the labellar hypochile are presented in Plate 2-15 (a), (b) and (c). These data confirm the non-papillate nature of this region. The origin of the labellar groove may be observed at X in (a) and (b). At which point there appears to be some differentiation of the cells and the formation of the grooved area is marked in (b). The cellular detail (c) illustrates the rounded shapes of the individual cells and further confirms a glabrous, non-papillate cellular structure for this area. Although the absence of vesicles in the cuticular layers of the cells is observed, there is some sculpting of the cuticular layer (c).

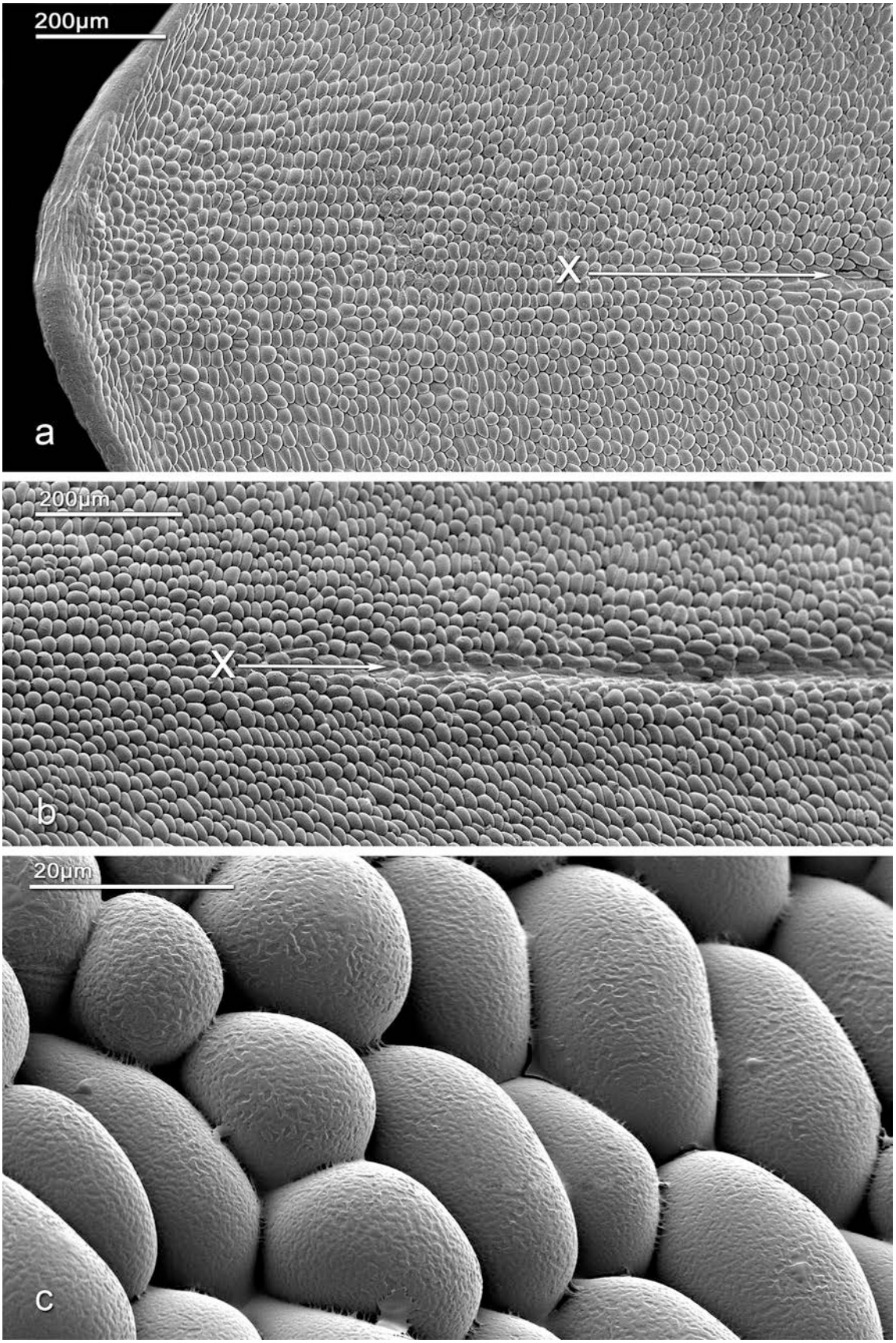


Plate 2-15: Detail of the hypochile - *R. brachypus* (Cryo Stage)
X = origin of the labellar groove,
(a) and (b) Non-papillate structure of hypochile; (c) detail of cells, smooth cuticles with no vesicles.

2.3.7 The column

All observations of the column were made on the Cool Stage, using *R. brachypus*, and are presented in Plate 2-16. The ventral view of the column illustrating the relative positions of the anther cap and stigmatic surface is shown in (a). Details of the anther cap, the relative positions of the viscidium (1) and the rostellum (2) are presented in (b), details of the stigmatic surface in (c) and (d) and details of the rostellum or rostellar flap in (e).

From the micrographs, changes in the nature of the stigmatic surface as it aged were observed. At anthesis (e) the stigmatic surface is prominent and appears swollen. One day post-anthesis (c) and (d) slight shrinkage of the area may be observed, although its viscid nature is more apparent (d). After four days (a) the area has shrunk and is no longer swollen or viscous in appearance. The position of the rostellum (2), in separating pollinia from the stigmatic surface and preventing self-pollinations is shown in (a), (b), (c) and (e). The smooth, flat, non-papillate cells of the outer column are shown in (f).

2.3.7.1 Anther caps

Anther caps from several species of *Restrepia* are presented in Plate 2-17A, in which some minor inter-specific differences were observed. Too few samples from different species were examined to determine whether these differences are species specific.

2.3.7.2 The pollinarium

A ventral view of the entire pollinarium is presented in Plate 2-17B. The position of the viscidium is shown at X, (a) and (b). The four, free, equal-sized, ovoid, laterally flattened pollinia in two pairs may be observed *in situ* (a) and *ex situ* (b). The empty anther cap is shown in (c).

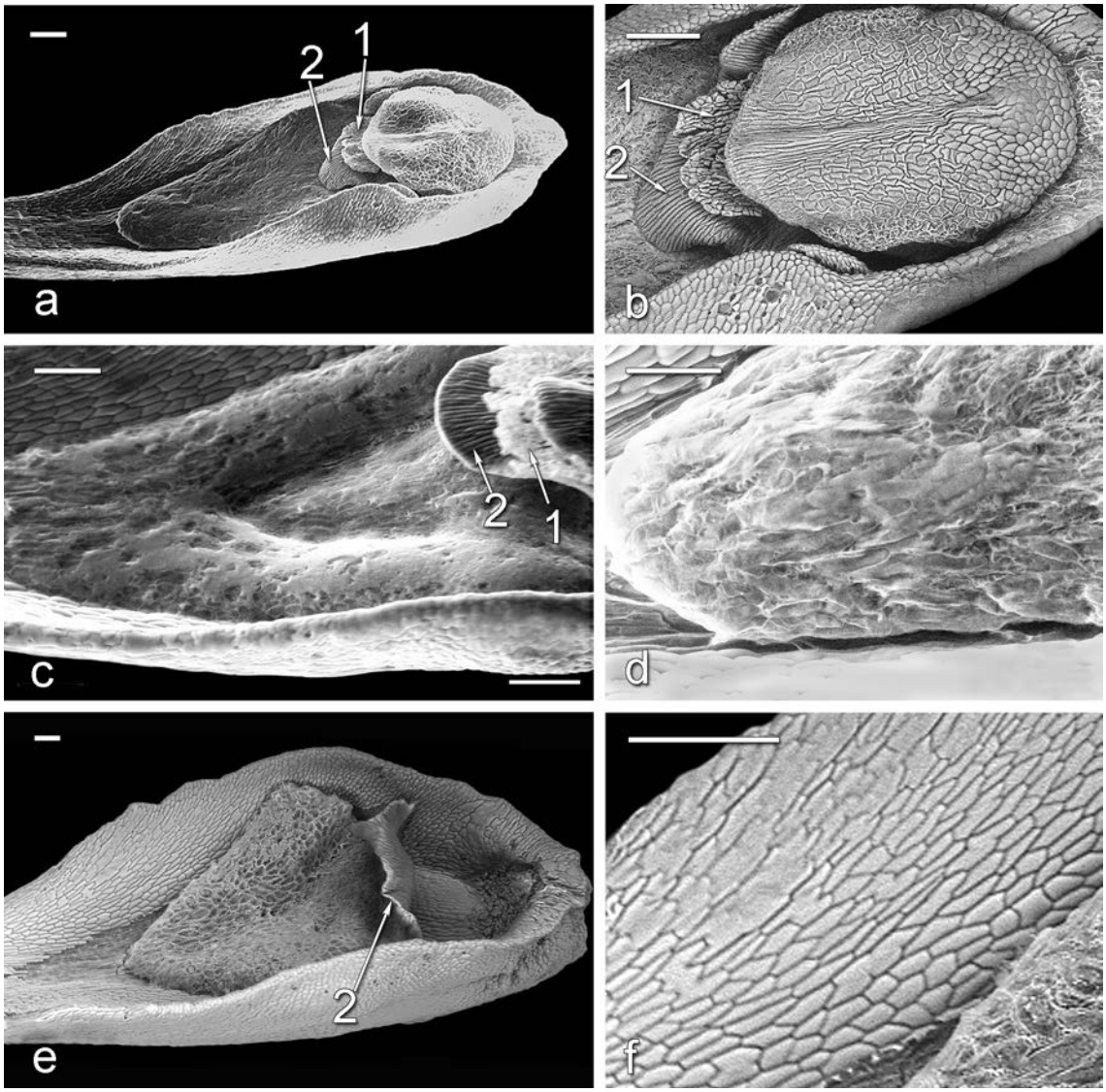


Plate 2-16: Ventral surface of the column - *R. brachypus* (Cool Stage)

(a) and (b) Anther caps, flower four days post-anthesis;
(c) and (d) stigmatic surface, flower one day post-anthesis;
(e) ventral surface of the column with the anther cap and pollinia removed, rostellum is visible as a protruding flap of tissue, partly covering the stigmatic surface;
(f) the smooth, non-papillate cells of the outer column, with no 'pale patches' on their cuticular surface.

1 = viscidium (a), (b) and (c); 2 = rostellum
(a), (b), (c) and (c) Internal scale bars represent 100µm.

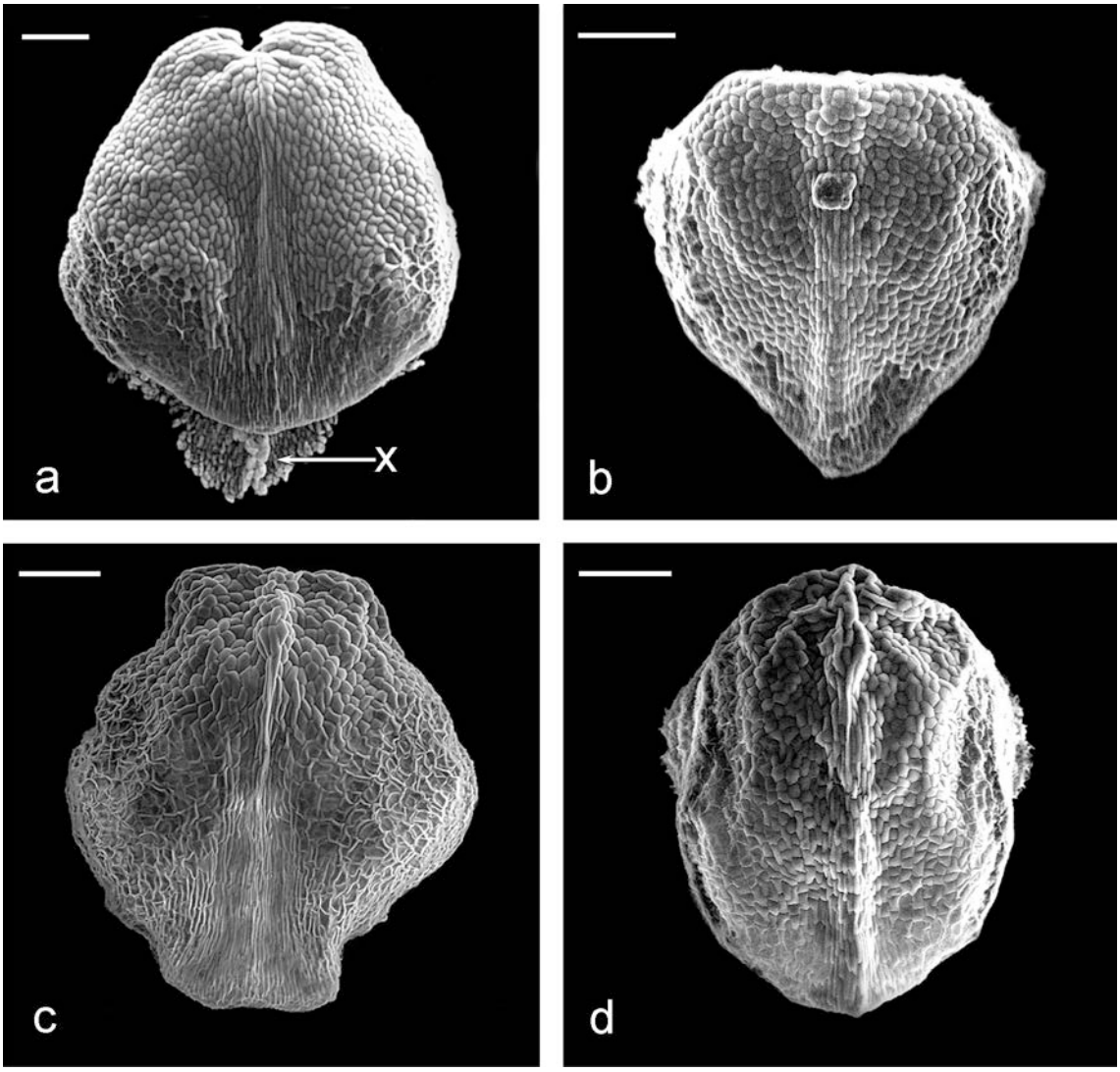


Plate 2-17A: Anther caps of different species (Cool Stage)
(a) *R. sanguinea,* X = viscidium; (b) *R. brachypus*, clone a; (c) *R. brachypus*, clone b; (d) *R. dodsonii*; internal scale bars represent 100μm

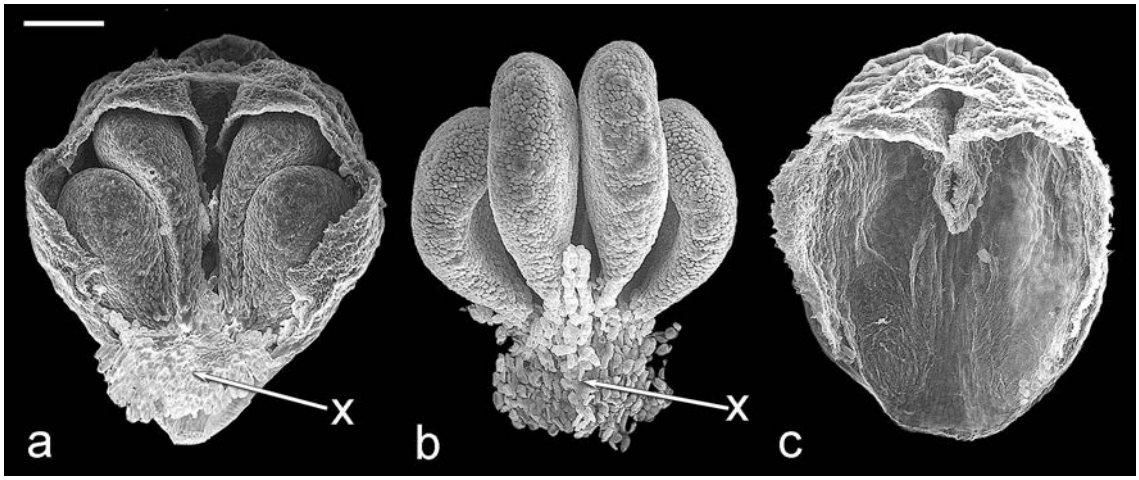


Plate 2-17B: The Pollinarium - *R. brachypus* (Cool Stage)
(a) Pollinarium, anther cap with pollinia *in-situ;* (b) pollinia removed from the anther cap; (c) empty anther cap; X = viscidium. Internal scale bar represents 100μm

2.3.8 The calli

2.3.8.1 Cool Stage

At the base of the labellum are two structures, the calli, Plate 2-2 (8) rounded in shape and bright yellow in colour, Plates 2-23 and 2-24. A flower of *R. brachypus* was examined on the Cool Stage and the papillate nature of the calli observed is presented in Plate 2-18. The presence of calli papillae was later confirmed in further specimens of *R. brachypus* together with *R. dodsonii*, *R. muscifera* and *R. sanguinea*.

The papillae were observed to cover the entire calli (a) radiating out from the apices (b) and (c). The papillae were conical in shape (b), (c), (d) and (e) with bright apices, X, (e) and (f). The bright apices in these images were first attributed to artefacts due to charging when using the Cool Stage. In none of the images was any evidence of the presence of exudate observed, either between, or on the cuticular surface of the papillae.

2.3.8.2 Cryo Stage

Further details of the cuticular structure of the papillae from images obtained using the Cryo Stage are presented in Plates 2-19. The bright apices of the papillae were found to consist of various cuticular folds and striations (a), (b) and (c), which radiated from the apices. Some electron charging, making the papillae appear bright was observed in (a), but this effect is less obvious in the more highly magnified images (b) and (c). The striations may be observed laterally on the papillae, and seem to continue across from one cell to another (b). Further details of the cuticular folds are shown (c). These structures were found to be unique to cells forming the calli and were not observed on any adjacent cells.

Details of the calli papillae apices are presented in Plate 2-20. Striations are observed extending laterally from one cell to another (a), while the apical striations of the papillae are shown to have a convoluted ‘tangled’ arrangement. This is illustrated further in (b).

The cuticular folds appear to be 'almost tangled' on top on the papilla. These images further confirm the absence of any exudate between the papillae which suggests the absence of nectar. All the images shown in Plates 2-19 and 2-20 are from calli found in *R. brachypus*.

2.3.8.3 Photographs of calli under different lighting conditions

The appearance of the calli of *R. brachypus* under different lighting conditions is presented in Plate 2-21. The photographic apparatus used for these observations is illustrated (a). The appearance of the calli under daylight may be observed (b), in which there is no reflection visible from either the calli, or the area underneath and around them. In contrast, when viewed under torchlight in a darkened room (c) the areas under the calli and the calli themselves appear to be reflective. Viewed under UV light, wavelength = 450nm (d), the calli fluoresced and appeared as two bright blue dots. There was also some fluorescence observed from the area underneath the calli.

2.3.9 The cirrhi

Attached to each side of the hypochile region of the labellum are two processes, the cirrhi. The position of these is shown in Plates 2-1 and 2-2 (6). The position of the cirrhi in different species may be compared in Plates 2-23, 24 and 25.

The surface morphology of the cirrhi as observed on the Cool Stage is presented in Plate 2-22. The lateral view (a) shows the hooked shape of the cirrhus, and (b) detail of the narrower tip. The smooth cuticular surface of the elongated cells may be observed in the more highly magnified images (b) and (c). No vesicles or exudate were observed in any images

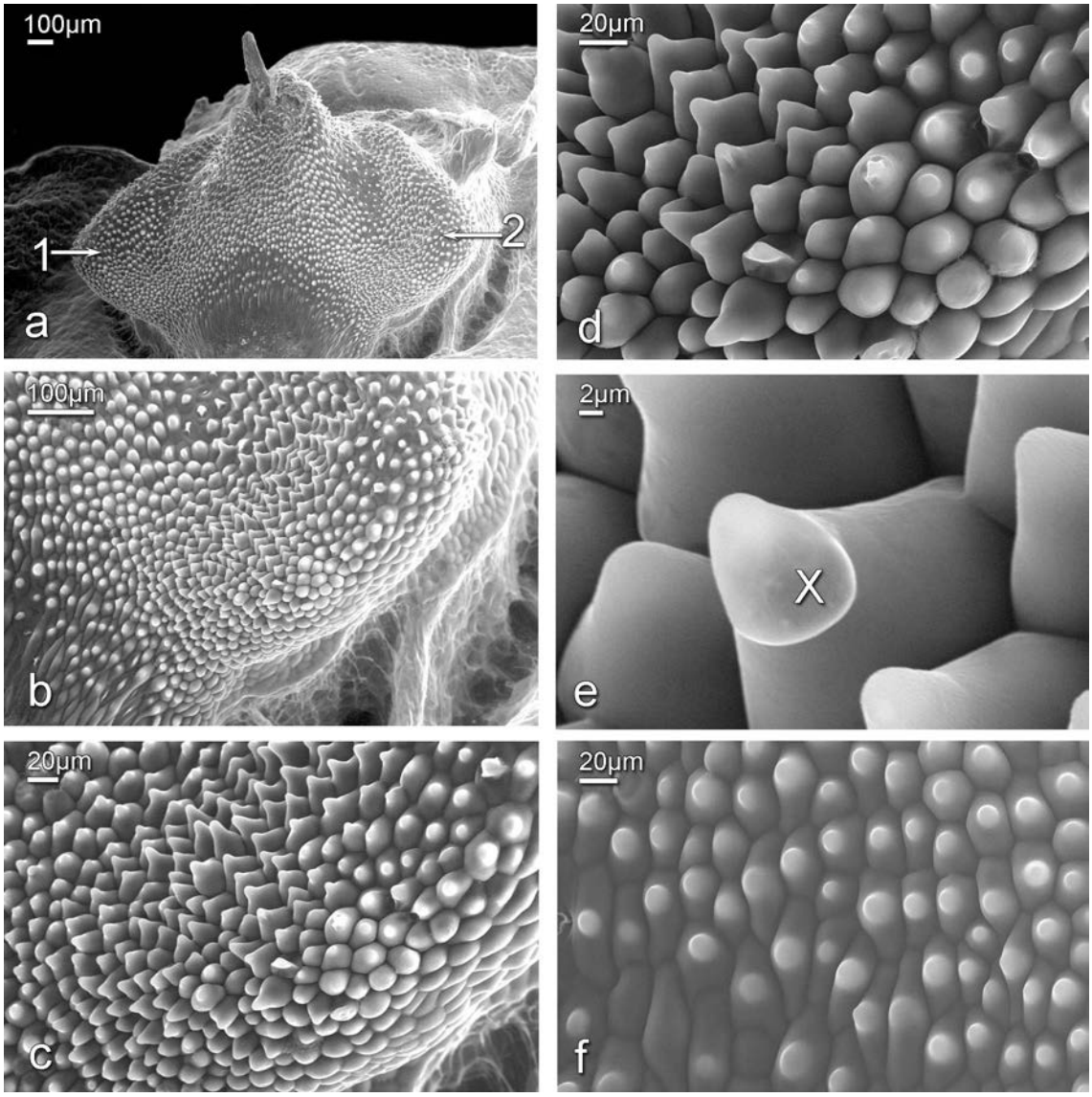


Plate 2-18: The structure of the calli - *R. brachypus* (Cool Stage)

(a) Base of the column, calli at 1 and 2;
(b), (c), (d) and (f) papillate structure of the calli. Papillae apices appear 'bright' in all images;
(e) detail of papilla apex, X, with distinct 'bright' appearance.

It was not possible to resolve further details using the Cool Stage, nor whether or not the bright apices were artefacts due to charging from the scanning electron microscope.

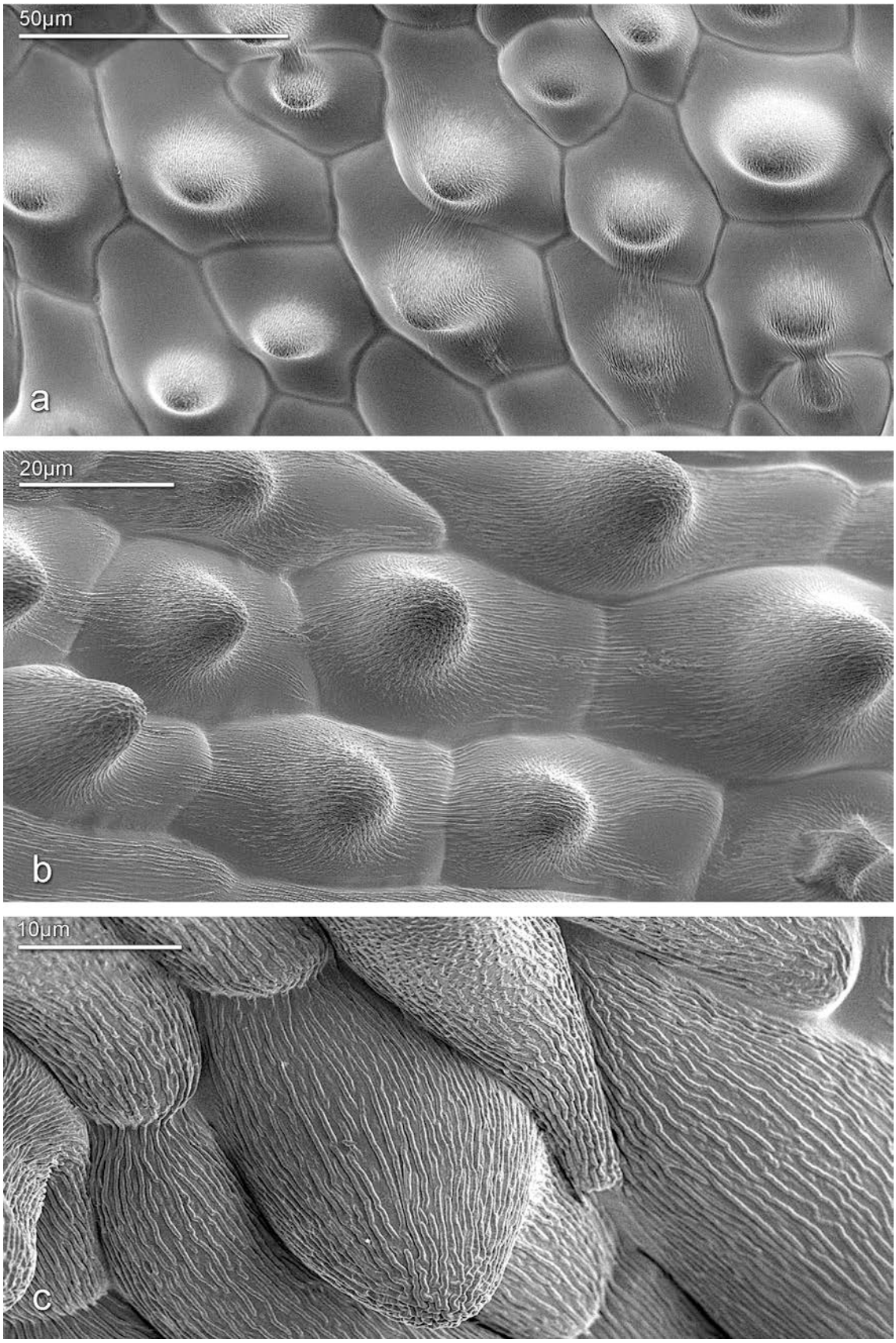


Plate 2-19: Detail of individual papillae of the calli - *R. brachypus* (Cryo Stage)
(a) Apices of the papillae appear 'bright' under VPSEM, due to some charging;
(b) and (c) cuticular details of the papillae.

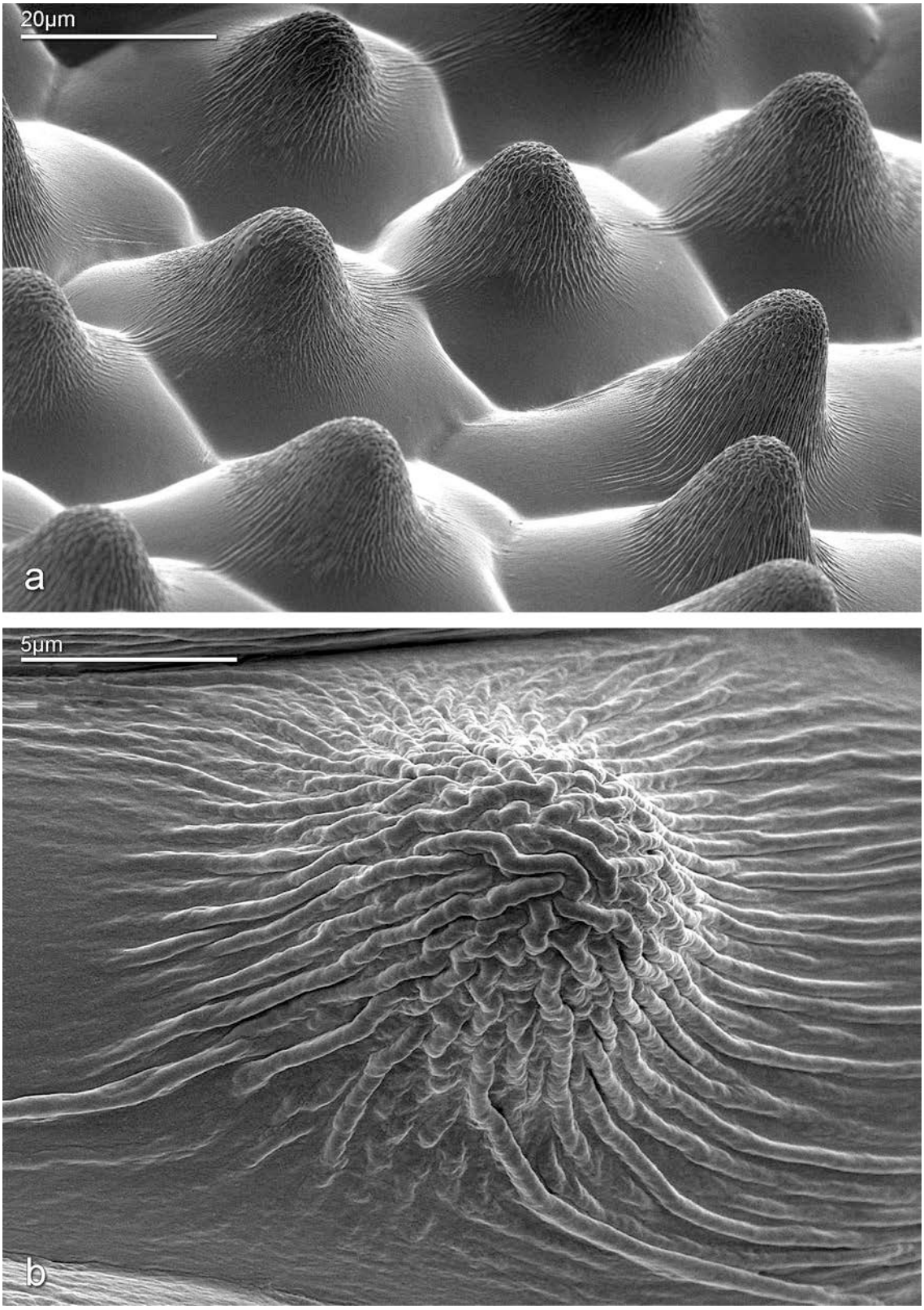


Plate 2-20: Apices of the calli papillae - *R. brachypus* (Cryo Stage)
(a) The distinctive structure of the tips of the calli papillae;
(b) details of an individual papilla. These 'structures' are found on all the papillae making up the calli and are unique to this area.

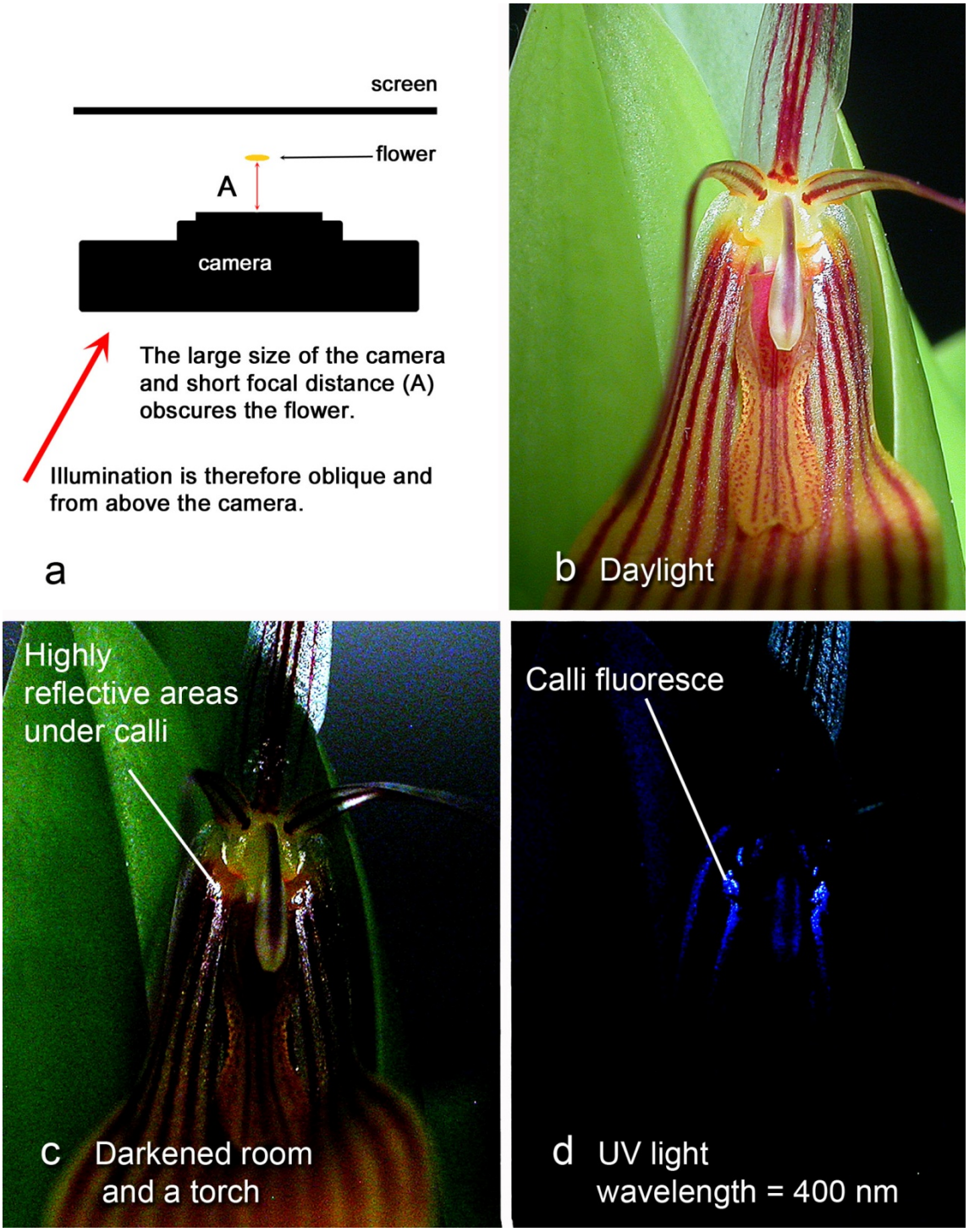


Plate 2-21: The appearance of the calli (*R. brachypus*) under different lighting conditions.

(a) Set–up used; (b) daylight, no reflection from calli; (c) under torch light in a darkened room, the cellular areas under the calli and the calli themselves are reflective; (d) UV light, wavelength = 450 nm, the calli fluoresced and appeared as two bright blue dots.

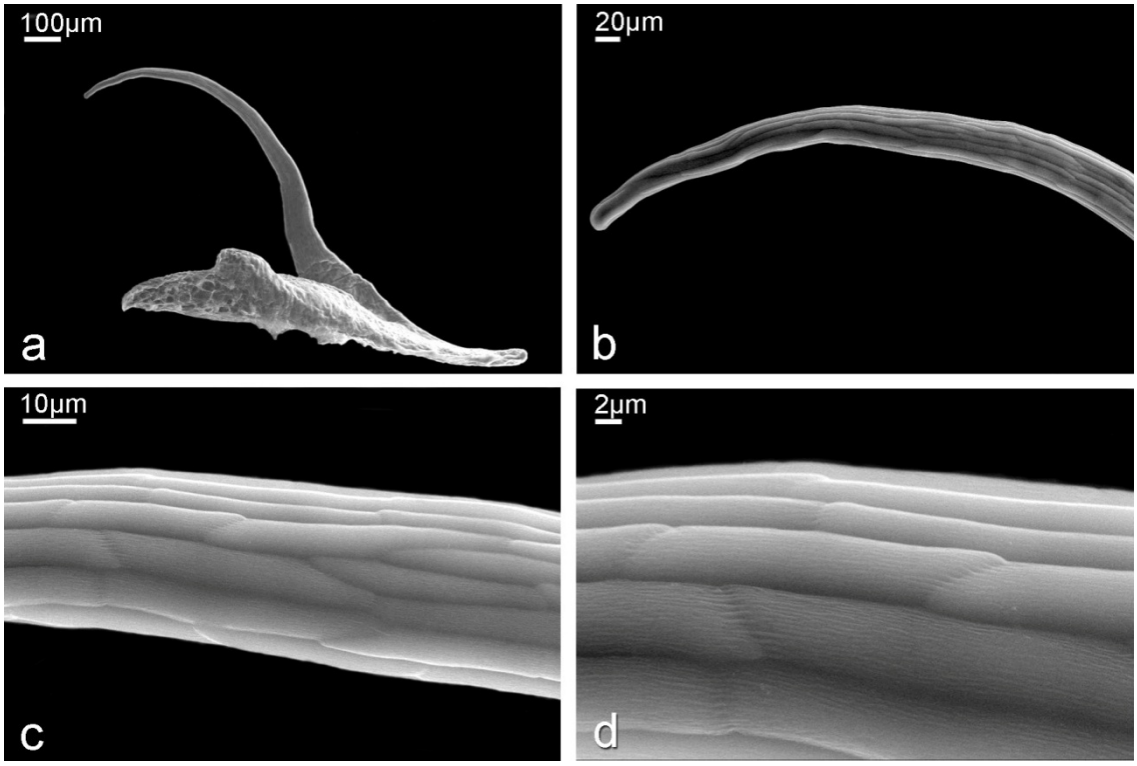


Plate 2-22: The structure of the cirrhi - *R. brachypus* (Cool Stage)
(a) Complete cirrhus; (b) detail of tip; (c) and (d) smooth cellular surface with no papillae or vesicles.

2.3.10 Photographic study

Frontal views of the flower, showing the relative positions of the cirrhi, the column and the labellum across a range of species, are presented in Plates 2-23 and 2-24. Lateral views from several species showing the relative position of the cirrhi to the column and anther cap are presented in Plate 2-25. The arrangement of these structures was found to be very similar in all species; i.e. the column is held above the hypochile region of the labellum, while the anther cap is 'flanked' by the two cirrhi, which extend upwards from the wings of the hypochile (Plate 2-25). The calli may be observed on either side at the base of the column (Plates 2-23 and 24) but are more prominent in some species than others (*cf. R. contorta, R. mendozae* and *R. antennifera* (*hemslyana*); Plates 2-23 and 24). The column, labellum and width across the cirrhi were measured with the measurement tool in Adobe Photoshop CS6, and the results are presented in Figure 2-2 and Table 2-1.

Ratios of the labellar and columnar lengths were calculated for all the species and these had a range from 1.9 to 2.1 (mean =2.0; se = 0.06; n =18). In comparison, ratios of the column length and width between the cirrhi ranged between 1.8 and 2.8 (mean = 2.3; se= 0.07; n =18).

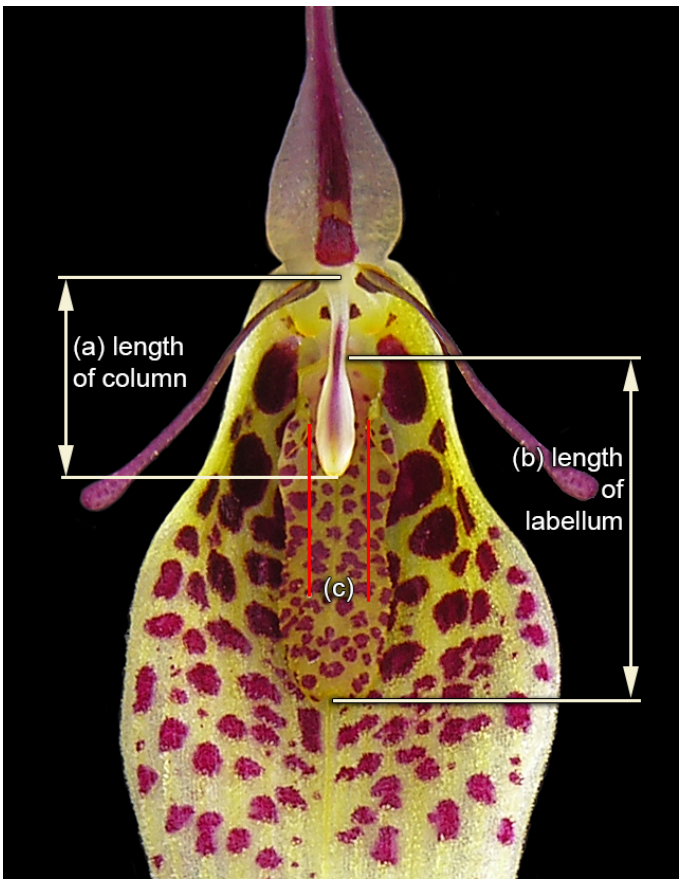


Figure 2-2: Relationship between column and labellum length and width between the cirrhi.

The length of the labellum (b) is approximately twice the length of the column (a). See notes below Table 2-1 for further details.

Table 2-1: Comparative mean length of the column, labellum and width between the cirrhi

Species	Column[1]	cv[2]	Labellum[1]	cv[2]	Lab/Col[3]	Cirrhi[1]	cv[2]	Col/Ci[4]
R. antennifera (hemslyana)	356.4	1.9	709.3	2.3	2.0	175.2	0.6	2.0
R. antennifera (Roseola)	361.6	2.4	702.0	2.5	1.9	157.7	1.1	2.3
R. brachypus	438.5	2.5	841.4	1.3	1.9	180.1	1.7	2.4
R. citrina	333.7	0.8	650.6	0.7	1.9	117.3	2.0	2.8
R. contorta 1	239.0	1.8	488.5	0.8	2.0	104.1	3.8	2.3
R. contorta 2	241.8	2.8	467.1	3.2	1.9	106.0	1.2	2.3
R. cuprea	309.4	2.3	609.9	0.9	2.0	137.0	4.3	2.3
R. dodsonii	226.6	2.2	476.9	1.2	2.1	97.6	4.8	2.4
R. echinata	271.6	2.9	568.5	2.7	2.1	107.2	0.7	2.5
R. elegans 1	328.0	3.5	672.2	0.8	2.0	150.2	1.6	2.2
R. elegans 2	238.4	2.0	466.0	2.3	2.0	114.7	3.1	2.1
R. guttulata 1	283.8	0.5	548.3	2.5	1.9	103.8	0.5	2.7
R. guttulata 2	263.7	2.5	527.8	2.1	2.0	103.5	2.2	2.5
R. mendozae	367.5	1.4	719.3	1.1	2.0	189.8	1.2	1.9
R. purpurea	254.2	2.2	489.4	3.2	1.9	138.1	1.2	1.8
R. schizosepala	312.5	1.1	642.4	1.3	2.1	167.8	1.5	1.9
R. seketii	260.0	2.6	536.8	1.0	2.1	112.2	1.4	2.3
R. vasquezii	261.7	3.3	505.9	3.8	1.9	99.6	0.5	2.6
Mean					2.0			2.3
n					18			18
se					0.02			0.07

Notes:

The values given are the pixel values from within the Plates 2-23 and 2-24, and do not represent a formal measurement such as mm. They are only used for comparative analysis within each flower and not for size comparisons between flowers.

[1]Mean values from ten repeated measurements of column, labellum and width between the cirrhi.

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

[3]Ratio of labellum length to column length, approximately 2:1, se = 0.06

[4]Ratio of column length to width across the cirrhi, se = 0.07 indicating greater variation between these values.

Plate 2-23: The cirri, column and labellum
Internal scale bars represent 2mm

Plate 2-24: The cirrhi, column and labellum
Internal scale bars represent 2mm

Plate 2-25: The position of the cirrhi
The position of the cirrhi (X) either side of the column is shown for different species. Main picture *R. guttulata;* (a) *R. antennifera;* (b) *R. mendozae;* (c) *R. purpurea.* Internal scale bars represent 2mm

2.4 Discussion

2.4.1 Osmosphores

Osmophores may be defined as floral tissues specialised for fragrance synthesis and secretion (Vogel, 1990; Dressler, 1993). Much of the work on the structure of osmophores was carried out comparatively recently. The structure of osmophores in orchids was studied using light microscopy by Vogel in 1963 (Vogel, 1990) and via scanning electron microscopy by Williams (1983). The term osmophore is derived from *osmophoro* (Greek, *osmo,* meaning odour) and *pherein* (Greek, to bear) and was first used in 1883 by G. Arcaneli.

The study by Williams demonstrated that the external morphology of osmophores varies from papillate to smooth and noted that "the structure of the osmophore regions is quite variable from species to species and from genus to genus" (Williams, 1983).

The most relevant work previously performed on osmophores in *Restrepia*, was published by Pridgeon and Stern (1983). These workers performed both SEM and TEM on these structures and thus formulated a hypothesis regarding the role of osmophore scent production in pollination. Subsequently, Vogel (1990) discovered the functional layering of structured osmophore structures into storage, production and emission layers. Sazima *et al.* (1993) further investigated the emission layer and reported an 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, shrinking and rupturing of the osmophore cuticle (Sazima *et al.*, 1993).

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, the papillae were

termed labellar trichomes, and similar indentations to those found in the cuticle layer in the current study were reported.

In light of the previous studies, the objective of the VPSEM study using the Cool Stage and Cryo Stage was to obtain higher resolution and more 'true to life' images than those obtained by Pridgeon and Stern (1983).

The micrographs obtained confirm the structures described by Pridgeon and Stern (1983), but provide additional details connected with papillae senescence. Plate 2-3A (a, b, c and d) shows the papillae less than 24 hours after anthesis, at which time the papillae are still turgid with the integrity of their structure still uncompromised. By contrast, two days after anthesis, Plate 2-3B (a, b and c) characteristic shrinking and indentations in the papillae are seen. These images are almost identical to those presented previously by Wiemer *et al.* (2009). In images, Plate 2-3B (b and c) the top of the papillae had shrunk, which agrees with previous descriptions (Sazima *et al.*, 1993; Wiemer *et al.*, 2009). This suggests that the fragrance compounds had already diffused through the cuticle.

Another feature of the papillae are the pale 'markings' or 'patches' on the surface of the cells, Plate 2-3A (d) 1, 2 and 3. These may be fragrance substances collecting on the cuticular surface of the osmophore. They are typically found in many of the floral papillae of *Restrepia;* see Plates 2-6 and 2-9.

The current investigation of *Restrepia* osmophores therefore confirms previous studies, with the exception that no cuticular pores were observed. The presence of vesicles on the osmophore surface supports the secretory function of the osmophores. These findings are in line with recent studies of the operation of osmophores in other orchid genera which illustrate the accumulation of fragrance substances in the outer cuticular layer (Sazima *et al.*, 1993; Wiemer *et al.*, 2009)

2.4.2 The synsepal

The synsepal is the brightest and most colourful component of the *Restrepia* flower and it is usually striped or spotted. Even under a low power dissecting microscope, papillae are visible on this structure which are arranged in rows and which correspond to the stripes of the synsepal, (Plate 2-6 (a) and inset photograph). The papillae are elongated (Plate 2-6 (b), (c) and (d)) and tiny 'bumps' are observed on their surfaces, (Plate 2-6 (d)). These are not visible on cells in between the papillae, (Plates 2-6 (e) 3 and 4; and 2-7 (c) 1 and 2, (d) 2) which suggest that these cells do not have a secretory function, whereas the papillae do. The precise arrangement and structure of these papillae are shown in the VPSEM (Cryo Stage) images, Plate 2-7 (a) and (b). These younger papillae do not have vesicles corresponding to the 'bumps' indicating that they may not have formed, since these papillae have not fully elongated (Plate 2-7 (a), (b), (e) and (f)).

To date no data has been published with regards the structure and function of the synsepal papillae in *Restrepia*. However, Davies and Turner (2004) described floral papillae in *Maxillaria* (Orchidaceae) and reported similar observations to those presented in this study. More recently, it has been postulated that conical cells are present on floral structures to enhance pollinator grip and to generate 'structural' colour, often in distinct patterns on the flower (Whitney *et al*., 2009a; Rands *et al.,* 2011). The proportion of conical cells to other surface morphologies could depend on the complex selective biotic and abiotic pressures occurring in each habitat (Whitney *et al.,* 2011).

2.4.3 The labellum

The morphology of the labellum is similar for all *Restrepia* species with the exception of *R. aberrans* (Luer, 1996a). The labellum always exhibits some degree of coloured spotting, though it may or may not also possess stripes. This is also true even in species

which have a striped synsepal (Plate 2-8(a)) and (*cf.* Plates 2-23and 2-24). The labellum of all species examined is oblong, obscurely or distinctly divided into an anterior epichile and a basal hypochile. The epichile is longer and is flattened into an oblong. In many species, the labellum is narrowed near the middle to create a pandurate appearance; this narrowing thereby creates the isthmus. The narrowing can be caused or exaggerated by the inward curving of the margins. A pair of calli extends onto the hypochile from the sides of the column (Section 2.4.5 and Plates 2-18, 2-19 and 2-20). The margins and surface of the epichile vary from glabrous (smooth) to coarsely denticulate or fimbriate and from verrucose to papillose. The hypochile in contrast is smooth and concave with a pair of marginal processes, narrowly triangular – the cirrhi, (Section 2.4.6, Plate 2-22, Plate 2-1 (6)). The base of the labellum is inflexibly united by a thick, cylindrical neck to the foot of the column.

2.4.4 The epichile

The margins of the epichile are coarsely denticulate and the surface is heavily papillose. The surface papillae are in a linear arrangement, (Plate 2-9 (b)) which follows the stripes of the labellum. When these papillae are examined at higher magnifications the surface of individual cells may be seen, (Plate 2-9 (c and d)). As observed on the surface of the cells of the osmosphores, there are numerous 'pale patches', together with evidence of some cells having ruptured, (Plate 2-9 (c) 1 and (d) 1). Cells in the region between the papillae (Plate 2-9 (e)) have fewer of these 'pale patches' on their surfaces. These 'patches' could indicate the presence of fragrance substances collecting in the cuticles of the cells, which later diffuse through the cuticle layer causing the cells to rupture or burst (Sazima *et al.*, 1993; Wiemer *et al.*, 2009). These cells may have a similar function to the osmosphores in regard to fragrance emission. This is supported by the images shown in Plate 2-10. Vesicles (*cf.* previous 'pale patches') may be observed in (a) 1 and 2, (b) 3, and exudate in (b) 4. This provides supporting evidence

that the 'pale patches' observed in various floral structures are cuticular vesicles and are secretory in function.

These features correspond with the general description from Luer (1996a); while features of the cuticular layer are similar to those found in the cuticular layer of the osmosphores. Both sets of results are in agreement with the features described by Sazima *et al.* (1993) and Wiemer *et al.*(2009).

2.4.5 The isthmus

The cellular morphology changes noticeably in this region. Individual papillae are absent and there were fewer 'pale patches' observed in the VPSEM images, (Plate 2-12A (b) and (c)), which suggests that this region does not serve a secretory function. Running through this region is the labellar groove, (Plate 2-12A (b)), the upper section of which also does not contain obvious vesicles, (Plate 2-13). The lower section of the labellar groove, (Plate 2-14) exhibits these features, ((a) 1 and 2, (b) 3 and 4, and (c)). Exudate was also observed ((c) 5 and (b)) close to the arrow. This groove may operate to channel any secretions formed towards the lower epichile and hypochile.

2.4.6 The hypochile

The concave nature of this region is shown in Plate 2-12 (c) and Figure 2-1. In living plants the three regions of the labellum are angled differently (Figure 2-1), with the hypochile being the steepest part of the flower presenting itself to a visiting insect. The complete absence of papillae and cuticular vesicles in this region (Plate 2-15 (a), (b) and (c)) confirms that this area is non-secretory. The difference in surface texture of this area provides a different surface to the visiting pollinator. This may be an example of the flower manipulating a pollinator through tactile signals from different surfaces as reported in work on other species (Glover and Martin, 1998; Whitney *et al.,* 2009a; Whitney *et al.,* 2009b).

2.4.7 The column

In the Orchidaceae the male (pollinia) and female (gynoecium, stigmatic surface) reproductive organs of the orchid flower are enclosed in a single structure, the gynandrium or column. In *Restrepia* the column is typically slender and clavate (Plates 2-1 and 2-2).

The stigmatic surface, of the *R. brachypus* column, one day post-anthesis, is shown in Plate 2-16 (c). The surface appears turgid, moist and ready to receive pollinia. Upon pollination, the pollinia would adhere to the stigmatic surface; the pollen grains would germinate and the resultant pollen tubes grow down the column.

There is an important difference between the stigmatic surface shown in Plate 2-16 (a) and that depicted in Plate 2-16 (c). The first is from a flower four days post-anthesis and the second from a flower one day post-anthesis. The stigmatic surface (a) has desiccated and does not appear viscid or swollen. Pollinia would be less able to adhere to this stigmatic surface, less able to germinate and hence fertilisation of the flower is less likely to occur. This sequence of images (e to c to a) illustrates the changes that take place in the stigmatic surface as floral senescence progresses, and suggests that pollination is more likely to be successful in a fresh flower.

The rostellum (Plate 2-16 (e) 2) is a projecting flap of tissue from the column found in orchid flowers. It serves the function of separating the male pollinia from the female gynoecium, or stigmatic surface, and commonly helps to prevent self-pollination. It is also a gland that exudes a sticky substance which sticks to any pollinia which are attached to a visiting insect and ensures that the pollinia attach to the stigmatic surface. This structure is very important in wild orchid populations to affect cross pollination and prevent inbreeding. As shown in the SEM images (Plate 2-16 (e)), the rostellum in *Restrepia* is a well developed flap of tissue, which hangs partly over the stigmatic

surface, thus ensuring that the pollinia cannot inadvertently adhere to it. The edge of the rostellum is also clearly visible (Plate 2-16 (a, b and c)) as it extends well below the edge of the 'sticky' viscidium, acting as a protective shield and preventing the viscidium from adhering to the stigmatic surface.

2.4.7.1 The anther cap

Although there are some differences between the anther caps illustrated, not enough specimens were examined to determine if this was a diagnostic feature which would enable species identification. The most marked difference illustrated is that between the two clones of *R. brachypus.* These are two of the clones used in the breeding experiments (Chapter 4) and these SEM images illustrate some of the minor morphological or phenotypical differences that occur between them.

2.4.7.2 The polliniarium

The entire pollinarium is shown in Plate 2-17B (a) and the position of the viscidium is shown at X. There are four, free, equal-sized, ovoid pollinia in two pairs (Plate 2-17B (b)) as previously described by Luer (1996a). The lateral flattening of the pollinia may be seen. Plate 2-17B (c) shows the empty anther cap.

2.4.8 The calli

The presence of calli in *Restrepia* has been recorded previously, but their structure and function have never been established. The presence of a callus on the labellum of an orchid species has been reported previously (Arditti, 1992) e.g. *Phalaenopsis, Maxillaria*, (Davies *et al.,* 2003) but usually the callus is 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 it is attached to the labellum (Luer, 1996a).

Nectaries in orchids 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). Davies *et al.* (2003) showed the papillate nature of the labellum in *Maxillaria* and established 'viscid secretions' from these structures. These secretions could be clearly seen in their published SEM images. The labellar callus in *Bulbophyllum* species was shown to exhibit a papillate form that collected nectar (Teixeira *et al,* 2004). *Bulbophyllum* comprise a Pan tropical genus, of over 2000 species and are myophilous in common with *Restrepia.* Taking these two studies into account, it was expected that *Restrepia* would be similar and initially it was concluded that the calli might function as nectaries and provide food reward for the pollinating insect.

The Cool Stage SEM images showed the calli in *Restrepia* to be papillate in structure. This was confirmed in *R. brachypus, R. dodsonii* and *R. sanguinea,* all species from the subgenus *Restrepia* and also in *R. muscifera,* a species from the subgenus *Pleurothallopsis.* These structures were thus shown to have the same structure in both subgenera which might serve the same purpose. None of the Cool Stage SEM images showed any evidence of secretions similar to those reported by Davies and his colleagues (Davies *et al.,* 2003). Therefore, in this genus, the calli may not be concerned with nectar secretion or collection. The 'pale patches' previously observed elsewhere on the labellum and synsepal papillae were also absent. The bright apices of some papillae, (Plate 2-18 (e) X), were initially thought to be artefacts due to charging of the electron microscope.

When further examination of these structures was carried out using the Cryo Stage, clear images of the structure of the papillae were obtained (Plates 2-19 and 2-20). The cuticle was observed to be variously folded and striated, radiating from the apex of the papillae. No evidence of nectar or any exudate was found on the cuticular surface, even at this higher resolution. Since *Restrepia* are fly pollinated, food rewards would have to

be nectar since flies have no biting or chewing mouthparts. One explanation for this lack of nectar is that *Restrepia* is a non-rewarding genus of orchids. Many orchids do not produce nectar and still more do not provide any reward at all (van der Pijl and Dodson, 1966; Ackerman, 1985). While therefore, it is not unusual for orchids to be non-rewarding, this represents the first evidence for this in the genus *Restrepia.*

The pattern of the cuticular folds in plant cells is associated with iridescence in plants, in which the image alters with the viewing angle. This has been attributed to the cuticular folds acting as diffraction gratings (Whitney *et al.,* 2009c; Glover *et al.,* 2012) but for this effect, the cuticular layer should be flat and striated. The generation of iridescence will only occur if the ridges are separated by gaps of the right size (Glover, 2009). Rounded or conical cells do not allow directional reflection since they scatter light (Glover *et al.,* 2012) and would not be associated with iridescence. Similar cuticular 'folding' has been reported in other studies on orchidaceous labellar spurs (Bell *et al.,* 2009) and petal surfaces (Glover, 2009). Bell *et al.* (2009) argued that the striated labellar spur papillae improved pollination because they were either, a tactile expectation of the pollinating insects, or were connected with nectar production by the spur. Glover (2009) concluded that these structures had some influence on the behaviour of light, acting as a scattering mechanism to evenly distribute all wavelengths leaving the petal surface.

The features observed on the calli therefore seem to be associated with the scattering of light (including UV radiation), while the striated flatter areas between the papillae, (Plate 2-19 (b)) may exhibit a small degree of iridescence in the UV region of the electromagnetic spectrum (Whitney *et al.,* 2009c). These structures could therefore present a different image to the insect depending on the viewing angle.

2.4.8.1 Photographs of calli under different lighting conditions

When the calli were illuminated under different lighting conditions, they exhibited different optical properties (Plate 2-21). They were highly reflective under torch light and fluoresced bright blue under UV light, wavelength = 450nm. Since flies are visually sensitive to radiation in the UV region, it is possible that they see the calli differently in UV light and act as a visual signal to the pollinating insect. The calli may attract the pollinator due to their colour or other visual properties but because there is no nectar reward this may contribute to the deception of the pollinating insect by the Restrepia flower.

2.4.8.2 Action of the calli

These data led to the conclusion that the calli may act as 'landing lights' for the pollinating insect, which operate to lure or guide the insect by their brightness. This is important as the fly has to enter the flower under the column in order to be in the correct position to bring about pollination. The compound eye of the insect will play a role in how the insect 'perceives' the calli. The visual properties of the callus are caused by its cuticular folds which produce complex structural reflection and scattering of both UV radiation and visible light. Possibly the calli will only appear 'bright' and attractive to the insect when it is in the correct position, or on the correct 'flight path', towards the flower. Furthermore, since no evidence of any exudate was found on the calli, they would not appear to be concerned with nectar production. The *Restrepia* species studied, from this evidence, would appear to be non-rewarding. Since all the species are so similar, and species from both sub-genera were examined, it is logical to deduce that this may be true for all species within the genus.

2.4.9 The cirrhi

The position of the cirrhi in the flower is illustrated in Plates 2-1 and 2-2 (6) and they are depicted in detail in Plate 2-22. They are also shown in Plates 2-23, 2-24 and 2-25.

While these structures have been recorded previously (Luer, 1996a; Pridgeon, 1983), their function has never been fully established. They are distinctive structures that are unique to *Restrepia* so, it would be logical to postulate that they may have a function specifically related to pollination in this genus.

Plate 2-22 (c) and (d) demonstrate that there are no secretory vesicles on their surface. So, although they do not therefore play a part in attracting a pollinator by scent they may assist in guiding pollinators towards the anther cap and the pollinia (see Section 2.4.12 and Plate 2-26).

2.4.10 Photographic study

It may be concluded from the photographic images (Plates 2-23, 2-24 and 2-25) that the floral organs have a similar arrangement in all the species illustrated, the difference between the species being the relative sizes of the flowers as indicated by the scale bars. Using the pixel measurements (Table 2-1) the ratio between the labellum length and the column length was found to be constant for all the species studied.

(Ratio labellum length to column length = 2.0; measurement range =1.9 to 2.1

In contrast the ratio of the column length to cirrhi width was found to vary.

(Ratio column length to cirrhi width = 2.3; measurement range = 1.8 to 2.8)

From this it may be concluded that while the labellum and column of different species may be different in size, they are in the same proportion to each other. This suggests that these species are pollinated by insects of similar proportions. The differences in the column length to width across cirrhi ratio between species indicates different pollinator species. For example, *R. citrina,* has a column length to width across cirrhi ratio of 2.8

compared to that of *R. purpurea,* which is 1.8. One possible interpretation is that the pollinators for *Restrepia* might have the same body proportions of head, thorax and abdomen, but different body widths. Only those of the correct width would be able to fit in between the cirrhi. This further suggests that the genus may be oligophilic; i.e. pollinated by a few related taxa of very similar proportions. The question as to whether each separate species may be pollinator specific also arises. This is contrary to the literature (Pridgeon, 1983; Luer, 1996a), in which *Restrepia* pollination is assumed to be carried out by various small Dipteran species, but is in agreement with the view that orchid floral morphology is highly adapted to its pollinators and is commonly achieved by a 'functional fit' between flower and pollinator (Benitez-Vieyra *et al.,* 2006).

2.4.11 Fly pollination in the Pleurothallidinae

Many orchids have highly specialised structures adapted to facilitate pollination. The consequence of these adaptations is that the pollinator species for orchids are often specific.

Fly pollination is divided into two categories - myophily and sapromyophily. In myophily, the pollinator behaviour may be of two forms. In one, visiting adults flies feed on nectar and visit flowers regularly. In the second, male fruit flies (Tephritidae) (are attracted by a specific floral attractant which acts as the fly's sex pheromone precursor or booster by flowers which do not produce nectar. An example of which is found in some *Bulbophyllum* species which have a highly mobile labellum. In these species these floral attractants have been identified as either methyl eugenol (Tan *et al.*, 2002), zingerone (Tan and Nishida, 2007) or raspberry ketone (Tan and Nishida, 1995). None of these substances have been identified in any Pleurothallid species to date. Given the secretory nature of the synsepal and labellum in *Restrepia*, the intriguing question arises as to whether the exudates observed on the micrographs of these areas might contain

any of these substances.

Flies will quickly leave the flower if they obtain no reward but the flower may have traps to slow them down. Myophilous plants tend not to emit/produce a strong scent and are often purple, violet, blue or white in colour. The flowers may be simple (cup-shaped) with exposed stamens and stigma, or may have complex traps. Some of these features were found in *Restrepia* flowers.

Restrepia, are thought to be myophilous (Pridgeon and Stern, 1983; Luer, 1996a), with Dipteran species postulated as the pollinators (Pridgeon and Stern, 1983). While there is much indirect evidence to support this, it has never been confirmed directly in the wild or in cultivation. Indeed, as mentioned by Luer (1996a), spontaneous capsule set was practically unknown in the collections he studied, and from personal observations made over the past ten years it is also very rare in UK collections. This suggests that the necessary pollinator is not present in either instance and that a specific relationship exists between flower and pollinator(s) in *Restrepia.*

Flies have often been considered 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). They tend to be important pollinators in high altitude systems where they are numerous and other insect groups may be lacking (Larson *et al.,* 2001). As *Restrepia* are typically found in montane rain forests (2000-3500m) this supports the hypothesis of fly pollination for this genus. However, as direct observation of the pollinator for wild populations of *Restrepia* is impossible *ex situ*, evidence from indirect sources such as comparison with fly-pollination in other Pleurothallidinae genera (Christensen, 1994; Borba *et al.,* 2001a; Borba *et al.,* 2001b; Borba *et al.,* 2002) and investigation of the floral features found in *Restrepia* is very useful and easier to obtain.

Plate 2-25 shows oblique views of the position of the cirrhi on either side of the column. Several different species are included for reference. The similarity between the images is remarkable, suggesting that the cirrhi perform the same function in all the species examined. Perhaps a pollinating insect would be effectively trapped between them. They are also positioned in such a way that they protect the anther cap and pollinia and thus prevent the pollinia from being 'robbed'.

One distinguishing feature of Dipteran species is the presence of *halteres*. These are believed to be the vestigial remains of a second pair of wings. They are said to have a gyroscopic action and stabilise the insect in forward flight. The precise size and position of these varies from species to species. Plate 2-26A shows a typical Dipteran species and the position of the halteres (Blake, 2012). When an image of a cirrhus is superimposed on that of a typical Dipteran, Plate 2-26B, it is observed that the tip of the cirrhus might fit between the wings and halteres and so disrupt their function. For this to function efficiently, a precise or 'functional' fit between the two would be required (Benitez-Vieyra *et al.,* 2006).

Elaborate 'trapping' mechanisms have been found in *Dracula,* another Pleurothallid genus (Endara *et al.,* 2010). In this genus pollinators' thoraces are trapped by the incurved flaps of the rostellum which creates an angle between the scutellum and the abdomen for the removal and deposition of the pollinia. A precise fit between flower and pollinator is required, (Benitez-Vieyra *et al.,* 2006) which provides further evidence for pollinator specificity in Pleurothallid genera and the operation of oligophily. The role of the rostellum is important, as it prevents self-pollination; in the case of *Dracula*, remaining partially attached to the fly and being pulled forward to cover the stigmatic cavity.

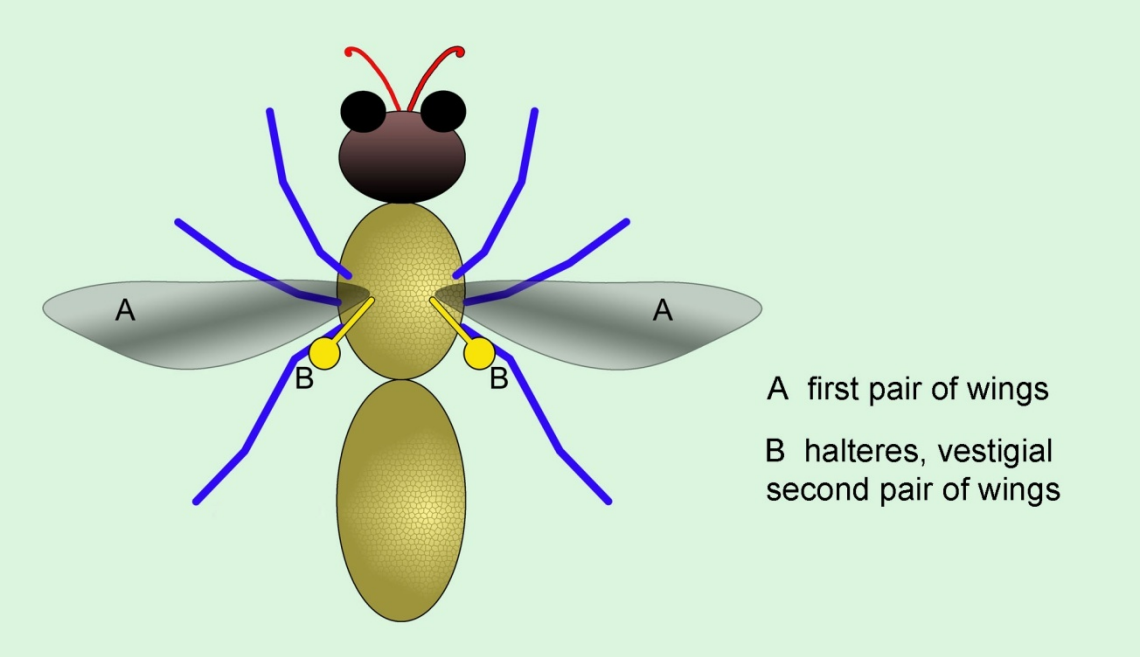


Plate 2-26A: Diagram of a typical Dipteran species. The position of the halteres is illustrated (B).

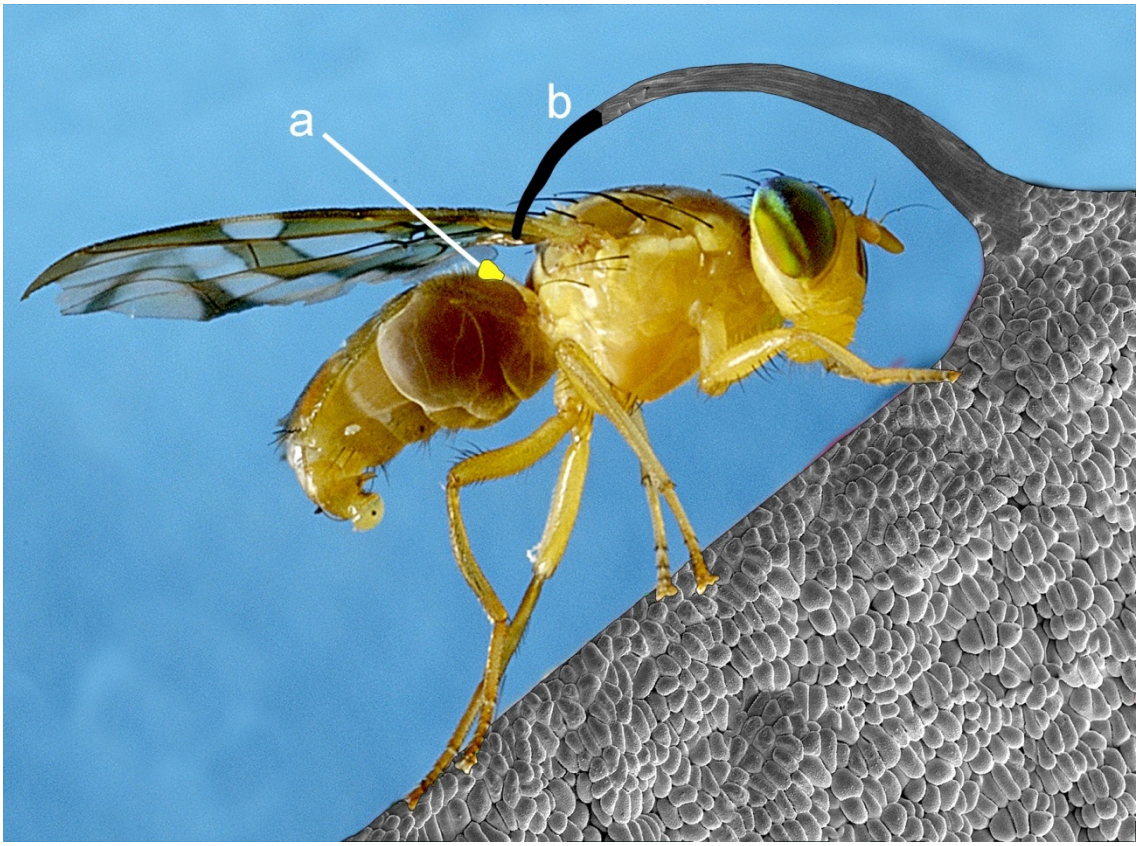


Plate 2-26B: Composite picture illustrating the proposed 'fit' of the cirrhi to the fly The cirrhi fit around the halteres (gyroscopic flight stabilisers) so interfering with their action and destabilising the fly in flight.
(a) haltere, shown in yellow and (b) tip of cirrhus, shown in black.

2.4.12 Pollination hypothesis

A tentative pollination hypothesis was formulated which postulates possible roles for the calli and cirrhi and includes evidence from the VPSEM studies for the roles of the other floral structures.

1. The fly (a small species of Diptera,) is attracted to the flower by scent produced by the osmophores (Pridgeon, 1983).

2. The fly is able to locate the flower by triangulation, due to the arrangement of the osmophores at the apices of the dorsal sepal and lateral petals.

3. The fly lands on the synsepal, where papillae arranged in lines lead it towards the labellum by scent. These conical papillae provide grip for the insect (Whitney *et al.,* 2009a; Rands *et al.,* 2011) and act as tactile clues to guide it towards the labellum.

4. When the fly reaches the labellum, the uneven surface provides grip and aids locomotion. The cells of the epichile (lower labellum) produce waxes and oils which the insect would be able to sense through its proboscis. These substances may be chemical precursors for the fly's male sex pheromone, as in *Bulbophyllum* (Tan and Nishida, 2007; Tan *et al.,* 2002).

5. As the fly progresses along the labellum, the cells of the isthmus become smoother and the surface steeper. This makes progress more difficult for the fly which at this point is positioned between the cirrhi and beneath the column.

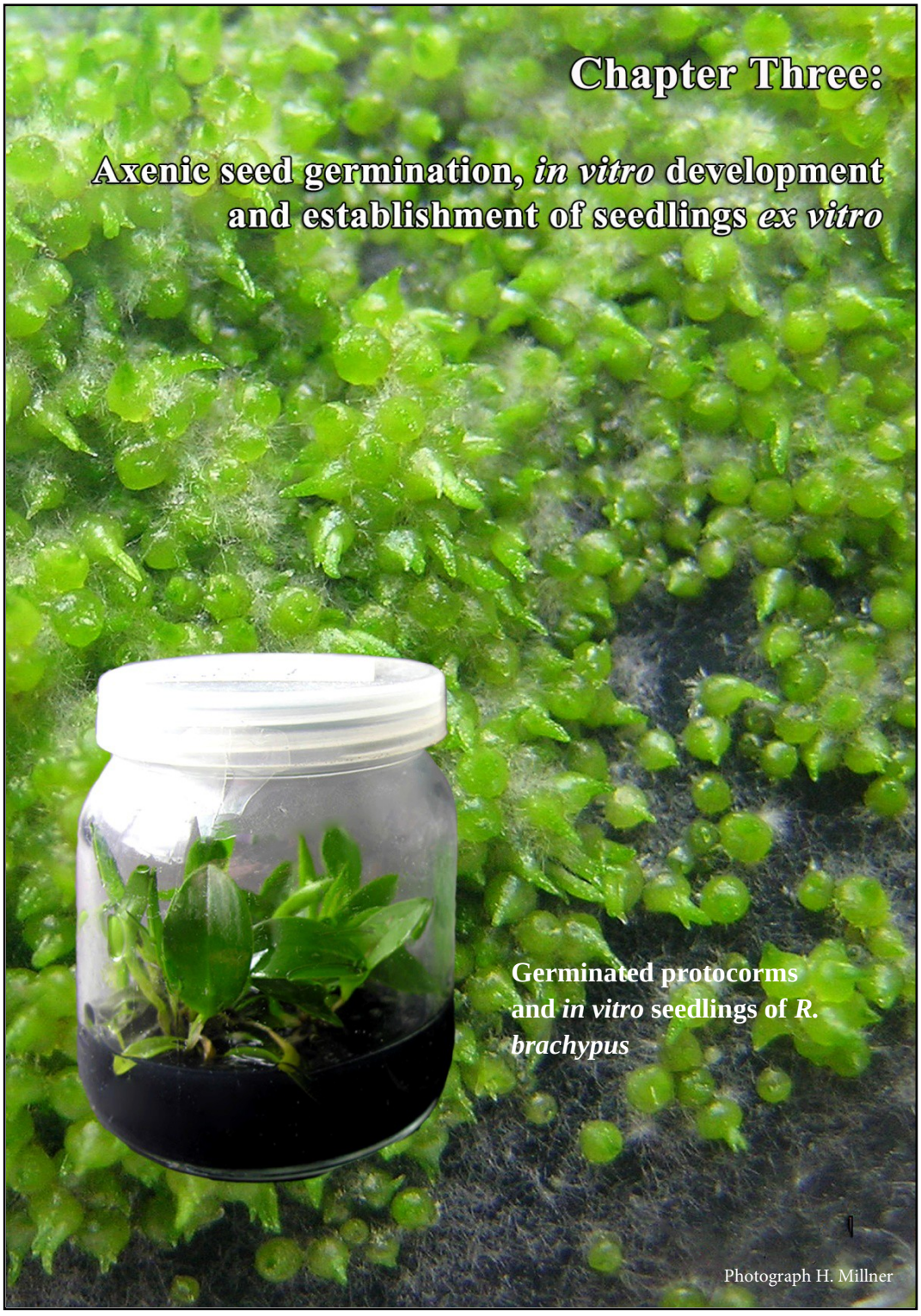
6. The cirrhi interfere with the action of the halteres, disrupt their gyroscopic action, and destabilise the fly in flight.

7. The fly's struggles bring about pollination by either depositing pollinia onto the stigmatic surface, or having pollinia from the flower becoming stuck onto it.

8. The fly is lured or attracted by the calli, and eventually progresses along the isthmus and onto the hypochile. As flies see in the UV region it is not possible to know precisely how it will observe the structural optical effects of the calli, but it may be guided in the correct direction by the fact that the calli will appear different in different directions.

9. None of the SEM pictures reveal evidence of food reward for the fly, and at this point it is able to leave the flower, albeit unrewarded.

This hypothesis leads to the conclusion that *Restrepia* species require pollinators of the correct proportions and that the genus as a whole is pollinated by very similar species, differing in size only. This would explain why very few spontaneous capsules are set in cultivation (Luer, 1996a) as the correctly sized pollinating species is not present. Pollination may only be brought about by the Dipteran species of the correct body dimensions to be 'disabled' in flight by the action of the cirrhi and to be able to fit between the column and the labellum and between the cirrhi.



"Conservation through cultivation…"

Plant Heritage (NCCPG)

3.1 Introduction and historical background

3.1.1 Introduction

All commercially available *Restrepia* plants have been propagated by either keiki production, leaf cuttings or by division. The ease with which some of the common species form keikis explains why, to date, very little effort has been made to propagate them from seed. Cultivated populations of *Restrepia* species have originated from a few 'founder' individuals with the result that remaining plants are genetically closely related to each other (Maunder *et al*., 1997). Furthermore, these plants will still closely genetically resemble their wild counterparts having only undergone vegetative reproduction. This feature can be used to study effects such as inbreeding depression (ID) and self incompatibility (SI) in small *ex situ* populations (Chapter 4). In order to facilitate these studies it was first necessary to establish reliable, replicable protocols for axenic seed germination of *Restrepia* which could be used subsequently across a range of *Restrepia* species.

While orchid seeds have been grown successfully on many different media, best results are only obtained when the correct balance of nutrients is present. This may vary from species to species. Some media are designed to be species or genera specific while others will support germination across a wide range of genera. All plant tissue culture media contain a carbohydrate source, a range of mineral salts and agar or other gel which solidifies the medium. In addition, many contain other additives including vitamins, amino acids or plant extracts such as banana pulp or potato extract. Consequently, the initial challenge for this investigation was that of establishing which media would support good germination rates for *Restrepia* seeds and which media would support subsequent good seedling growth and development.

In order to decide on culture media and to develop protocols for axenic cultivation it was necessary to consider the following aspects: the historical background of orchid seed culture and the role of Lewis Knudson, the important differences between symbiotic and asymbiotic germination, additions to orchid media, micro propagation techniques and the work of Morel together with what is currently known regarding axenic seed propagation of genera within the Pleurothallidinae.

While the techniques presented in the later sections of this chapter were vital to facilitate later parts of this project (Chapter 4), they are also of importance with regards to *ex situ* conservation strategies of *Restrepia* and related genera. The role of such propagated plants in living collections, seed banks and other initiatives is considered later in this chapter.

3.1.2 Orchid seed germination

3.1.2.1 Moore and Bernard

When orchids were first brought into cultivation, they were difficult to propagate from seed. Orchid seeds are minute, lacking an endosperm and there was little understanding of their germination requirements. Moore (1849) based his work on the fact that orchid seeds were reported to germinate if scattered at the base of a mature plant. Using this information he succeeded in germinating seeds of several orchid species, most notably *Epidendrum crassifolium* and *Phaius albus*. His method involved scattering the seed on the surface substrate of orchid pots at Glasnevin Botanical Gardens, Dublin, Ireland and maintaining subsequent high temperatures, heavy shade and moisture (Moore, 1849).

The first method for the production of any plant *in vitro* (Bernard 1899, 1900; Arditti and Yam, 2010) was postulated by Bernard in 1899. The term ‘protocorme’ for an early stage in the germination of lycopods (club mosses) had been used by Treub (1890) and this term was adopted by Bernard to describe the early stage of orchid seed germination.

This term is now used exclusively for orchids (Arditti, 1989, 1990; Yam and Arditti, 2009). Prior to the work of Bernard, the role of fungi in orchid seed germination had not been established (Yam and Arditti, 2009).

Bernard (1899, 1900) drew correct conclusions about the nature of the fungus seen in *Neottia nidus-avis* seedlings and their function in orchid seed germination (Yam and Arditti, 2009). He concluded that the fungal hyphae entered the seeds prior to germination and found that orchid seeds when sown 'with the germs of the appropriate fungus' germinated 'in a very regular manner' (Bernard, 1899, 1900). Symbiotic orchid fungi were described by Frank (1885, 1892) who first used the term *mycorrhiza.* During the period following these discoveries, growers and orchid breeders in the UK and France sowed seeds on the surface of potting mixtures in pots which supported orchids. However, the method was not very efficient and germination was uncertain (Arditti, 1984, 1990).

3.1.2.2 Commercial symbiotic germination of orchids

The first commercially successful method for *in vitro* symbiotic orchid seed germination was developed at Charlesworths, an orchid firm in the UK which specialised in the production of *Odontoglossum* hybrids. By 1924 the Charlesworths catalogue listed 2,422 *Odontoglossum* hybrids raised by this method, which gained widespread use throughout the globe until Knudson developed his medium in 1946 (Yam and Arditti, 2009; Greatwood, 2010). It is important to note that Charlesworths' *Odontoglossum* hybrid plants typically took 7 to 9 years to reach maturity by this method (Greatwood, 2010), compared to commercial methods today, which typically take 12-18 months to produce a mature *Phalaenopsis* (Riley, 2012).

3.1.2.3 Asymbiotic germination of orchids – Lewis Knudson

Lewis Knudson (1922) concluded that germination might be induced not by the action of the fungus within the embryo, but by external products of the fungus. He further deduced that germination of orchid seeds might be obtained in the absence of fungi by the use of certain sugars (Knudson, 1922, 1924) and both his media (B and C) contain 2% sugar (Yam and Arditti, 2009).

Knudson B medium (KB) was a modification of Pfeffer's solution devised by Wilhelm Pfeffer as a medium for orchid seed germination. Knudson further improved it by the addition of microelements (boron, copper, iron, manganese and zinc) and published his solution (Knudson C, KC) in 1946 (Knudson, 1946). His method for the asymbiotic germination of orchid seeds of *Cattleya, Laelia* and *Epidendrum* (Knudson, 1922; Seaton apgnd Ramsay, 2005) was the first practical procedure for the *in vitro* propagation of any plant in axenic culture.

His work paved the way for the germination of seeds and the growth of seedlings for a wide variety of orchid species on prepared sterile media without the need for fungal symbionts. After the formulation and publication of these media, orchid growing and hybridisation became widespread.

3.1.2.4 More recent additions to orchid media

Orchid media have been further developed by various modifications and additions (Arditti and Yam, 2010). Ernst originally added activated charcoal to seedling culture media to improve the aeration of the medium (Arditti and Ernst, 1984). He found that *Paphiopedilum* and *Phalaenopsis* seedlings grew well in media darkened with charcoal. His findings resulted in the formulation and widespread use of media containing activated charcoal for orchid seed germination, seedling culture and micro propagation (Arditti, 2008; Yam and Arditti, 2009). Its addition to culture media may promote or

inhibit *in vitro* growth, depending on the species and tissues used. The effects of activated charcoal may be attributed to establishing a darkened environment; adsorption of undesirable/inhibitory substance; adsorption of growth regulators and other organic compounds, or the release of growth promoting substances present in or adsorbed by activated charcoal (Pand and van Stade, 1998).

Incorporation of banana in culture media also became widespread shortly after its first addition to orchid media in Brazil (Arditti, 1968). Since which time enhanced growth of various genera has been reported to occur in the presence of a large number of complex additives, including coconut water, banana pulp, peptone, apple juice and peptone, fish extract and peptone, pineapple and tomato fruit (Ernst, 1967; Arditti and Ernst, 1993). The most common practice at present is to add pulp of ripe bananas to media (Yam and Arditti, 2009).

3.1.3 Orchid micropropagation

3.1.3.1 Early workers

Dr Gavin Rotor published the first paper on orchid micro propagation (Rotor, 1949), although it was not widely cited at the time of publication. He used Knudson C medium to culture *Phalaenopsis* nodes. This was the first tissue culture (*in vitro*), clonal propagation method developed for orchids, although he did not use an explant as the term is understood today (Rotor, 1949; Arditti and Yam, 2010).

German nursery owner Hans Thomale clonally propagated both tropical orchids and species native to Germany using shoot tip culture for micro propagation (Thomale, 1956). This was the first clonal propagation method of orchids involving a bud or tip explant and from this work Thomale hypothesised that tissue culture had the potential of being used for mass, rapid, clonal propagation of orchid species (Thomale, 1956; Arditti and Yam, 2010).

3.1.3.2 Morel

Professor Georges Morel (1916-1973) is widely celebrated as being the first to culture an orchid explant *in vitro*, although this has been refuted by some authorities (Arditti and Yam, 2010; Yam and Arditti, 2009). His contributions to orchid micropropagation are important because he made the mass propagation of orchids possible by showing that shoot tip culture could be used to produce virus-free orchid plants (Morel, 1960) and coined the term 'protocorm-like bodies' (PLBs) for bodies that could be subcultured. He popularised mass, rapid, clonal propagation through tissue culture, thus bringing the attention of commercial growers to the method. One such company was Vacherot and Lecoufle who made the first commercial use of shoot tip cultures for clonal propagation. Morel estimated that it was possible to obtain more than four million plantlets in a year from a single explant (Morel, 1965).

3.1.3.3 Other advances

The requirements for auxin and vitamins for plant tissue culture were already well understood when cytokinins were discovered. Cytokinins were shown to stimulate cell division and morphogenesis (shoot initiation and bud formation) and as such they were included in tissue culture media when the Murashige and Skoog (MS) medium was formulated by Toshio Murashige and Folke Skoog (Murashige and Skoog,1962). This medium was originally produced to grow *Nicotiana* (tobacco) callus *in vitro* (Murashige and Skoog, 1962) and is now the most widely used medium for plant tissue culture. It is unique compared to other media in that it has a high nitrate, potassium and ammonium content (Smith and Gould, 1989). There are dozens of variations on the original formulation, one of which is used in this study.

Since these discoveries, asymbiotic axenic seed germination has been used extensively to propagate many species of orchids. Some current examples include: *Laelia speciosa* (Avila-Diaz *et al.,* 2009); *Broughtonia lindenii*, *Cattleya aclandiae*, *C. granulosa*, *C.*

percivaliana, *Dendrobium parishii*, and *Guarianthe bowringiana*, (Buyun *et al.*, 2004), *Arundia graminifolia* (Bhadra and Bhowmik, 2005), *D. tosaense*, *D. moniliforme* and *D. linawianum* (Lo *et al.*, 2004) and *Habenaria radiata* (Shimada *et al.*, 2001).

Many other species, in particular terrestrial orchids, have been propagated using symbiotic axenic seed germination - these include: species of European terrestrial orchids (Clements *et al.*, 1986), Australian terrestrial orchids (Warcup, 1973) *Platanthera leucophaea* (Zettler *et al.*, 2001) and *Habenaria macroceratitis* (Stewart and Kane, 2006).

Micropropagation techniques have revolutionized the commercial production of orchid hybrids for the home, in particular the production of *Phalaenopsis* orchids. In 2000, commercial orchid sales were approximately $100,000,000 in the US and had increased significantly by 2002 (Greisbach, 2002). In 2005, commercial sales had risen to $145,000,000 but this is misleading, as it does not include companies with sales of less than $100,000 per year; the actual sales would be substantially higher than this (Runkle *et al.*, 2007). This commercial production is occurring in the Netherlands, Germany, China, Taiwan, the USA and Japan. Taiwan specializes in *Phalaenopsis* production and is the largest exporter of orchids; Thailand is noted for *Dendrobium* exports (McKrath, 2004). The global expansion of tissue culture for the rapid production of *Cymbidium*, *Phalaenopsis*, *Dendrobium* and *Oncidium* (Chen and Chang, 2000) far exceeds Morel's original prediction of four million plantlets in a year from a single explant (Morel, 1965).

3.1.4 Propagation of genera within the Pleurothallidinae

3.1.4.1 Masdevallia *and* Dracula

Few genera within the Pleurothallidinae have been routinely propagated via axenic seed culture; with the notable exceptions of some *Masdevallia*, *Dracula*, *Pleurothallis*

species and hybrids. In addition, the genera *Masdevallia* and *Dracula* have been hybridised to produce a new intergeneric hybrid genus, *Dracuvallia*. These hybrids are all recorded on the RHS International Register of Orchid Hybrids (RHS, 2013). There are currently 1193 *Masdevallia* hybrids, 34 *Dracula* hybrids, 56 *Dracuvallia* intergeneric hybrids and 8 *Pleurothallis* hybrids recorded.

3.1.4.2 **Restrepia**

With regard to the propagation of *Restrepia* species the situation is unusual. Although the genus is considered to be of horticultural interest (Bechtel *et al.,* 1992; Pridgeon, 1992; O'Shaughnessy, 2010; Plant Heritage, 2013) and possesses small but attractive flowers (Rice, 2006; Howe, 2013; Phafl, 2013), axenic seed germination, *in vitro* protocorm and seedling development of *Restrepia* were not reported until 2008 (Millner *et al*., 2008). Some *Restrepia* species are easy to propagate by plantlets that form on the base of the leaves, called 'keikis', and from leaf cuttings (Webb, 1985). This is also true of some *Lepanthes* and *Pleurothallis* species, but to date none of these species have been micro propagated by tissue culture procedures for leaf explants (Yam and Arditti, 2009) and very few have been propagated via seed (Millner *et al*., 2008).

Cultivated *Restrepia* species propagated either by keiki production, leaf cuttings or division are genetically closely related to each other (Maunder *et al*., 1997) and will still closely genetically resemble their wild counterparts having only undergone vegetative reproduction. These characteristics make this genus ideal subject material for investigating ID and SI.

3.1.5 Chapter Objectives

1. To establish a simple, reliable and reproducible protocol for the asymbiotic, axenic seed germination of *Restrepia* so that species and hybrids of this genus can be propagated from seed, to help maintain genetic diversity within cultivated populations.
2. To develop protocols that will facilitate the investigation of the breeding barriers within the genus.
3. To establish cultivation protocols for seedlings by investigating seedling development *ex vitro*. The resulting methodologies could then be applied to the *ex situ* conservation of *Restrepia* and related genera.

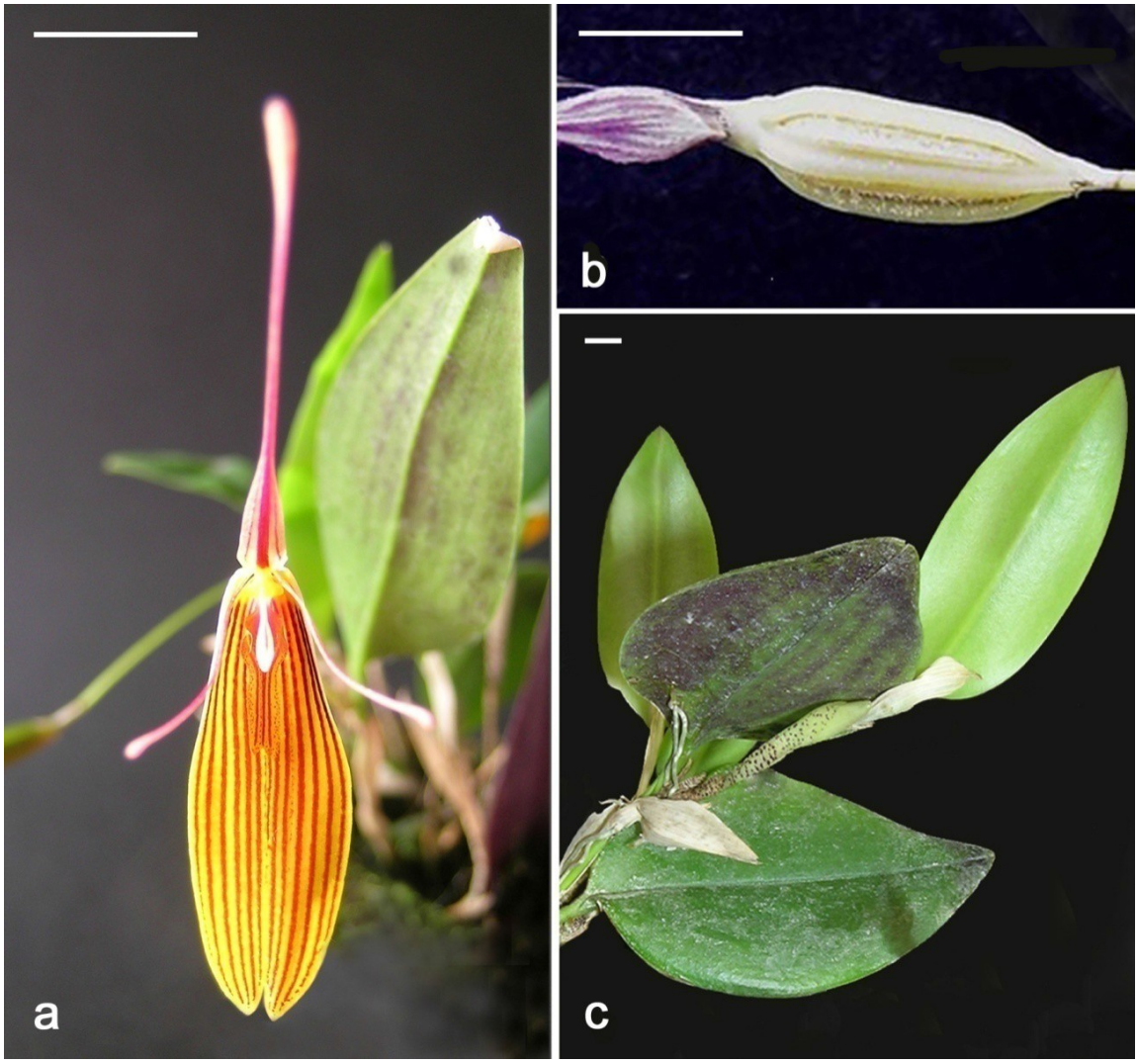


Plate 3-1: ***Restrepia brachypus***

(a) *Restrepia brachypus* flower; (b) seed capsule at dehiscence; (c) leaf with keiki forming in the leaf axil.

Internal scale bars represent 1cm

3.2 Materials and methods

3.2.1 Axenic seed germination and initial protocorm development

3.2.1.1 Plant Material

When working with rare species of any plant genus, due to the shortage of material, it is often impossible to perform the requisite number of replicates required for statistical analysis. The experimental design of the current study was constructed to overcome this problem. Since *Restrepia* set hundreds of seeds per capsule (Arditti, 1992; Arditti and Ghani, 2000), statistical evaluation of results from a comparatively small number of seed capsules is feasible.

In this investigation all plant material had been greenhouse grown and came from the personal collection of H. Millner (see also Chapter 2, Materials and methods, Plant Material). The seeds used were from capsules produced by hand, cross-pollinations of two different, unnamed clones of *R. brachypus* (Plate 3-1a). Previous trials had shown that this cross-pollination produced seeds that would germinate readily on Western medium. Only seed from well formed, healthy capsules, produced from hand pollinations that had dehisced naturally were used, Plate 3-1(b). All seeds and plant material for germination and subsequent *in vitro* protocorm and seedling development were incubated in an illuminated growth chamber at 21°C with a 16 hour day length.

3.2.1.2 Media used

The media used in the current study are shown in Table 3-1.

Two of the media were commercially prepared and modified by Duchefa labs: Vacin and Went (VW); (Vacin and Went, 1949) and Murashige and Skoog (MS); (Murashige and Skoog, 1962). The MS medium was used at half strength (Kyte and Kleyn, 1999; PhytoTechnology Laboratories, 2013; Seaton and Ramsey, 2005) and both MS and VW were further modified by the addition of 3% sucrose. The P668 medium was supplied

by PhytoTechnology Laboratories and W medium was supplied by Western Laboratories, Australia.

Table 3-1: Media used in the study

Media and abbreviations		Modifications in study	Original citations
PhytoTechnology P 668 (668)	Used commercially by orchid flasking companies in the UK, very similar to 1/2 strength MS and identical to Sigma P-6668	0.5% TC agar	Phytotechnology Laboratories, 2013; Murashige and Skoog, 1962
Murashige and Skoog (MS)	Preliminary expts. showed *Restrepia* seeds would germinate on 1/2 strength macro elements MS (Thompson, 1980; Seaton and Ramsey, 2005).	½ strength macro nutrients (Seaton and Ramsay, 2005) 0.5% TC agar, 3% sucrose	
Vacin and Went (VW)	Used by RBG Kew in propagation of orchids (McMichen, 2005)	0.5% TC agar, 3% sucrose	Vacin and Went, 1949
Western (W)	A comparatively new medium, developed to overcome changes in pH of medium during growth and development of seedlings.	None	Proprietary brand, supplied by Western Orchid Laboratories, Australia

3.2.1.3 Methodology

Media were adjusted to pH 6.0 after the addition of sucrose and agar with 0.1N KOH and were dispensed into 300ml medical flats before autoclaving for 20 min at 120^{o} C. Four replicates were then prepared for each medium in 90mm diameter sterile plastic Petri dishes, giving 16 plates in total.

Seeds from four, hand, cross-pollinated capsules of *R. brachypus* were mixed together to ensure the viability of the seed was homogeneous. Seeds were then surface sterilised in 0.5% v/v sodium hypochlorite solution for 10 minutes and allowed to settle. This solution was then decanted in the LAF and the seeds washed twice in SDW. The seeds were then resuspended in 5ml SDW and spread evenly across the sixteen, prepared media plates. Germination was recorded using a dissecting microscope.

Since *Restrepia* seeds do not display synchronous germination, germination rates were recorded every week for five weeks. A count of >150 filled (containing embryos, Stage

2, Table 3-2 and Figure 3-1) seeds per plate was sampled each time. The number of swollen embryos (Stage 2) was recorded, and full germination (Stage 3) was assumed when the developing protocorms had split their testae. Final full germination rates were calculated for all replicates of each medium each week.

The diameters of the protocorms after five weeks were recorded using the Spot RT Colour camera (with integrated software Version 4.02) manufactured by Diagnostic Instruments Inc., Michigan, USA, mounted on a Nikon 'Eclipse' ME600 (Nikon Corp., Tokyo, Japan). The image analysis software used was Image Pro Plus (Version 5.0.1) manufactured by Media Cybernetics Inc. Maryland. All statistical analysis was performed on SPSS (Statistical Package for the Social Sciences) Version 19 (SPSS Inc., Chicago, USA). The Kolmogorov-Smirnov test and Levene's test were used to test for normality and homogeneity of variances respectively. A Kruskal-Wallis test was used to compare the germination percentage means, as the sample sizes were too small to ensure normality. Initial protocorm diameters were transformed ($\log_{10}$) for normality and analysed by one-way ANOVA; Tamhane's T2 was used *post hoc* where differences were highly significant, but variances were not homogeneous.

Table 3-2: Seed germination and protocorm development in *Restrepia brachypus,* (Millner *et al.*, 2008)

Stage	Description
1	Empty seed, no embryo
2	Viable embryo, no germination
3	Swollen embryo, testa not split
4	Further swelling, testa split, embryo (protocorm) is photosynthetic (=germination)
5	Leaf primordia visible
6	First rhizoids emerge

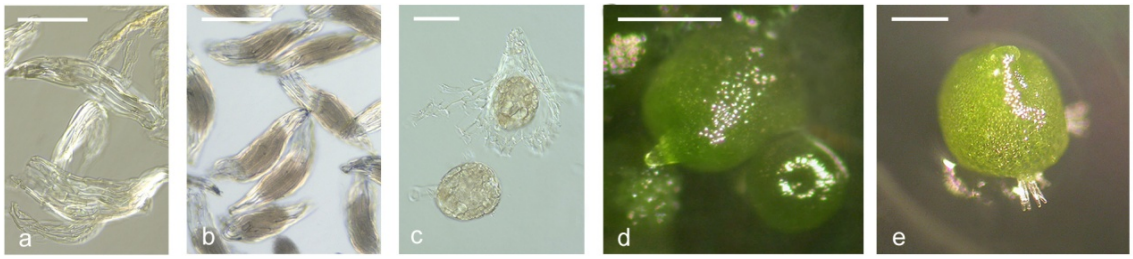


Figure 3-1: Stages of germination, (Millner *et al.*, 2008).
(a) Stage 1, empty embryos;
(b) Stage 2 with viable embryos;
(c) Stage 3 embryos have begun to swell, and Stage 4 one embryo has burst its testa;
(d) Stage 5 leaf primordial just visible;
(e) Stage 6 rhizoids have emerged.
Internal scale bars (a), (b) and (c) represent 50µm and (d) and (e) represent 5mm

3.2.2 Seedling development *in vitro*

3.2.2.1 Replating protocorms and seedling development

Once the optimal medium for axenic seed germination had been established (see Results section) the following protocol was used to determine the best medium on which to replate protocorms for subsequent seedling development. Seeds from the same interclonal cross of *R. brachypus* were therefore pre-germinated on Western medium for this part of the study.

The media used for replating were the same as used for the axenic seed germination except that each medium was also trialled with the addition of banana pulp at the rate of 60 g/l of medium. Banana supplementation was trialled (despite being considered to be an undefined additive) because banana is commonly used in orchid replate/subculture media and has been consistently shown to promote rooting and development (Arditti, 1967, 1982).

When protocorms were large enough to be transferred, replicates were replated/ subcultured onto this range of media. Microscopic examination of the individual protocorms confirmed that they were all at a similar stage of development, i.e. Stage 5, (Table 3-2 and Figure 3-1); they had initiated roots and leaf primordia and were undergoing photosynthesis. Twenty five protocorms per standard 90mm Petri dish were arranged in a numbered grid formation. This enabled random selection of individual protocorms later in the study. Duplicate grids of 25 protocorms were prepared for each medium, plus and minus banana.

The length of each protocorm was measured weekly using the Spot RT Colour camera. The image analysis software used was Image Pro Plus (Version 5.0.1). These measurements were continued for four weeks (Results section: Figure 3-2, Weeks 1 –4).

By this time the vertical growth of the seedlings made valid comparisons of the measurements impossible.

After five weeks, ten seedlings were randomly selected from each plate, removed, laid horizontally and measured, (Results section: Figure 3-2, Week 5). Mean protocorm lengths on the different media were calculated and the effects of media and banana were compared using two-way ANOVA. Tamhane's T2 was used *post hoc* where differences were highly significant, but variances were heterogeneous, and *t*-tests were used to distinguish the different effects of banana additions for each medium, where these were obscured in two-way ANOVA by an interaction between factors. The remaining seedlings were replated onto their respective media in single culture vessels and allowed to develop further in the growth chamber for another six months (Plate 3-2).

3.2.3.2 Recording seedling development in vitro

In addition, images of viable embryos, germination and protocorm development using light microscopy were recorded to show the non-synchronous nature of their germination and development (Plate 3-3).

VPSEM images using the Cool Stage were produced of early protocorm and seedling development (see Plate 3-4). The methodology employed was that detailed in Chapter 2, (see Materials and Methods, 2.2.2).

3.2.3 Establishment of seedling *ex vitro*

Various procedures for establishing orchid seedlings *ex vitro* are well documented elsewhere (Thompson, 1980; Seaton and Ramsey, 2005), for this reason comparative statistics between methods were not undertaken. However, the growth and development of the seedlings was recorded so that a timeline for the development of seedlings into mature plants could be established. There follows a description of the methodology

used for the establishment of seedling *ex vitro* used in this study and illustrations of the plants obtained are included in the results.

When the *Restrepia brachypus* seedlings were large enough (Plate 3-5: a, b and c), they were removed from the culture vessel by firstly tipping the contents out of the culture flasks and then rinsing the medium off the seedlings using SDW. The roots of the seedlings were very fine and easily damaged (Plate 3-5: a) so if they could not be separated easily at this stage, they were left as a 'clump' until they were established. Unlike other protocols, no fungicides or insecticides were used at this stage. The seedlings were then potted very loosely in damp *Sphagnum* moss and placed in an unheated propagator. The seedlings in the propagator were maintained in a heated greenhouse with a minimum night temperature of 16°C. The propagator maintained humidity around the seedlings of >80%. High humidity levels are required in the propagator to reduce 'transplant shock' for the seedlings at this stage. The humidity in the culture vessels is a constant 100%, and newly 'deflasked' seedlings are very sensitive to changes in humidity levels. In addition, great care was taken that the seedlings themselves did not become too wet, due to over-watering, as they can rot and die very quickly. It is for this reason that *Sphagnum* moss was used as a substrate at this stage as it reduced the need for watering.

Once new growth had commenced, the seedlings were gradually moved on as they grew, until eventually they reached flowering size. Although plants can survive some level of dehydration due to 'succulence' in their tissues, lack of humidity is still an important factor in the success or failure of their culture, and growth rates of seedlings are greater when higher levels of humidity are maintained.

A timetable of all stages of development is included in the Results section (Table 3-4).

3.3 Results

3.3.1 Axenic seed germination and initial protocorm development

A summary of germination rates and means is presented in Table 3-3. Comparison by Kruskal-Wallis (Table 3-3a) of germination percentages showed a highly significant difference between means: χ^2 (3) = 12.794, p = 0.005. The media W (53.05%) and P668 (26.66%) gave the largest means, with much smaller means from VW (16.61%) and MS (7.96%).

A mixed model one-way ANOVA (Table 3-3b) of initial protocorm diameter with 'media' as the fixed factor and 'plate' as a random factor, indicated a highly significant difference between media means: F (3,302) = 27.755; p = 0.000. The medium P668 produced significantly larger protocorms at the end of the germination period than any other (410.01µm; Tamhane's T2, $p < 0.05$). The medium W produced the next largest (325.10µm), which were significantly larger than those produced by either MS (235.20 µm) or VW (225.32µm). The means of these two were not significantly different from each other.

Since W medium supported the highest mean germination rates and protocorms at the most consistent stage of development (Stage 5, Table 3-2), it was chosen as the medium of choice to pre-germinate seeds for the seedling development study. The non-synchronous nature of the germination and development of viable embryos and protocorm development is illustrated in Plate 3-3.

Table 3-3: Results of media comparisons for germination and diameter of *Restrepia* protocorms after four weeks' germination on media: P668, MS, VW and W.

Media Comparisons					
a) Germination rate (%)					
Medium	*Mean length*	*SE*	*df*	χ^2	*P*
P668	26.66	3.35	3	12.794	0.005**
MS	7.96	2.17			
VW	16.61	4.25			
W	53.05	6.38			
b) Protocorm diameter (µm)					
Medium	*Mean length*	*SE*	*df*	*F*	*P*
P668	410.01 c	34.15	3, 302	27.755	0.000***
MS	235.20 a	10.52			
VW	225.32 a	9.12			
W	325.10 b	26.47			

Notes:

** and *** indicate the significance level when *P* values are <0.05; **, *p*<0.01; ***, *p*<0.001. Comparison of percentage germination (Kruskal-Wallis; p <0.01) showed a very significant difference between media. Mixed model one-way ANOVA on transformed ($\log_{10}$) data with 'plate' as a random factor also indicated very significant differences between media (p < 0.001). Media means with the same label (a, b or c) are not significantly different from each other (Tamhane's T2; p <0.05).

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VW	225.32 a	9.12			
W	325.10 b	26.47			

Notes:

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3.3.2 Seedling development *in vitro*

3.3.2.1 Replating protocorms and seedling development

Growth rates for the different media, with and without the banana addition, are presented in Figure 3-2, (a-d). A mixed model two-way ANOVA on transformed (log_{10}) data with 'media' and 'banana' as fixed effects and 'plate' as a random effect showed a significant main effect due to the growth media: F (3,143) = 21.154, p = 0.016. The overall effect of adding banana to the media was not significant: F (1,143) = 0.742, p = 0.547; however, a significant interaction (p = 0.044) between 'banana' and 'media' indicated the likely confounding effect of media on banana. The mean protocorm lengths at the end of the treatment were smallest for MS (1.82mm) and greatest for W (3.23mm); P668 (2.78mm) and VW (2.32mm) were intermediate. All were significantly different from each other (Tukey, $p < 0.05$). The separate effects of banana additions on the different media investigated by *t*-test showed a very significant difference for MS: t (37) = 3.687, p = 0.001; VW: t (29.07) = -4.04, p = 0.000 and W: t (38) = -3.72, p = 0.001. However there was no significant difference for P668: t (38) = 1.99, p = 0.054. Mean protocorm lengths were increased by the addition of banana to VW and W but decreased by addition to MS.

3.3.2.2 Seedling growth in vitro *six months after final replating*

The seedlings cultured on media without banana supplement (Plate 3-2 (a)) are markedly smaller than those cultured on media containing banana supplement. For the seedlings cultured without banana, the seedling on P668 medium shows much better development than the others. For the seedlings cultured with banana supplement, the seedling on W medium shows the best overall development. Of all the seedlings the one cultured on W medium had the best overall development both of leaves and roots.

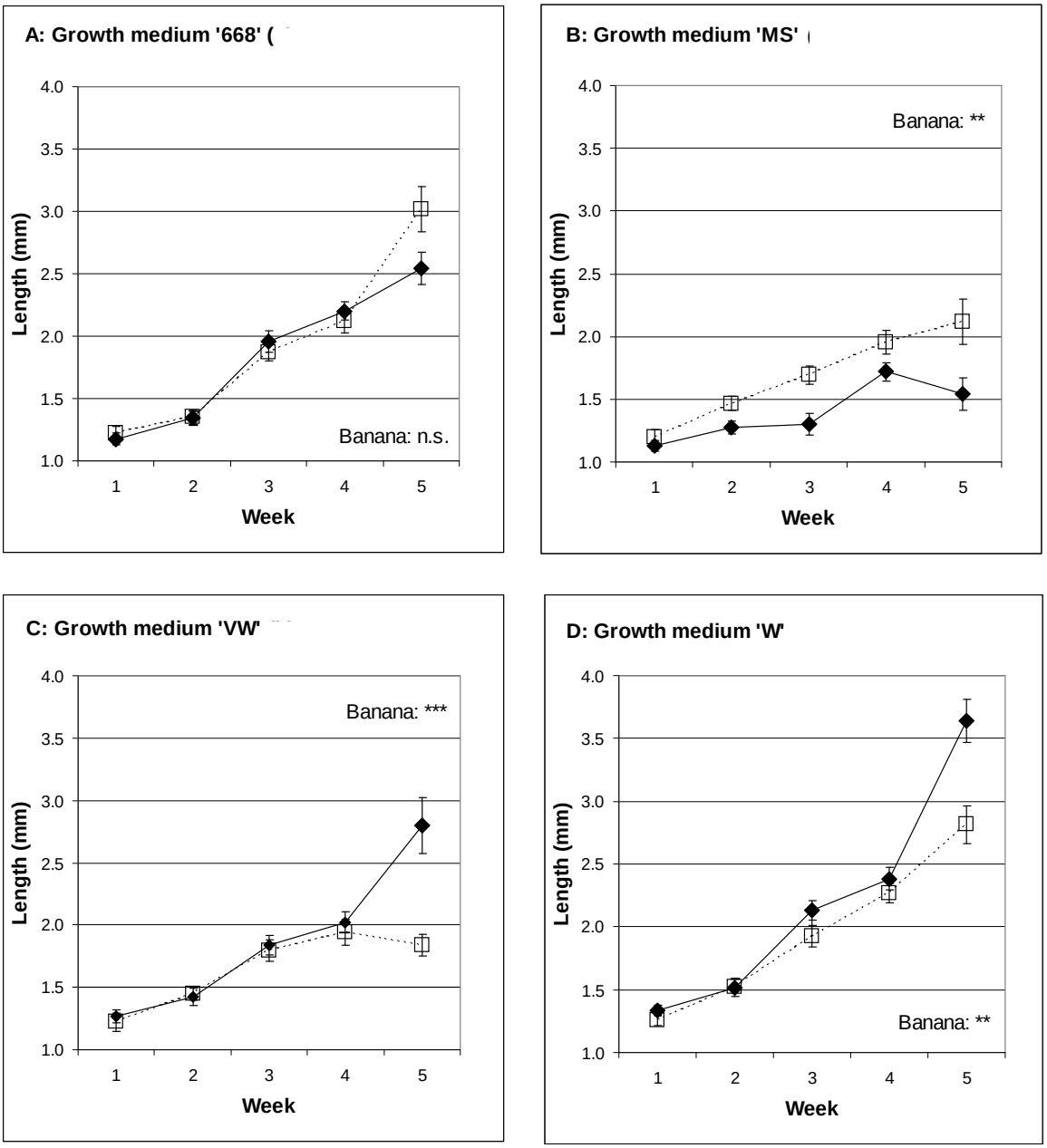


Figure 3-2: Growth rates of *Restrepia* protocorms on four different media:

A, 'P668'; B, 'MS'; C, 'VW' and D, 'W' for the five weeks following germination. Banana additions are depicted as ◆, with banana and □, without banana; errors bars represent ± 1 SE. Two-way ANOVA on transformed (log_{10}) data indicated a significant difference between media (p <0.016); all media means are significantly different from each other (Tukey, p <0.05).

Results of media-specific *t*-tests on the banana additions are displayed as: 'n.s.', non-significant; **, p <0.01; ***, p <0.001.

Plate 3-2: Comparison of seedling growth on all media after six months

(a) Media without banana supplement; (b) media with banana supplement.

Internal scale bar represents 1 cm

3.2.3.2 Recording seedling development in vitro

Light microscopy images of seeds with viable embryos and germinated protocorms are presented in Plate 3-3. (a) Two viable seeds with large, dark embryos may be observed. In this image the embryo are swollen as they have imbibed moisture. (b) The embryo germinates as the cells begin to multiply, so splitting the testa and releasing the young protocorm. (c) Embryos at different stages of development may be distinguished by the differing development of the leaf primordia. The protocorms have become photosynthetic at an early stage of development.

Scanning electron micrographs of protocorms and early stages of seedling development are shown in Plate 3-4. (a) Illustrates a young protocorm before the leaf primordia have begun to develop. There is an indentation in the top of the protocorm, from which the leaf primordia will emerge as shown in (b). This feature was observed in many *Restrepia* protocorms over the course of the study. (c) The leaf primordia have lengthened further, the lower region of the protocorm has now also lengthened and distinct regions are apparent. (d) The lengthening has continued, the young shoot can be seen as two leaves at the top. The lower section of the protocorm has lengthened further and rhizoids may be seen emerging from this area. These structures were too fragile to survive using the Cool Stage of the VPSEM. (e) The position of stomata on the young shoots are shown (arrowed). As the young protocorms became photosynthetic at an early stage, the concomitant development of stomata is essential for gaseous exchange to take place. (f) Details of two stomata are shown. In (1) the stoma is closed while in (2) the stoma is open.

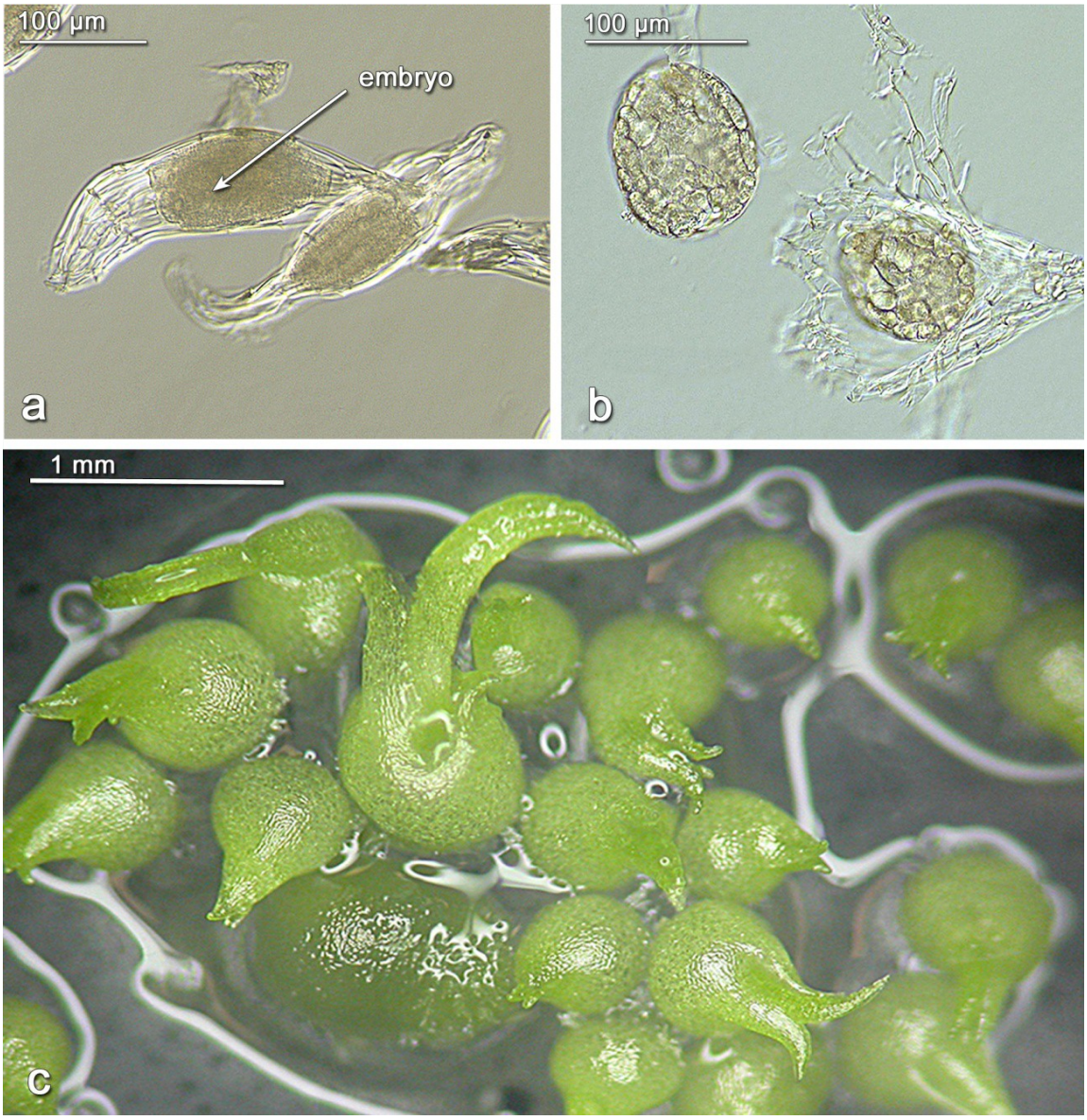


Plate 3-3: Seeds with viable embryos, germination and protocorm development, using light microscopy

(a) Viable seeds with embryo; (b) embryo germinates and testa splits; (c) embryos develop into protocorms which are photosynthetic at a very early stage;
(c) protocorms on agar medium, at various stages of development, showing the non-synchronous nature of their germination and development.

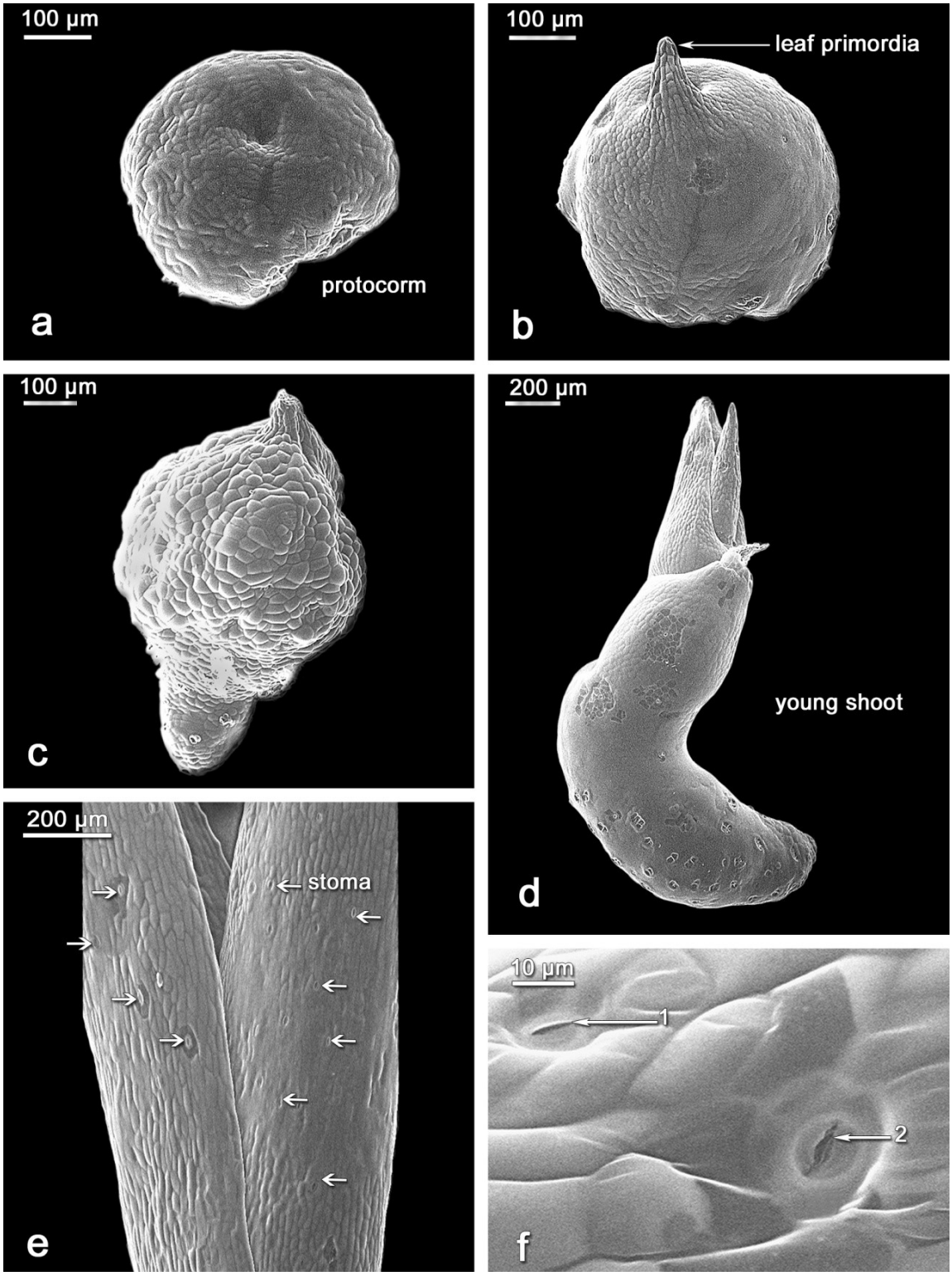


Plate 3-4: Protocorm and early seedling development, using scanning electron microscopy.

(a) Embryo develops into a ball of cells, called protocorm; (b) leaf primordia form on the protocorm; (c) protocorm lengthens, distinct regions now evident; (d) leaf primordia lengthen and the young shoot emerges; (e) stomata can be seen on the surface of the leaves, indicated by arrows; (f) details of stomata, shown at 1 (closed) and 2 (open).

3.3.3 Establishment of seedlings *ex vitro*

The timescale from seed germination through to flowering plant is presented in Table 3-4. These times are approximate. Seedlings ready to deflask are shown in Plate 3-5 and seedlings/plants subsequently established *ex vitro* in Plate 3-6.

Table 3-4: Timetable of seedling development

Stage	Feature	Time scale
Pollination		Day 0
Fertilisation	Flower collapses	1 day
Capsule formation and dehiscence	Figure 3-5 (a)	56 – 84 days, determined by environmental conditions
Seeds sown on agar germinate	Figure 3-5 (b)	14 – 21 days
Protocorm development	Figure 3-5 (c) Figure 3-6 (a, b, c, d and e)	21-56 days
Seedlings replated		3 -6 months
Seedlings ready to 'deflask'	Figure 3-7 (a, b and c)	1-2 years
Seedlings establish *ex vitro*	Figure 3-8 (a)	3 – 6 months
Plants reach flowering size	Figure 3-8 (b)	2 – 3 years

Notes:

The data above show that from pollination to capsule dehiscence takes typically 56 - 84 days; from seed to germination takes 14 - 21 days; from germination to tiny 'plantlet' takes 3-6 months; from 'plantlet' to large seedling takes 1-2 years and development *ex situ* to mature plant takes 3-4 years. This gives a time scale of 4-5 years, in some cases longer, for the entire process from pollination to flowering plant.

Plate 3-5: Development of seedlings in flask, prior to deflasking

(a) Extensive development of fine roots visible through the base of the flask; (b) shoot (leaf) development, showing strong growth; (c) seedling removed from flask, remains of agar medium can be seen, with the extensive roots and shoots.

Plate 3-6: Growth and development of seedlings of *R. brachypus, ex vitro*

(a) Seedlings potted into damp *Sphagnum* moss after deflasking; (b) seedling 2-3 years later, flowering size; (c) *inset*- details of flower.

3.4 Discussion

Very little has been published on the growth and development of *Restrepia* in its natural habitat, and nothing regarding these processes *in vitro.* The current study is therefore important since it represents the first media trial for axenic seed germination and *in vitro* seedling development for any species in this genus. The results demonstrate that W medium produced the highest axenic seed germination rates (53%; Table 3-3); the second largest (325 μm) and third most consistent (SE = 26.5) protocorm growth (Table 3-3); the best early seedling growth (3.6 mm; Figure 3-2) and subsequently the best seedling development after six months in culture (Plate 3-2).

These data demonstrate that *R. brachypus* seeds can be effectively and efficiently propagated *in vitro*; using W medium for germination and with banana pulp supplement for ongoing growth and development, and that P668 medium would be an adequate alternative without the need to add banana (Table 3-3, Figure 3-2 and Plate 3-2). Alternative concentrations of these media or other media may also produce acceptable results, but the current data provide a valuable starting point for axenic seed protocols within this genus and its subtribe.

The horticultural methods and protocols established in the later part of this study will assist in the cultivation of the genus and may provide a starting point for the culture of other Pleurothallid genera that originate from similar habitats. Establishing cultural protocols is necessary to address one of the criticisms of *ex situ* collections, which is that in such collections there may be little information on the history of the taxa in cultivation and no satisfactory horticultural protocols established (Maunder *et al.,* 1997). These protocols are important as different media will give different percentage germinations for the same species and all media are not equally suitable for all species (Seaton *et al.,* 2007).

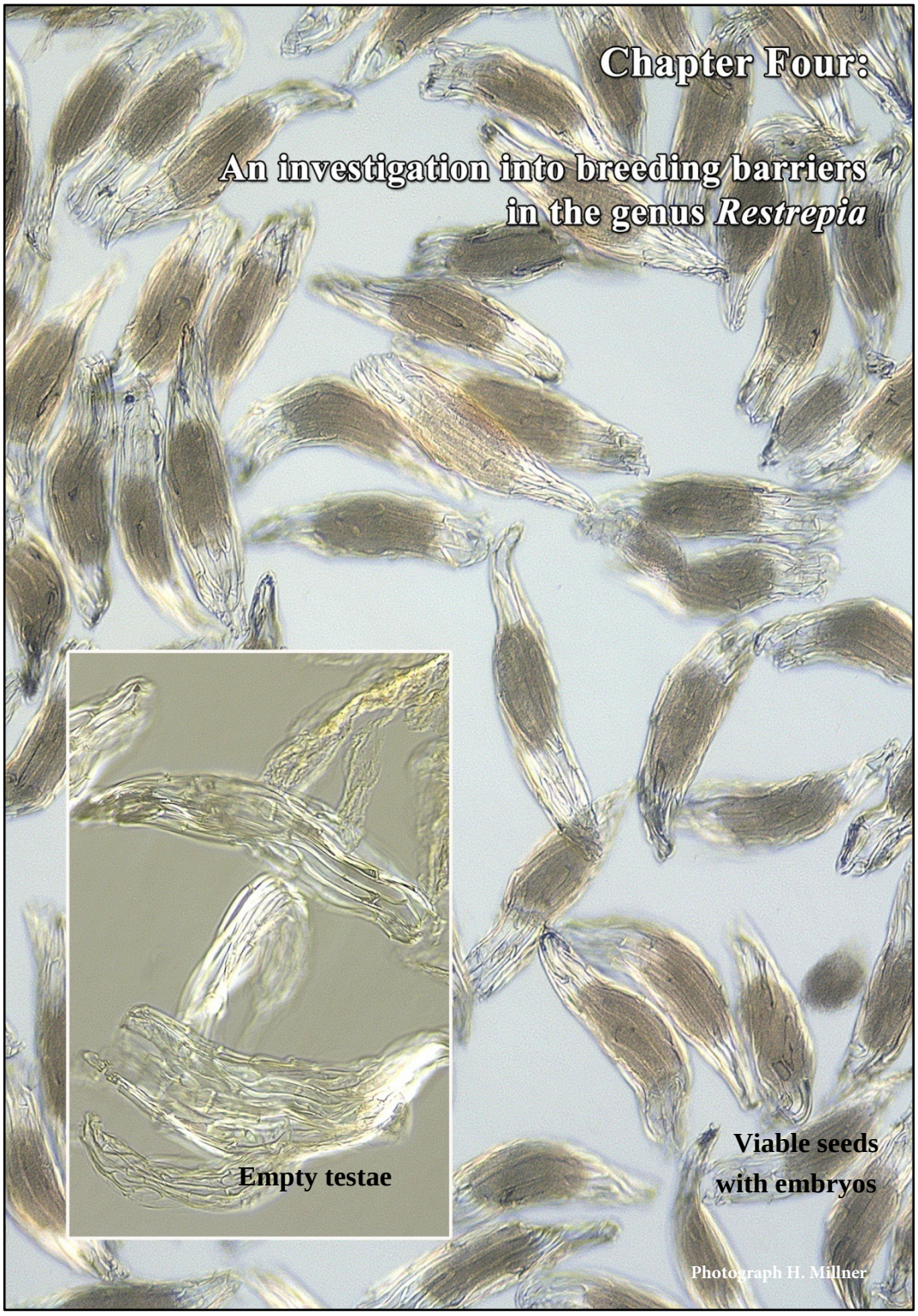
A species may be 'Critically Endangered', as indicated by its Red List status, but common in cultivation and hence its culture is well understood. However, many such species are also rare in cultivation because they lack commercial importance, are new to cultivation or because trade in that species is strictly controlled by CITES (Convention on International Trade in Endangered Species) regulations. Cultivation protocols for these species are essential for their *ex situ* conservation, but may not have been established.

To put the current study into the broader context of *ex situ* conservation of orchid species, there are several aspects to be taken into consideration. The primary objective of such programmes is to cultivate plants outside their natural habitat in order to preserve them for posterity. In consequence, seedlings of *Restrepia brachypus* produced using the methodology described, have been grown successfully in flask and sent to the Plant Heritage Collection (NCCPG) of *Restrepia*, UK and distributed to members of the Pleurothallid Alliance UK. *Ex situ* conservation also aims to relieve pressure on wild populations by producing material for scientific research and for horticultural purposes. This study has provided the methodology to supply *Restrepia* material for both these purposes.

Lastly, this approach to conservation has led to ongoing initiatives to develop worldwide seed banks of orchid and other plant species for which it is particularly important to develop suitable symbiotic and asymbiotic germination techniques (Seaton, 2007). The Millennium Seed Bank Project, Kew, is the most well known of these. It initially aimed to have banked seed from 10% of the world's wild plant species by the end of 2010 and has been described as the 'largest *ex situ* conservation project ever conceived' (RBG Kew, 2013c). The Darwin Initiative (DEFRA, 2013) assists countries that are rich in biodiversity, but poor in financial resources to implement the CBD by funding collaborative projects, which draw on UK biodiversity expertise. Another

current project is the creation of orchid seed banks in their countries of origin for 'sustainable' use (Pritchard, 2007). Orchid Seed Stores for Sustainable Use (OSSSU) is a three-year UK Darwin Initiative project with the primary objective of setting up a global network of orchid seed banks, focusing initially on orchid biodiversity hotspots in Asia and Latin America (Seaton *et al.*, 2007). They initially planned to use plants under cultivation in living collections, cross-pollinating different clones where possible (Seaton *et al.*, 2007).

Reintroducing plants from such seed banks into reclaimed habitats will require expertise derived from research into cultivation techniques such as described here. This work complements the seed bank approach and can therefore make an invaluable contribution to *ex situ* conservation.



"Nature….abhors perpetual self-fertilisation."

Charles Darwin (1876)

4.1 Introduction

4.1.1 Background to the study

Self-incompatibility (SI) and inbreeding depression (ID) are known to variously influence seed set and fruit set (Richards, 1997; Neiland and Wilcock, 1998; Borba *et al.,* 2011), seed filling (Borba *et al.,* 2001b), germination and subsequent seedling development (Borba *et al.,* 2001b). Although they have been studied extensively in other angiosperm families, less is known about their influence in the Orchidaceae and nothing at all in *Restrepia.* A brief review of SI and ID with a consideration of their perceived influence in the Orchidaceae is therefore presented.

4.1.2 Self-incompatibility (SI)

Darwin (1876) concluded that plant species favoured out-breeding and that fertilisation was prevented when the 'sexual elements' were identical. Later, in 1917 Stout coined the term 'self-incompatibility' (SI) to describe this phenomenon.

De Nettancourt (1977) famously defined SI as the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination. More recently, Richards (1997) further refined this definition stating that SI is a mechanism that ensures obligate out-breeding but may carry the penalty of reproductive inefficiency. Typically SI is regarded as operating before zygote formation, preventing fertilisation from taking place and therefore affects fruit and seed formation (Richards, 1997). The term is now used for the various genetic mechanisms occurring in nearly half of angiosperm families (Travers *et al.,* 2004), which prevent self-fertilization (Nasrallah, 2000; Franklin-Tong and Franklin, 2003; Goldberg *et al.*, 2010), encourage outbreeding and prevent inbreeding (Silva and Goring, 2001; Wheeler *et al.,* 2009; Goldberg *et al.,* 2010).

The best understood mechanisms of SI act either by inhibiting the germination of pollen on the stigma, or by inhibiting the elongation of pollen tube in the style. In both cases the pollen/pollen tube is recognised by the pistil and is rejected prior to fertilisation (de Nettancourt, 1977; Cock *et al.,* 1999; Snowman *et al.,* 2000; Franklin-Tong and Franklin, 2003; Travers *et al.,* 2004). The SI response is typically controlled by one or more multi-allelic S loci (Silva and Goring, 2001; Travers *et al.,* 2004; Wheeler and Franklin-Tong, 2007; Wheeler *et al.,* 2009).

The genetic basis of SI was first established by Correns (1913) and later work by East and Mangelsdorf on *Nicotiana* in 1925 identified a recessive polyallelic, SI gene, at the S locus, which meant that plants expressing this gene would breed true. Independent work by Lundquist (1956) and Hayman (1956) identified a second Z locus in grasses, which provided an explanation of gametophytic self-incompatibility, or GSI, in Graminaceous species. Many bifactorial systems have since been identified in both monocotyledonous and dicotyledonous genera (Brewbaker, 1957; Lewis, 1979; Franklin-Tong, 2008).

Incompatible pollen recognition systems preventing self-fertilization have evolved independently several times in different lineages of Angiosperm plants (Mau *et al.,* 1991; Matton *et al.*, 1994; Charlesworth, 2006; Wheeler and Franklin-Tong, 2007; Wheeler *et al.,* 2009). Despite their similar morphological and genetic manifestations, they are based on different cellular components; therefore, each mechanism has evolved its own, unique S-genes (Charlesworth, 2006).

Pollen tube growth may be inhibited by various mechanisms in different genera (Silva and Goring, 2001). In the Solanaceae incompatible pollen tube growth is blocked by a multi-allelic RNase in the pistil. In the Papaveraceae complex cellular responses such as calcium fluxes, actin rearrangements and programmed cell death appear to occur in the

incompatible pollen tube. As a final example the Brassicaceae have a receptor kinase-signalling pathway activating in the pistil, leading to pollen rejection (Silva and Goring, 2001).

Differing SI systems are found in homomorphic and heteromorphic flowering plants (Charlesworth, 2006). The incompatibility system in homomorphic species can only be established by testing the compatibility of different individuals whereas, in heteromorphic plants, the floral morphology usually indicates the incompatibility type as in heterostylous species e.g. *Primula vulgaris* (Charlesworth, 2010).

Homomorphic flowers all have the same phenotype and self-fertilisation is avoided by utilising genetic/biochemical mechanisms. Two distinct types of self-incompatibility in homomorphic flowers are recognised – sporophytic self-incompatibility (SSI) and gametophytic self-incompatibility (GSI).

Gametophytic self-incompatibility (GSI), is more common than SSI, but is not as well understood, although it is known to occur in approximately 60 (over half) angiosperm families (Richards, 1997) compared to 6 families for SSI. These two types are not related, having evolved independently (Wheeler and Franklin-Tong, 2007; Wheeler *et al.,* 2009). In GSI, the S loci are multiallelic but the pollen incompatibility is controlled by the single S allele in the haploid pollen. The pollen will not grow on any pistil that contains the same allele. However, all pollen will germinate (incompatible and compatible) and pollen tubes will begin to grow down the style (Franklin-Tong and Franklin, 2003). The growth of incompatible pollen tubes is arrested in the style, while compatible tubes continue to grow and eventually fertilise eggs in the ovary.

Sporophytic incompatibility (SSI) has been studied extensively in members of the Brassicaceae. Rejection of pollen from the same plant is controlled by the diploid genotype of the sporophyte generation. This is under the control of the 'S-locus', which

in these species is actually a cluster of three tightly linked loci. Pollen (haploid) will not germinate on the stigma (diploid) of a flower that contains the same S allele as the pollen (Hiscock and Tabah, 2003). There may be many different S alleles within a population, as the S-locus is polymorphic. In the multiallelic 'homomorphic' SSI systems of the Brassicaceae and Asteraceae, between 30 and 40 S-alleles are typically found within natural populations (Lawrence 2000).

Travers *et al.* (2004) noted that in some species the strength of SI was influenced by environmental conditions such as temperature, by internal stylar conditions such as the age of the flowers, by mutations that directly affected the strength of the S-alleles (e.g. weak and strong S-alleles), by mutations that rendered a specific S-allele functionless, and by unlinked genetic modifiers that could affect the strength of S-alleles in the population. Their study revealed genetically and environmentally induced variation in the strength of SI in natural populations (Travers *et al.*, 2004).

There is agreement in the literature that SI has a strong influence on the breeding system, inhibits self-pollination, and allows a hermaphrodite plant to avoid the adverse effects of inbreeding (Richards, 1997; Silva and Goring, 2001; Travers *et al.*, 2004). The huge success of the angiosperm plant families has been attributed to the operation of SI (Franklin-Tong and Franklin, 2003).

4.1.3 Inbreeding depression (ID)

The harmful effects of inbreeding were first documented in detail and quantified by Charles Darwin who found that it lowered vigour and fertility (Darwin, 1876; Charlesworth and Willis, 2009). Inbreeding may be defined as the reproduction of two genetically related parents, which can increase the chances of the offspring being affected by recessive, deleterious traits. This lowers fitness-related characters, such as survival, growth rate and fertility in a population. This phenomenon is termed

inbreeding depression (Lynch, 1991; Charlesworth and Willis, 2009). Genetic fitness may be defined as the ability of an individual or population to both survive and reproduce.

The deleterious alleles which cause inbreeding depression are quickly removed or purged from a population through natural selection. Purging becomes more efficient as homozygosity increases and the deleterious alleles are increasingly exposed to selection (Wright *et al.,* 2008). In general, the higher the genetic variation within a breeding population, the less likely it is to suffer from inbreeding depression. Inbreeding depression, although present in most groups of organisms, is of more importance in hermaphrodite species. The majority of plant species are hermaphrodite and are thus susceptible to inbreeding depression as a consequence of self-pollination.

Deleterious genes arise constantly through mutation within a population and, if inbreeding occurs frequently, most offspring are likely to inherit some of these recessive deleterious traits. However, very few individuals will have more survival fitness than others as these recessive deleterious traits will be "masked" by heterozygosity. If the population size continues to decrease and inbreeding continues, these deleterious traits may no longer be masked phenotypically.

Introducing alleles from a different population, via outbreeding, can reverse inbreeding depression. Different populations of the same species may have different deleterious traits; therefore cross-breeding will result in a reduction of homozygosity in most loci and produce phenotypic heterosis (or 'hybrid vigour') in the offspring. This strategy is practised by conservation and captive breeding managers to prevent homozygosity and is known as 'outbreeding enhancement'.

Intermixing two different populations may also give rise to unfit polygenic traits via 'out-breeding depression', as offspring may lack the genetic adaptations for specific

environmental conditions. Such offspring will have lower fitness than pure-bred individuals; e.g. a particular subspecies that has adapted to its local environment (Tremblay and Otero, 2009). Inbreeding depression will not inevitably continue since deleterious alleles may be eliminated by natural selection and genetic drift.

Since inbreeding may result in a higher phenotypic expression of deleterious recessive genes, first-generation inbred individuals are more likely to show reduced levels of fitness-related traits related to survival, growth rate and fertility (Charlesworth and Willis, 2009). For plant populations, these fitness-related traits may be expressed via seed set, seed filling, germination rates, growth rates and plant size. These effects are utilised in the evaluation of inbreeding depression via biomass production (van Treuren *et al.,* 1993; Zhang *et al.,* 2004; Busch, 2005).

Heterosis, (hybrid vigour, or out-breeding enhancement), is the improved or increased function of a hybrid offspring that is genetically superior as a result of the recombination of its parents' genes. In plant breeding, inbred lines are used as stocks for the creation of hybrid lines to make use of the effects of heterosis. Heterosis is the opposite of inbreeding depression, with two contrasting explanatory hypotheses - the dominance theory which attributes the superiority of hybrids to the suppression of undesirable (deleterious) recessive alleles from one parent by dominant alleles from the other; and the overdominance theory which states that some combinations of alleles (obtained by outbreeding) are especially advantageous when paired in a heterozygous individual (Charlesworth and Willis, 2009).

4.1.4 SI and inbreeding depression in the Orchidaceae

Although SI is thought to occur in 10% of orchid species, its full extent is not known (Dressler, 1993). Many studies consider SI to be rare in the Orchidaceae (Dressler, 1993; Borba *et al.,* 2001b; Roberts, 2003; Alcantara *et al.,* 2006; Gontijo *et al.,* 2010).

However, for a family that is thought to be mainly self-compatible, a surprising number of genera have been shown to exhibit SI - *Coelogyne* (Cheng *et al.*, 2009), *Oncidium* (Charanasri and Kamemoto, 1977), *Dendrobium* (Johansen, 1990), in 30% of species in the former subfamily Vandoideae, a commercially important group (Agnew, 1986), and in the Laeliinae (Stort and de Lima Galdino, 1984). The effect of SI on orchid conservation is not fully known (Roberts, 2003) and to date few studies have considered the link between SI, conservation biology and the maintenance of *in* and *ex situ* populations.

Alongside the increased awareness of the importance of *ex situ* orchid collections and their role in conservation programmes such as the Darwin Initiative and the Millennium Seed Bank (Defra, 2013; Pritchard, 2007; RBG Kew, 2013c), the significance of the pollination biology and related self-incompatibility/compatibility systems operating within orchid species has also become evident.

To date, two studies on SI within the Pleurothallidinae have revealed the existence of weak SI systems operating in five *Pleurothallis* species (Borba *et al.,* 2002) and 'functional' SI in three *Octomeria* species (Barbosa *et al.,* 2009), both of which link SI to myophily. Other studies have identified SI in four *Anathallis* species (Gontijo *et al.,* 2010), in *Stelis argentata* (Christenen, 1992) and *Lepanthes* species (Blanco and Barbosa, 2005; Tremblay and Ackerman, 2007). *Restrepia,* another genus within the subtribe, has had neither its breeding systems, nor pollination biology studied in any detail to date. Commercially available *Restrepia* species have been propagated either vegetatively from leaf offsets, called keikis (Webb, 1985), or by repeated divisions from original collections (Millner *et al.,* 2008). This has resulted in cultivated plants whose genotype most probably still closely resembles those found in the wild, compared to orchid genera that have undergone artificial selection, line breeding or extensive inter-specific and inter-generic hybridisation. However, these cultivated populations have

been developed from very few original plants and for some of the rarer species in cultivation they may all be divisions or offsets of the original plant. So, while it is possible to use *ex situ* collections of *Restrepia* as model systems with which to conduct pollination and breeding studies (the logistics of which would be very difficult in their native habitat), great care must be taken in the subsequent use of resultant data by extrapolation to evaluate the effect(s) of self-incompatibility and inbreeding depression on already dwindling wild populations. However, such information could help inform future guidelines to conserve wild and cultivated populations of the genus.

Since *Restrepia* species readily set fruit after hand pollination they do not exhibit dichogamy. However, they do not spontaneously set fruit in the absence of pollinators, a fact commented upon by Luer (1996a), and self-set seed capsules are virtually unknown in private collections (Luer, 1996a; Howe, 2010) and as observed in the course of this study. This suggests that they are herkogamous which is in agreement with Richards (1997), who stated that the majority of orchids exhibit either herkogamy or dichogamy. Nothing is known of SI within the genus, with the exception of *R. aberrans* from Panama - an extremely rare species known to be self-compatible - which exhibits autogamy (Luer, 1996b).

From observations made over the period of this study (eight years), it became possible to construct a set of developmental criteria (Results section: Table 4-2) that was based on fitness-related traits connected to survival, growth rate and fertility. These were used to study the growth and development of seeds and seedlings from various pollinations.

4.1.5 Chapter Objectives

1. To study the breeding system of *Restrepia.*

2. To determine the presence or absence of genetic barriers to self-pollination such as self-incompatibility (SI).

3. To determine if inbreeding depression operated post fertilisation and zygote formation following self-, inter-clonal and inter-specific pollinations.

4.2 Materials and methods

4.2.1 Plant material

All the plant material used in the current study to produce inter-specific cross pollinations was obtained from private, greenhouse grown collections belonging to members of the Pleurothallid Alliance UK and the former National Collection of *Restrepia,* U.K., held by Mr Colin Howe under the Plant Heritage Scheme (Plant Heritage, 2013). All the plant material for the self-pollinations and inter-clonal pollination in *R. brachypus* came from the private collection of H. Millner and were cultivated in the conditions described previously in Chapter 2.

Only fully mature, naturally dehisced capsules were used in the study. Pollinations that failed to initiate either fruit set, capsule formation or development for most species during this study were not recorded for two reasons. Capsule set and development may be influenced firstly by environmental factors and secondly by the variable success of hand-pollination due to human error (see 4.2.1.1). This made capsule formation an unreliable criterion for most of the species studied. In addition, of the total hand pollinations performed, very few failed to set capsules when pollination had been correctly implemented. The exception to this was *R. chocoënsis,* which failed to form capsules (Plate 4-1: a, b and c) on all occasions, and this was recorded (see Tables 4-2 and 4-3).

When working with rare species of any plant genus, it is often impossible to perform the requisite number of replicate measurements or counts required for statistical analysis because of the shortage of material. However, since *Restrepia* forms hundreds of seeds per capsule (Arditti, 1992; Arditti and Ghani, 2000), the statistical evaluation of results from a comparatively small number of seed capsules is feasible (Millner *et al.,* 2008). In this part of the study it was not possible to carry out the type of crosses regarded as

standard practice in, for example, investigations on crop species. Many species of *Restrepia* (and small/young plants) may only produce one flower at a time, thus making inter-ramet pollinations impossible. To compensate for this, self-pollinations (within-flower) were performed across as many different species as possible

4.2.2 Self-pollination and inter-specific pollination within the genus

In the initial phase of the investigation, 127 within-flower self-pollinations were performed by hand. These represent 26 out of a total of 52 species in the genus and included three clones of *R. antennifera*. In addition, self-set seed from *R. aberrans*, the only species known to be self-compatible (Luer, 1996a, 1996b), was included in this section of the study, making the total number of species tested 27. This represents 53% of known species in the genus. Subsequently, 20 cross-pollinations were performed by hand in order to produce inter-specific crosses (or primary hybrids). This produced 20 different inter-specific crosses and which provided results that could be compared with the self-pollinations. For the self-pollination results the capsules were produced over a long period of time (several years) it was possible to repeat the seed filling and germination counts several times for some species as more capsules became available (see Table 4-3, column A). The final seed filling and germination results represent the mean values obtained from within-flower pollinations for a particular species over time.

4.2.2.1 Hand pollination of Restrepia *flowers*

Due to the small size of the floral parts of *Restrepia* species, hand pollinations required great care. The entire procedure was most successful when performed under an illuminated magnifying viewer. The anther cap containing the pollinia was first removed using a long pin to gently prize it free, as the traditionally used wooden tooth pick would have been too large. At this point, the pollinia were usually attached via the viscidium to the point of the pin. Then, the pollinia (still within the anther cap) were

carefully removed from the pin onto a piece of paper. The anther cap was then separated from the pollinia using the point of the pin. This proved to be the most difficult part of the operation, as the pollinia would often 'ping' away and be lost. Once the anther cap and pollinia were separated, the pollinia were then picked up on the point of the pin and placed carefully onto the stigmatic surface (located on the ventral side of the column) of the flower intended as the seed (female) parent. The pollinia readily attached to the stigmatic surface which confirmed correct placement. If they failed to attach, then either the placement of the pollinia had been wrong, or the receptor flower was too old. All flowers in this study were hand-pollinated within 2 days of anthesis as this ensured maximum capsule formation.

Once the seed capsules were approaching dehiscence (Plate 4-1 (b)), they were inspected daily and seeds were collected just as the capsules began to dehisce (Plate 4-1 (c)). Capsules had been previously found to dehisce from 70 days onwards post-pollination, depending on species and environmental conditions, with the majority dehiscing after 85 days (Howe, 2006). Dehiscence is often preceded by the capsules changing colour, becoming yellow or darkening and occasionally becoming slightly ribbed in appearance. However, these signs did not always occur and often the seeds were lost if the capsules dehisced unexpectedly.

Seeds were sown within 24 hours of collection to ensure that storage had a minimal effect on the germination results. The Western medium (W) used for this experiment had previously been shown to support effective asymbiotic, axenic seed germination for *Restrepia* species (Millner *et al.,* 2008). The medium was autoclaved for 20 minutes at 120^{o}C and four 30 ml replicates per pollination were prepared in 90mm diameter, sterile, plastic Petri dishes.

Seeds were surface sterilized in 0.5% v/v sodium hypochlorite solution for 10 minutes and then allowed to settle. This solution was decanted in a LAF and the seeds washed twice in SDW. They were resuspended in 5ml SDW and spread evenly across the prepared Western media. All seeds were incubated in an illuminated growth chamber at 21^{o}C with a 16 hour day length.

Visual estimates of seed filling were made immediately after sowing using a dissecting microscope with a count of >150 seeds per plate sampled. At this stage the embryos were clearly visible, which gave more accurate initial embryo counts than by using dry seed.

Since *Restrepia* seeds do not display synchronous axenic germination (Millner *et al.*, 2008), germination rates were recorded every week for five weeks. The numbers of empty seeds (Plate 4-1 (d)), filled seeds (Plate 4-1 (e)) and germinated seeds (Plate 4-1 (f)) were recorded. Germination was assumed to have occurred when the developing embryos had split their testae. Final, full germination rates were calculated for all replicates of each medium, each week. Seed filling ratios (SFR self) for all the self-pollinations were calculated using the formula:

$$\text{SFR self} = \frac{\text{average \% filling} \times \text{self}}{\text{average \% filling all outcrosses}} \qquad \textbf{Equation 1}$$

Where 'all outcrosses' represent all the primary hybrids for the same species

A previous study of axenic seed germination and seedling development (Millner *et al.,* 2008) made it possible to devise a set of developmental criteria (Results section: Table 4-2). Each self-pollination, intra-specific and inter-specific cross was then given a cumulative developmental score based upon these criteria.

4.2.3 Self-pollination and inter-clonal pollination in *R. brachypus*

In order to compare intra-specific pollinations and self-pollinations of clones within a single species, *R. brachypus* was selected (Introduction Plates 1-5 to 1-8 and Plate 4-1). *R. brachypus* was chosen as the model species for these experiments as it is a commonly cultivated species in the UK, several clones of which were readily available. The seeds used in this part of the investigation were harvested from capsules produced by hand cross-pollination of three clones of *R. brachypus,* designated Clones 1, 2 and 3 (Table 4-1). Each clone was self- pollinated and also cross-pollinated with each of the other clones.

Table 4-1: Pollinations performed between clones of *R. brachypus*

	Clone 1	**Clone 2**	**Clone 3**
Clone 1	Clone1 × 1	Clone 1 × 2	Clone 1 × 3
Clone 2	Clone 2 × 1	Clone 2 × 2	Clone 2 × 3
Clone 3	Clone 3 × 1	Clone 3 × 2	Clone 3 × 3

Key:
- Self-pollinations
- Reciprocal crosses (i)
- Reciprocal crosses (ii)

Note:
In each pollination (above), the seed parent or pollen receiver (female) is first and the pollen parent or pollen donor (male) is second. E.g. Clone 1 × 2; Clone 1 is the seed or female parent or pollen receiver and Clone 2 the pollen parent or pollen donor.

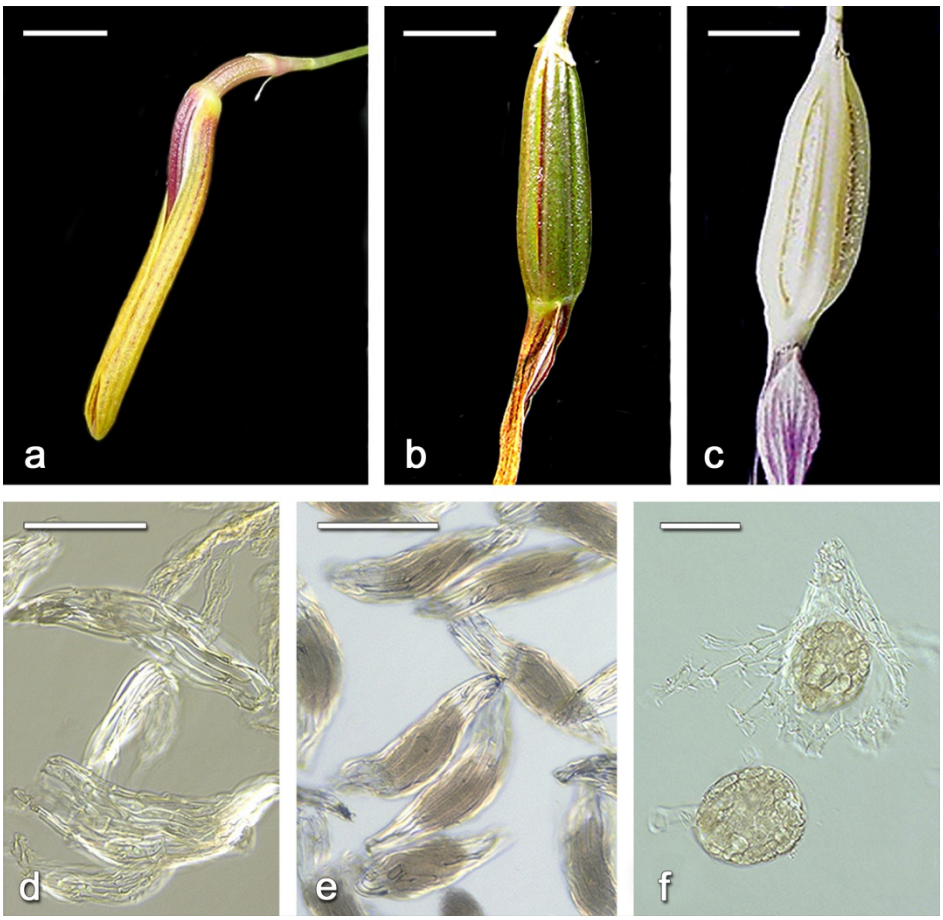


Plate 4-1: *R. brachypus* capsule development and seed filling

(a), (b) and (c) capsule development;

(a) flower one day after pollination, (b) capsule prior to dehiscence, (c) seed capsule about to dehisce. Internal scale bars represent 5mm.

(d), (e) and (f) seed filling;

(d) empty seeds, no embryos, (e) filled seeds containing embryos, (f) germinated seeds. Here, twin embryo seed has burst its testa. Internal scale bars represent 50µm.

Visual estimates of seed filling were made using the same methodology as described previously. The numbers of empty seeds (Plate 4-1 (d)), filled seeds (Plate 4-1 (e)) and germinated seeds (Plate 4-1 (f)) were recorded each week for five consecutive weeks. Final full germination rates were calculated each week for all replicates. Seed filling ratios (SFR self) were calculated for the self-pollinations using Equation 1.

Seed filling ratios (SFR hybrids) between the crosses were calculated by the formula:

$$\text{SFR hybrids} = \frac{\text{average \% filling outcross 1}}{\text{average \% filling all outcrosses}} \qquad \textbf{Equation 2}$$

Where 'outcross 1' represents any outcross for a particular species and 'all outcrosses' represent all the other primary hybrids for the same species.

Using the set of development criteria (Results section: Table 4-2) each self-pollination and inter-clonal cross was also given a cumulative developmental score.

Statistical analyses (mixed model one-way ANOVA) to compare the percentage germination rates of self-pollinations and reciprocal crosses (i) and (ii) were performed on SPSS for total embryos and for filled embryos. The Kolmogorov-Smirnov test and Levene's test were used to test for normality and homogeneity of variances respectively.

4.2.3 Examination of pollen tube growth

Pollen tube growth was visualised using a modification of the procedure of Stoddard (1986) and Chen (2006). *R. brachypus* flowers, one day post anthesis, were hand pollinated. Self-pollinations and cross-pollinations with *R. purpurea* were carried out. The flowers were subsequently harvested at intervals from 1 day to 3 weeks. The column and ovary were dissected out from each flower and then placed in vials containing 70% (aq.) ethanol. They were then immediately suspended in a boiling water

bath for 45 seconds, just long enough to bring the alcohol to the boil. This prevented discolouration of the tissue. The vials were then drained and filled with 80% (aq.) lactic acid. They were again suspended in the boiling water bath for 15 seconds and then allowed to cool for 1 minute. The lactic acid was drained and the tissues were rinsed twice in tap water and once more with distilled water and left overnight to soak. The vials were drained and filled with 0.1M K_3PO_4, for a minimum of 12 hours to raise the pH, and drained again. The tissues were then covered with 0.2% aniline blue (water soluble; batch 72270, Riedel-di Haën Laborchemifalien GmbH & Co KG, Germany) in 0.1 M K_2HPO_4 for a minimum of 1 day. The timings proved to have a fair degree of latitude and successful results were obtained even when the timings were not optimal.

The ovary and column were dissected (Figure 4-1) and the stylar tissue placed onto a slide; a cover slip was mounted with glycerol and a squash of the tissue was made. The ovary was dissected longitudinally into two or three sections and the same procedure performed. The tissue was then examined using a Nikon ME600 Eclipse fluorescence microscope (Nikon Corporation, Japan). The positive reaction to aniline blue by callose in the walls and plugs of pollen tubes and in the sieve plates of phloem was indicated by yellow-green fluorescence.

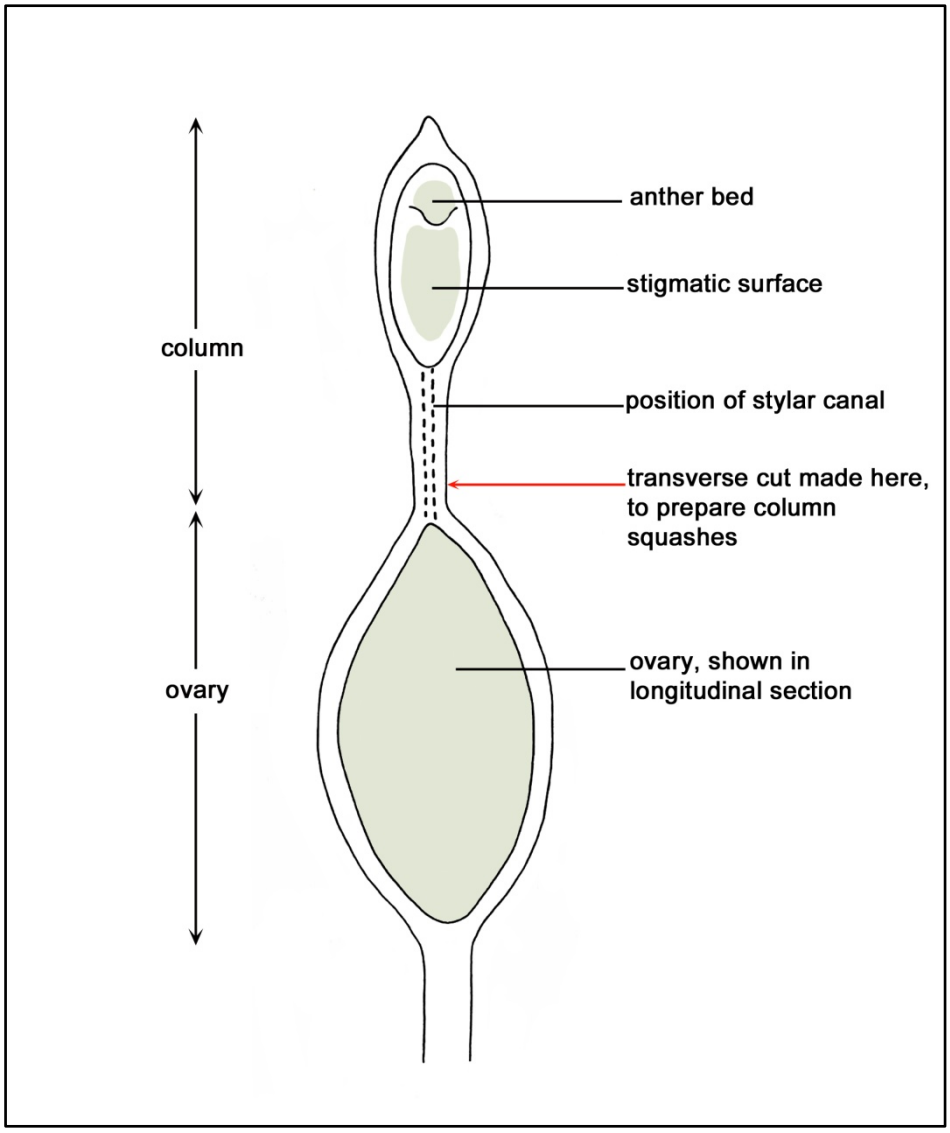


Figure 4-1: Diagrammatic representation of the column and ovary in *Restrepia*, showing position of transverse cut made when preparing the tissue squashes.

4.3 Results

4.3.1 Development criteria scores

The development criteria scores are presented in Table 4-2. These scores represent the variations in seed set, germination and subsequent growth and development that were observed over the course of the current study (2003-2011).

Table 4-2: Developmental criteria scores

Capsule and seed set	S[1]	Germination[2]	S	Subsequent growth	S
Capsule fails to set	**0**	Germination <1%	**5**	Protocorms fail to thrive	**8**
Capsule sets, no seed formed	**1**	Germination 1-30%	**6**	Plantlets do not thrive	**9**
Capsule sets with empty seed	**2**	Germination >30%	**7**	Slow growing, weak plantlets	**10**
Capsule sets: <20% filled seed	**3**				
				Normal plantlets	**11**
Capsule sets: >20% filled seed	**4**				

Notes:

The criteria were based on fitness-related traits connected to survival, growth rate and fertility. The developmental scores represent critical stages in pre- and post-fertilisation development that can be influenced by self-incompatibility (SI), cross-incompatibility (CI) and inbreeding depression (ID). The seeds/plantlets were scored at the highest stage they reached in all sections, producing a cumulative developmental score.

[1]Cumulative score. The score represents the developmental stage that was reached.

[2]Germination is % of total seed count.

The cumulative scores may be interpreted as follows:

Cumulative score < 4 indicates incompatibility, either SI or CI.

Cumulative score = 4 indicates no incompatibility but post-fertilisation effects due to ID then prevent seeds developing further.

Cumulative score > 4 but <11 indicates that inbreeding depression is in operation affecting fitness-related traits. Cumulative score = 11 indicates no inbreeding depression.

4.3.2 Self-pollination and inter-specific pollination within the genus

The combined developmental criteria scores for 27 self-pollinated species are presented in Table 4-3, of which 25 species have a cumulative score ≤4 (Table 4-2). Of these, only three species (*R. dodsonii, R. mohrii and R. muscifera*) demonstrated any germination (score = 1; <1%), but the resultant protocorms failed to develop. Only two species (*R. aberrans* and *R. schizosepala*) scored 4 for seed formation. This suggests that *R. aberrans* and *R. schizosepala* do not exhibit SI, since by definition SI operates prior to fertilisation. *R. trichoglossa* scored 3 for seed formation with a cumulative score of 9; this suggests ID in this species, as expressed by subsequent very slow growing plantlets failing to mature. It is worth noting that no self-pollinated species seedlings were ever raised beyond the *in vitro* stage during the entire period that this study was running. This is in sharp contrast to the successful inter-specific and intra-specific hybrids raised - see section 4.3.4. In all cases the methodology employed was identical.

The comparison between developmental scoring for self-pollinated species and their inter-specific crosses is presented in Tables 4-4 and 4-5. These results show a pattern of low cumulative scores for self-pollinated species compared to high cumulative scores for inter-specific cross-pollinations. One explanation for this is possible high levels of SI within the species studied and low levels of ID in inter-specific hybrids (primary hybrids), due to heterosis.

Table 4-3: The developmental stages for self-pollinated species

		Capsule and seed set				Germination and subsequent growth							
Score:	N[5]	1	2	3	4	5	6	7	8	9	10	11	CS[6]
Species													
R. chocoënsis[1]	7												0
R. antennifera (gigantea)	2	*	*										2
R. aristulifera	4	*	*										2
R. brachypus	12	*	*										2
R. citrina	2	*	*										2
R. condorensis	4	*	*										2
R. contorta	6	*	*										2
R. howei	2	*	*										2
R. mendozae	6	*	*										2
R. antennifera	4	*	*	*									3
R. antennifera (hemslyana)	4	*	*	*									3
R. cloesii	2	*	*	*									3
R. cuprea	6	*	*	*									3
R. elegans	6	*	*	*									3
R. falkenbergii	2	*	*	*									3
R. guttulata	8	*	*	*									3
R. iris	4	*	*	*									3
R. purpurea	6	*	*	*									3
R. sanguinea	6	*	*	*									3
R. seketii	6	*	*	*									3
R. vasquezii	4	*	*	*									3
R. wageneri	2	*	*	*									3
R. dodsonii	6	*	*	*		*							4
R. mohrii	4	*	*	*		*							4
R. muscifera	4	*	*	*		*							4
R. schizosepala[2]	2	*	*	*	*	*	*	*	*				8
R. trichoglossa[3]	4	*	*	*		*	*	*	*	*	*		9
R. aberrans[4]	2	*	*	*	*	*	*	*	*	*	*	*	11

Notes:

The stages completed by each species are indicated by *. Species have been sorted by cumulative score (CS). Total number of self-pollinations performed were 127, representing 26/52 species (Luer, 1996a) together with 3 clones of *R. antennifera.* The capsules were produced over a period of several years and the seed set and germination scores are the mean results for that species.

[1]*R. chocoënsis* scored 0 as it failed to set capsules.

[2,3]*R. schizosepala* and *trichoglossa* both scored highly, compared to other species.

[4]*R. aberrans* is the only species known to self-pollinate successfully and had the highest cumulative score, as its seedlings developed normally.

N[5] number of capsules harvested, CS[6] cumulative score

Table 4-4: Seed filling ratios for species and their primary hybrids

Crosses	a[1]	b[2]	c[3]	Capsule and seed filling				Score[4]	SFR 'selfs'	SFR hybrids
				1	2	3	4			
R. aristulifera × self	1	0	0	*	*			2	0.01	
R. aristulifera × *R. chameleon*	94	64	60	*	*	*	*	4		1.09
R. aristulifera × *R. citrina*	80	62	50	*	*	*	*	4		0.86
R. aristulifera × *R. guttulata*	93	65	61	*	*	*	*	4		1.07
R. mendozae × self	1	0	0	*	*			2	0.01	
R. mendozae × *R. brachypus*	87	51	44	*	*	*	*	4		0.96
R. mendozae × *R. dodsonii*	84	48	40	*	*	*	*	4		0.91
R. mendozae × *R. guttulata*	98	61	60	*	*	*	*	4		1.15
R. sanguinea × self	2	0	0	*	*	*		3	0.02	
R. sanguinea × *R. chameleon*	80	35	28	*	*	*	*	4		0.85
R. sanguinea × *R. Matthew Howe*	96	67	64	*	*	*	*	4		1.12
R. sanguinea × *R. antennifera*	92	63	58	*	*	*	*	4		1.05
R. cuprea × self	1	0	0	*	*	*		3	0.01	
R. cuprea × *R. sanguinea*	95	59	56	*	*	*	*	4		1.08
R. cuprea × *R. citrina*	96	34	33	*	*	*	*	4		1.10
R. cuprea × *R. guttulata*	80	45	36	*	*	*	*	4		0.84
R. condorensis × self	1	0	0	*	*			2	0.01	
R. condorensis × *R. lansbergii*	95	42	40	*	*	*	*	4		
R. guttulata × self	1	0	0	*	*	*		3	0.01	
R. guttulata × *R. Matthew Howe*	87	45	39	*	*	*	*	4		
R. muscifera × self	2	45	1	*	*	*		3	0.10	
R. muscifera × *R. chameleon*	20	29	6	*	*	*	*	4		
R. falkenbergii × self	1	0	0	*	*			2	0.01	
R. falkenbergii × *R. cloesii*	96	68	65	*	*	*	*	4		
R. antennifera × self	1	0	0						0.01	
R. antennifera × *R. citrina*	97	56	54	*	*	*	*	4		1.05
R. antennifera × *R. chameleon*	92	31	29	*	*	*	*	4		0.95
R. brachypus × self (Clone 1)	0	0	0	*	*			2	0.00	
R. brachypus × self (Clone 2)	54	32	17	*	*	*	*	4	0.62	
R. brachypus × self (Clone 3*)*	1	0	0	*	*			2	0.01	
R. brachypus × *citrina*	89	45	40	*	*	*	*	4		1.03
R. brachypus × *purpurea*	86	55	47	*	*	*	*	4		0.97

Notes to Table 4-4:

Developmental stage scores are presented for self-pollinations of 10 species representing 20% of the genus, plus 20 interspecific crosses. The stages completed by each cross are indicated by an asterisk *.

Capsule and seed filling effects ranged from capsule setting with empty testae, to capsule setting with >20% filled seed, producing scores from 2 - 4. Capsule failure could not be estimated, with the exception of *R. chocoënsis,* which failed on every occasion to set a capsule from self-pollination.

[1] % filled seeds. Where % filling was <1% it was rounded to 1% (see Tables 4-5 and 4-6, for comparison with *R. brachypus* results).

[2] Germination of filled seeds (%) [3]Germination of total seed count (%) When seed filling % is high, these two germination rates are similar, but if seed filling % is low, then the two germination rates are very different, it is therefore important to distinguish between the two when quoting germination rates.

[4] Score for capsule and seed filling results

[5] Seed filling ratios (SFR) for self-pollination (selfs), calculated using Equation 1

If seed filling % is similar for self-pollinations and cross-pollinations for a species, then the SFR 'self' value should be approximately 1. However, low seed filling % values for self-pollinations compared to cross-pollinations will produce low SFR 'self' value s. Comparing SFR 'self' values is useful when comparing differing values of seed filling percentages resulting from self-pollinations.

[6] SFR hybrids – seed filling ratio of hybrids for any one species, calculated using Equation 2.

If seed filling % is similar for all the outcrosses of a species then the SFR hybrid value should be approximately 1. However, if one outcross produces a higher seed filling % than other outcrosses, the SFR hybrid value will be >1 and correspondingly if one outcross produces a lower seed filling % than the other outcrosses, the SFR hybrid value will be <1.

Table 4-5: Developmental stages for species compared to interspecific crosses (primary hybrids)

Crosses	Capsule and seed set				Germination and subsequent growth							Cumulative score[8]
	1	2	3	4	5	6	7	8	9	10	11	
R. aristulifera × self	*	*										2
R. aristulifera × *R. chameleon*	*	*	*	*	*	*	*	*	*	*	*	11
R. aristulifera × *R. citrina*	*	*	*	*	*	*	*	*	*	*	*	11
R. aristulifera × *R. guttulata*	*	*	*	*	*	*	*	*	*	*	*	11
R. mendozae × self	*	*										2
R. mendozae × *R. brachypus*	*	*	*	*	*	*	*	*	*	*	*	11
R. mendozae × *R. dodsonii*	*	*	*	*	*	*	*	*	*	*	*	11
R. mendozae × *R. guttulata*	*	*	*	*	*	*	*	*	*	*	*	11
R. sanguinea × self	*	*	*									3
R. sanguinea × *R chameleon*	*	*	*	*	*	*	*	*	*	*	*	11
R. sanguinea × *R. Matthew Howe*	*	*	*	*	*	*	*	*	*	*	*	11
R. sanguinea × *R. antennifera*	*	*	*	*	*	*	*	*	*	*	*	11
R. cuprea × self	*	*	*									3
R. cuprea × *R. sanguinea*	*	*	*	*	*	*	*	*	*	*	*	11
R. cuprea × *R. citrina*	*	*	*	*	*	*	*	*	*	*	*	11
R. cuprea × *R. guttulata*	*	*	*	*	*	*	*	*	*	*	*	11
R. condorensis × self	*	*										2
R. condorensis × *R. lansbergii*	*	*	*	*	*	*	*	*	*	*	*	11
R. guttulata × self	*	*	*									3
R. guttulata × *R. Matthew Howe*	*	*	*	*	*	*	*	*	*	*	*	11
R. muscifera × self	*	*	*	*								4
R. muscifera × *R. chameleon*	*	*	*	*	*	*	*	*	*	*	*	11
R. falkenbergii × self	*	*										2
R. falkenbergii × *R. cloesii*	*	*	*	*	*	*	*	*	*	*	*	11
R. antennifera × self	*	*										2
R. antennifera × *R. citrina*	*	*	*	*	*	*	*	*	*	*	*	11
R. antennifera × *R. chameleon*	*	*	*	*	*	*	*	*	*	*	*	11
R. brachypus × self (Clone 1)	*	*	*									3
R. brachypus × self (Clone 2)	*	*	*	*	*	*	*	*	*			9
R. brachypus × self (Clone 3*)*	*	*	*									3
R. brachypus × *citrina*	*	*	*	*	*	*	*	*	*	*	*	11
R. brachypus × *purpurea*	*	*	*	*	*	*	*	*	*	*	*	11
R. chocoënsis												0
R. chocoënsis × *chameleon*	*	*	*	*	*	*	*	*	*			9
R. chocoënsis × Matthew Howe	*	*	*	*	*	*	*	*	*	*		10
R. aberrans × self	*	*	*	*	*	*	*	*	*	*	*	11

Notes to Table 4-5:

In total, 56% of the genus was tested in this way (Table 4-3).

Germination and subsequent growth effects ranged from germination >1% (score = 5) to normal vigorous plantlets (score = 11). These effects are attributable to ID and not SI or CI (Richards, 1997).

4.3.2.1 New Restrepia hybrids

The inter-specific crosses performed in the course of this study (Section: 4.3.2 and Table 4-5) resulted in the production of new *Restrepia* hybrid seedlings, some of which have subsequently reached maturity. The *Restrepia* hybrids that have now flowered and been registered on the International Orchid Register (RHS, 2013) are presented in Table 4-6.

Notes to Table 4-6:

Before 2005, there was little primary hybridisation between species carried out. Hybrids registered by C. Howe are the ones which provided some of the data for this study. New hybrids may only be registered on the RHS orchid data base after their initial flowering; this means that hybrids produced later in the course of this study have not been registered yet, as they have not flowered at the current time. In order to register a new hybrid, flowering must have taken place, and a detailed photograph has to be submitted to the society.

The International Orchid Register (RHS, 2013) is searchable online and is a continuation of the original work started in 1906 by Henry Sander, in which a record of all orchid hybrids was kept (Sander, 1906). New hybrid additions are published bi-monthly in the Orchid Review (UK), Orchids (AOS publication, USA) and online.

[1] The International Orchid Register (RHS, 2013)

[2] *Restrepia hemselyana* is now regarded as a synonym for *Restrepia antennifera.*

[3] The only intergeneric hybrid recorded for this genus. These plants are not available.

[4,5]These are the first second generation hybrids using *Restrepia* 'Matthew Howe' as one parent.

[6] Registration pending

Table 4-6: Hybridisation using *Restrepia species* as recorded on the RHS International Orchid register[1].

Hybrid epithet	Seed parent	Pollen parent	Registrant	Originator	Date of registration
Eleana	*R. hemsleyana*[2]	*R. elegans*	Jerry Matthews	Jerry Matthews	01/01/1984
Tattoo	*R. antennifera*	*R. guttulata*	C. Withner	C. Withner	17/04/1990
Samantha[3]	*Myoxanthus serripetalus*	*R. falkenbergii*	Hoosier	W. Kilikunas	18/08/2003
Frank Feysa	*R. sanguinea*	*R. guttulata*	M. Ferrusi	F. Feysa	05/02/2004
Sangflosc	*R. sanguinea*	*R. flosculata*	E.S. Eyre	E.S. Eyre	22/06/2004
Coup D'Etat	*R. cuprea*	*R. dodsonii*	Trop. O. Farm	Trop. O. Farm	01/02/2005
Matthew Howe	*R. cuprea*	*R. chameleon*	C. Howe	C. Howe	19/09/2005
Beryl	*R. schizosepala*	*R. tabeae*	C. Howe	D. Read	17/07/2007
Chloe Howe	*R. antennifera*	*R. condorensis*	C. Howe	C. Howe	24/08/2007
Mary Smallman	*R. brachypus*	*R. antennifera*	C. Howe	C. Howe	11/09/2007
Orange Pixie	*R. flosculata*	*R. tabeae*	C. Howe	C. Howe	15/10/2008
Alice Howe	*R. contorta*	*R. chameleon*	C. Howe	C. Howe	19/12/2008
Bjorn	*R. jesupiana*	*R. flosculata*	C. Howe	C. Howe	15/04/2009
Strawberry Pixie	*R. lansbergii*	*R. condorensis*	C. Howe	C. Howe	15/04/2009
Megan Amy	*R. cuprea*	*R. sanguinea*	C. Howe	C. Howe	21/09/2009
Carole Howe	*R. aristulifera*	*R. guttulata*	C. Howe	C. Howe	09/11/2009
Gwenie	*R. renzii*	*R. chameleon*	C. Howe	C. Howe	09/11/2009
Julia Howe	*R. cuprea*	*R. guttulata*	C. Howe	C. Howe	09/11/2009
Karen Howe[4]	Matthew Howe	*R. guttulata*	C. Howe	C. Howe	09/11/2009
Citari	*R. aristulifera*	*R. citrina*	C. Howe	C. Howe	23/11/2009
Golden Pixie	*R. citrina*	*R. cuprea*	C. Howe	C. Howe	23/11/2009
Sarah Ruth[5]	*R. sanguinea*	Matthew Howe	C. Howe	C. Howe	27/11/2009
Alan F Garner	*R. brachypus*	*R. trichoglossa*	P.F. Garner	O/U	01/04/2011
Helen Millner[6]	*R. pelyx*	*R. schizosepala*	C. Howe	C. Howe	01/07/2011
Stephanie[6]	*R. mendozae*	*R. guttulata*	H. Millner	H. Millner	02/08/2011

4.3.3 Self-pollination and inter-clonal pollination in *R. brachypus*

The combined developmental criteria scores for self-pollinations and intra-specific crosses of *R. brachypus* are presented in Tables 4-7 and 4-8.

4.3.3.1 Seed filling

In all cases, self-pollinations produced less seed filling than cross-pollinations. Clone 1 self-pollination produced no filling, compared to 98% (Cross 1 × 2) and 76% (1 × 3) for the cross-pollinations (Table 4-7). Clone 3 self-pollination produced 1% filling compared to 44% (3 × 1) and 72% (3 × 2) for the cross-pollinations. Although Clone 2 self-pollination produced 54% filling, much higher than the other self-pollinations, its cross-pollination rates were correspondingly higher at 98% (2 × 1) and 100% (2 × 3). Cross 3 × 2 produced 72% filling, but Cross 3 × 1 produced a lower filling of 44% indicating that Clone 3 and Clone 1 are less cross compatible than Clones 3 and 2. These results suggest SI in two clones, (Clone 1 and Clone 3) because of the low seed filling rates found, with apparent semi-incompatibility or 'leaky' self-incompatibility in Clone 2, which produced a higher seed filling rate. There may be CI between Clone 3 and Clone 1, which had a lower seed filling rate. The seed filling ratios for self-pollinations, intra-specific pollinations and inter-specific pollinations of *R. brachypus* are presented in Table 4-7.

Table 4-7: Seed filling ratios for self-pollinations, intra-specific pollinations and inter-specific pollinations of *R. brachypus*

Crosses	a[1]	b[2]	c[3]	Capsule and seed set				Score[4]	SFR 'Selfs'[5]	SFR Hybrids[6]
				1	2	3	4			
Clone 1 × Clone 1	1	0	0	*	*			2	0.00	
Clone 1 × Clone 2	98	59	57	*	*	*	*			1.28
Clone 1 × Clone 3	76	63	55	*	*	*	*	4		0.78
Clone 2 × Clone 2	54	32	17	*	*	*	*	4	0.54	
Clone 2 × Clone 1	98	85	82	*	*	*	*	4		0.98
Clone 2 × Clone 3	100	0	0	*	*	*	*	4		1.02
Clone 3 × Clone 3	1	0	0	*	*			2	0.01	
Clone 3 × Clone 1	44	63	62	*	*	*	*	4		0.61
Clone 3 × Clone 2	72	10	7	*	*	*	*	4		1.63
R. brachypus × *citrina*	89	45	40	*	*	*	*	4		1.03
R. brachypus × *purpurea*	86	55	47	*	*	*	*	4		0.99

Notes:

The stages completed by each cross reached are indicated by *. Two interspecific crosses, *R. brachypus* × *citrina* and *R. brachypus* × *purpurea*, are included for comparison.

[1] % of filled seeds,

[2] % germination of filled seeds

[3] % germination of the total seed count

[4] Score for capsule and seed filling effects

[5] SFR 'selfs' seed filling ratios for self-pollination

[6] SFR hybrids - seed filling ratio of hybrids for any one species

Table 4-8: Germination and subsequent growth developmental score for self-pollinations, intra-specific pollinations and inter-specific pollinations of *R. brachypus*

	Germination and subsequent growth							
Crosses	5	6	7	8	9	10	11	Cumulative score[1]
Clone 1 × Clone 1								2
Clone 1 × Clone 2	*	*	*	*	*	*	*	11
Clone 1 × Clone 3	*	*	*	*	*	*	*	11
Clone 2 × Clone 2	*	*	...[2]	*	*			8
Clone 2 × Clone 1	*	*	*	*	*	*	*	10
Clone 2 × Clone 3								4
Clone 3 × Clone 3								2
Clone 3 × Clone 1	*	*	*	*	*	*	*	11
Clone 3 × Clone 2	*	*	...[2]	*	*	*		9
R. brachypus × *citrina*	*	*	*	*	*	*	*	11
R. brachypus × *purpurea*	*	*	*	*	*	*	*	11

Notes:

[1] Cumulative score for all developmental stages reached

[2] Germination < 30%, Clone 3 × Clone 2 did not achieve this score, although the remaining protocorms continued to develop and completed further stages.

4.3.3.2 Seed filling ratios of self-pollinations (SFR self)

For Clones 1 and 3 the ratio of the self-pollination to cross-pollinations. SFR was <0.01, indicating the possibility of SI in these clones due to the low seed filling (Table 4-6). However, SFR Clone 2 is equal to 0.54, indicating increased seed filling and reduced or leaky 'SI'. Thus, comparing 'SFR self' values is useful when comparing differing seed filling percentages resulting from self-pollinations.

4.3.3.3 Seed filling ratios of hybrids (SFR hybrids)

The expected value if all outcrosses are equally viable is ~1 (Table 4-7). However, slight variations, especially in the reciprocal crosses can be shown by comparing these values. Clones 2 × 1, 2 × 3 and outcrosses with *R. citrina* and *R. purpurea* all produced values close to 1, showing that these crosses were all equally viable. This indicates increased viability and improvement in fitness-related traits equal to the out-cross (inter-specific) values, which are likely to be increased by heterosis. The reciprocal crosses 1 × 3 and 3 × 1 have lower values of 0.78 and 0.61 respectively, indicating that these clones are less compatible.

4.3.3.4 Germination rates

Incompatibility effects (both SI and CI) are apparent in percentage seed filling (Table 4-7), while percentage germination rates and subsequent seedling development are indicators of ID (Richards, 1997). Clone 1, when crossed with Clones 2 and 3, produced the highest germination rates (85% and 63% respectively for germination of filled seeds). Clone 3 produced a high germination rate when crossed with Clone 1, although not with Clone 2 (63% and 10% respectively for germination of filled seeds). This nevertheless suggests decreased ID/increased heterosis with intra-specific pollinations. Clone 2 produced anomalous results, with a higher germination rate for self-pollinations than in crosses with Clone 3 (32% and 0% respectively for germination of filled seeds). The self-pollination produced lower percentage seed filling than in

intra-specific pollinations, suggesting 'leaky' SI. However, Clone 2 × 3 produced high filling (100%), indicating no cross-incompatibility, but these seeds did not germinate, which indicates ID.

Since these results seemed anomalous, the pollinations for Cross 2 × 3 and its reciprocal Cross 3 × 2 were repeated using the same *R. brachypus* clones. However, similar results were obtained, which supported the validity of the original findings. There was no direct correlation between seed filling ratios and subsequent germination rates (see Table 4-8).

Lower germination rates might be expected for Crosses 1 × 3 and 3 × 1 as they had lower seed filling. However germination rates were 63% for both crosses (germination of filled seeds) compared to 59% for 1 × 2, 10% for 3 × 2 and 0% for 2 × 3. The highest germination rate of filled seed was 85% for 2 × 1. The lowest germination rate was 0% for 2 × 3, which also had the highest value for seed filling (100%).

The reciprocal crosses 1 × 3 and 3 × 1 (SFR hybrid = 0.78 and 0.61) show less compatibility than the reciprocal crosses 1 × 2 and 2 × 1 (SFR hybrid = 1.63 and 1.28), with only small differences occurring between the reciprocal crosses. The reciprocal crosses 2 × 3 and 3 × 2 both indicated compatibility with high seed filling rates, but produced very little germination (0% and 10%). This cannot be explained by CI, which like SI would affect pre-fertilisation, but may be attributable to ID between the clones. All other germination rates for the inter-clonal crosses are high, indicating reduced ID.

One-way ANOVA (mixed model with 'plate' as the random factor) to compare the mean percentage germination rates of all self-pollinated clones (Table 4-1) with all those for both types of reciprocal cross (i) and (ii) showed highly significant differences. For filled embryos only: F (2, 18) = 1213.8, p = 0.000 and means were 9.90a, 40.56a, b and 52.71b for selfs and reciprocal crosses (i) and (ii) respectively. For total germination rates: F (2, 18) = 529.2, p = 0.000 and means were 5.84a, 37.25a, b

and 50.48b. In each analysis, means with the same letter (a) or (b) were not significantly different from each other, indicating that overall germination rates for self-pollinated clones were significantly smaller than those for the reciprocal ii crosses but not from those for the reciprocal i crosses.

4.3.4 Pollen tube growth and development

Photographs taken of the different stages of pollen tube growth in self- and cross-pollinations are shown in Plates 4-2 to 4-7 and a diagrammatic representation of these is presented in Figure 4-2. A considerable difference was observed between the growth of pollen tubes following self- and cross-pollinations. There were no pollen tubes observed in the stylar tissue of self-pollinations after 24 hours; and after nine days no pollen tubes had penetrated into the ovary. After three weeks, the pollen tubes from self-pollinations had only grown into the upper third of the ovary and exhibited a haphazard and irregular growth pattern. In contrast, pollen tubes were found in the stylar tissue of cross-pollinations after 24 hours and after nine days they had grown into the top of the ovary and into the ovules. After three weeks they had grown fully along the length of the ovary and displayed a more regular growth pattern than that observed following self-pollinations.

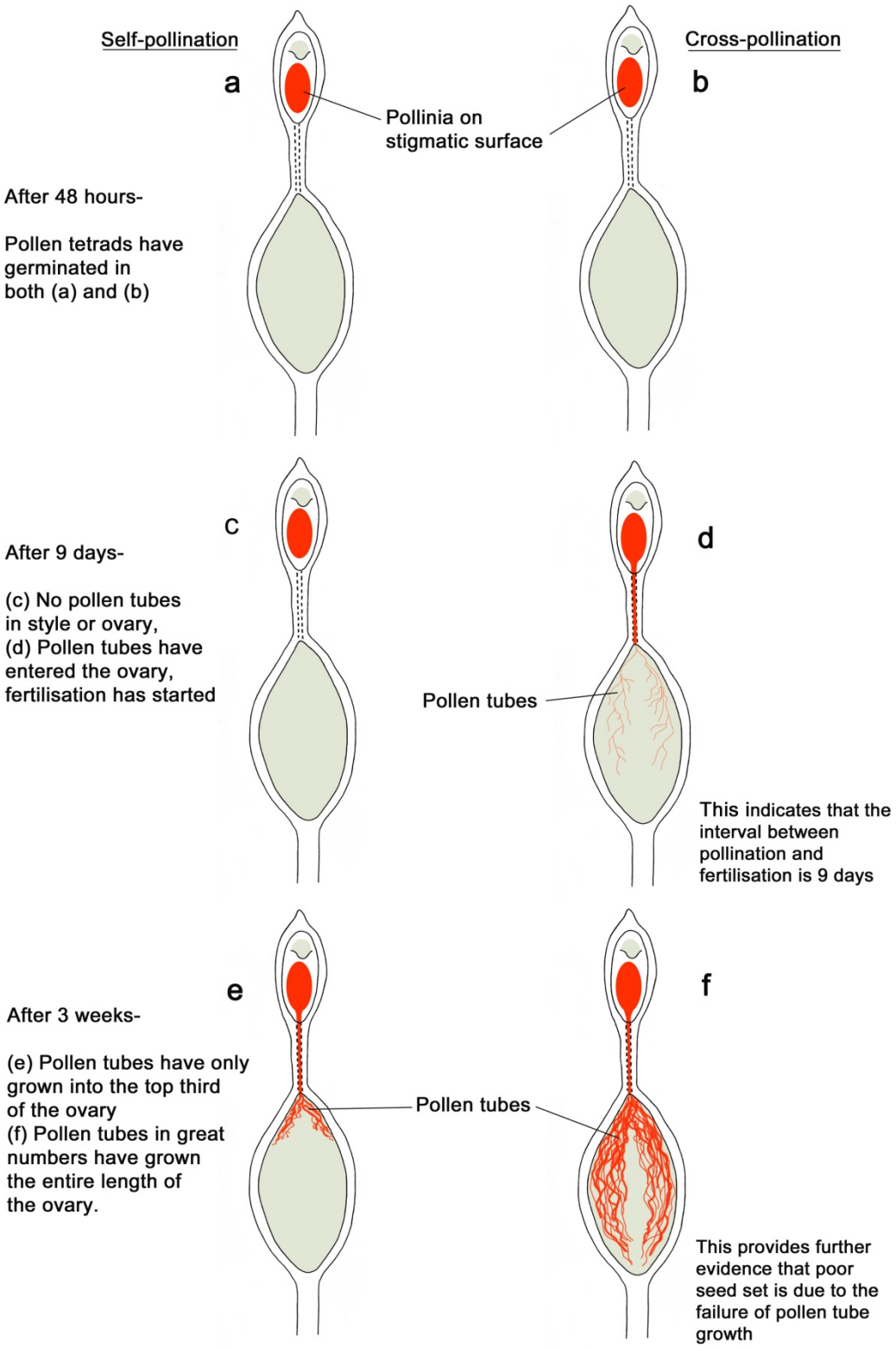


Figure 4-2: Diagrammatic representation of pollen tube growth in self- and cross-pollination

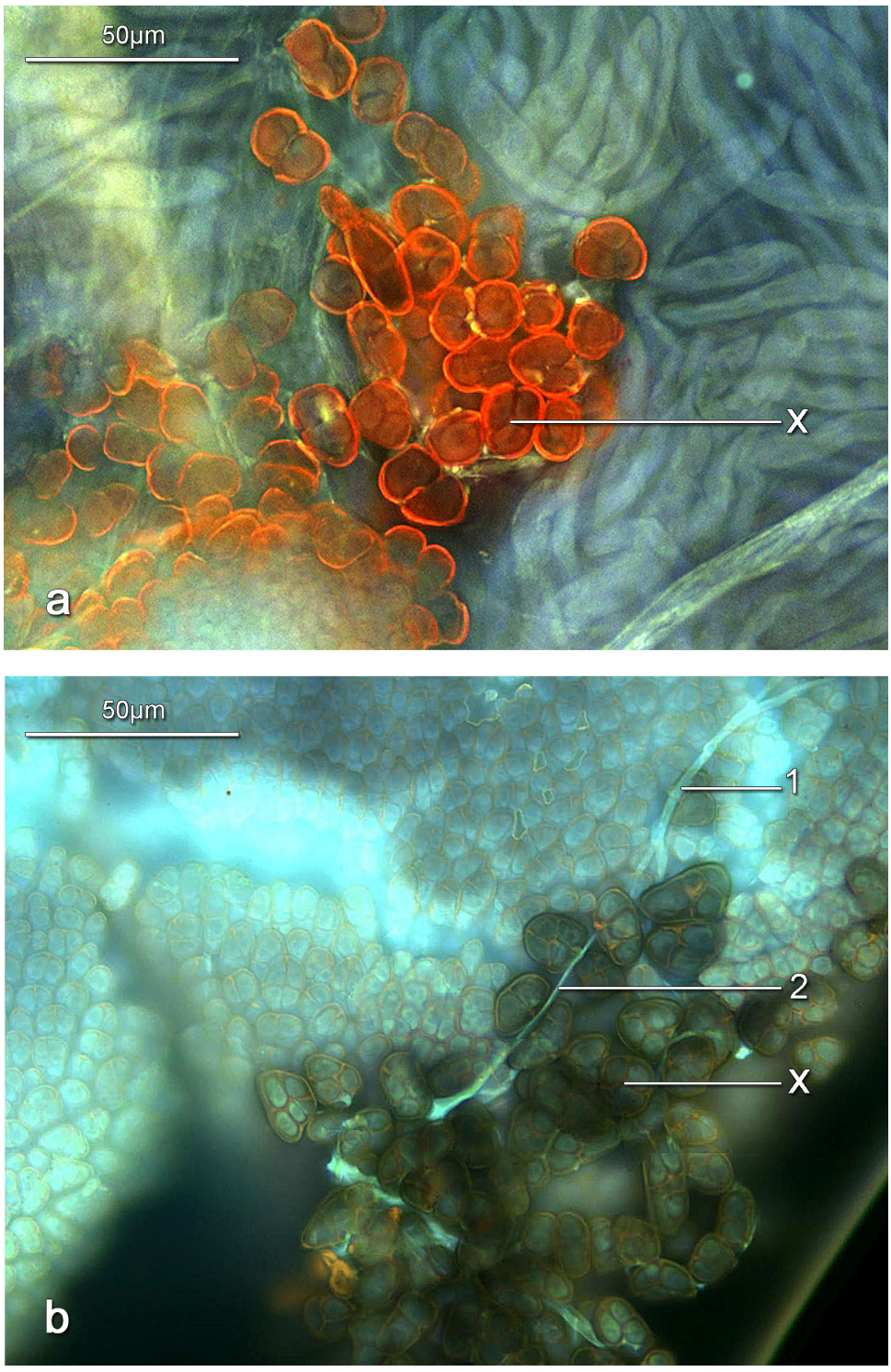
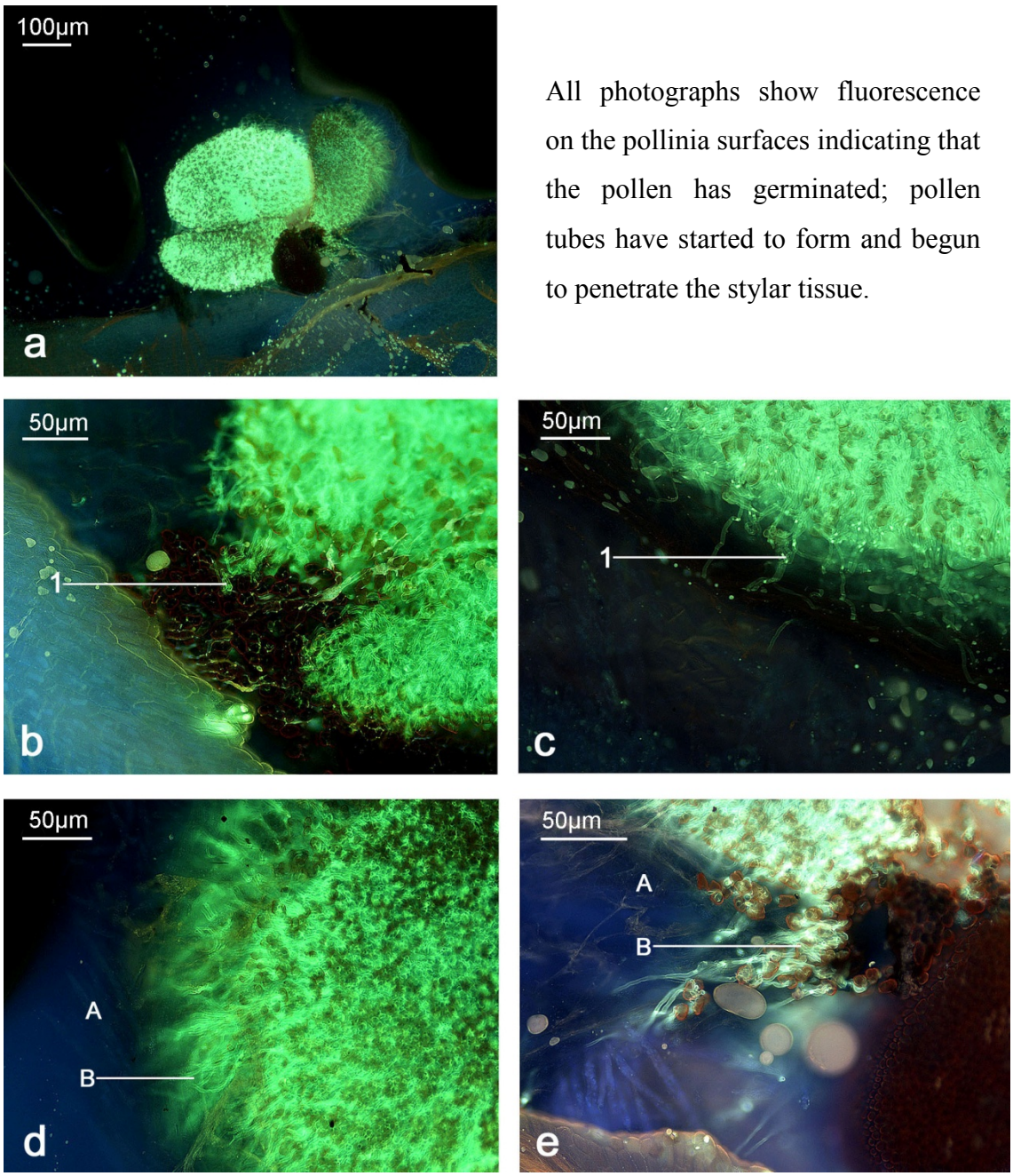


Plate 4-2: Pollen tetrads 24 hours after hand pollination

(a) *R. brachypus* × self, (b) *R. brachypus* × *purpurea*

In both cases some of the tetrads (X) have separated from the main body of the pollinium, but only the cross pollination (b) has produced any visible (i.e. fluorescing) pollen tubes (b) 1 and 2. The pollen tubes have not grown any distance into the stylar tissue or stylar canal.



All photographs show fluorescence on the pollinia surfaces indicating that the pollen has germinated; pollen tubes have started to form and begun to penetrate the stylar tissue.

Plate 4-3: Pollinia 48 hours after hand pollination.

(a) *R. brachypus* × *purpurea*, there is little difference between the self- and cross-pollination at this magnification.

(b) and (c) *R. brachypus* × self. Individual pollen tubes may be observed at the edge of the pollinium.

(d) and (e) *R. brachypus* × *R. purpurea.* Pollen tubes have grown a greater distance into the surrounding stigmatic tissue (A) and (B) detail of the pollen tubes.

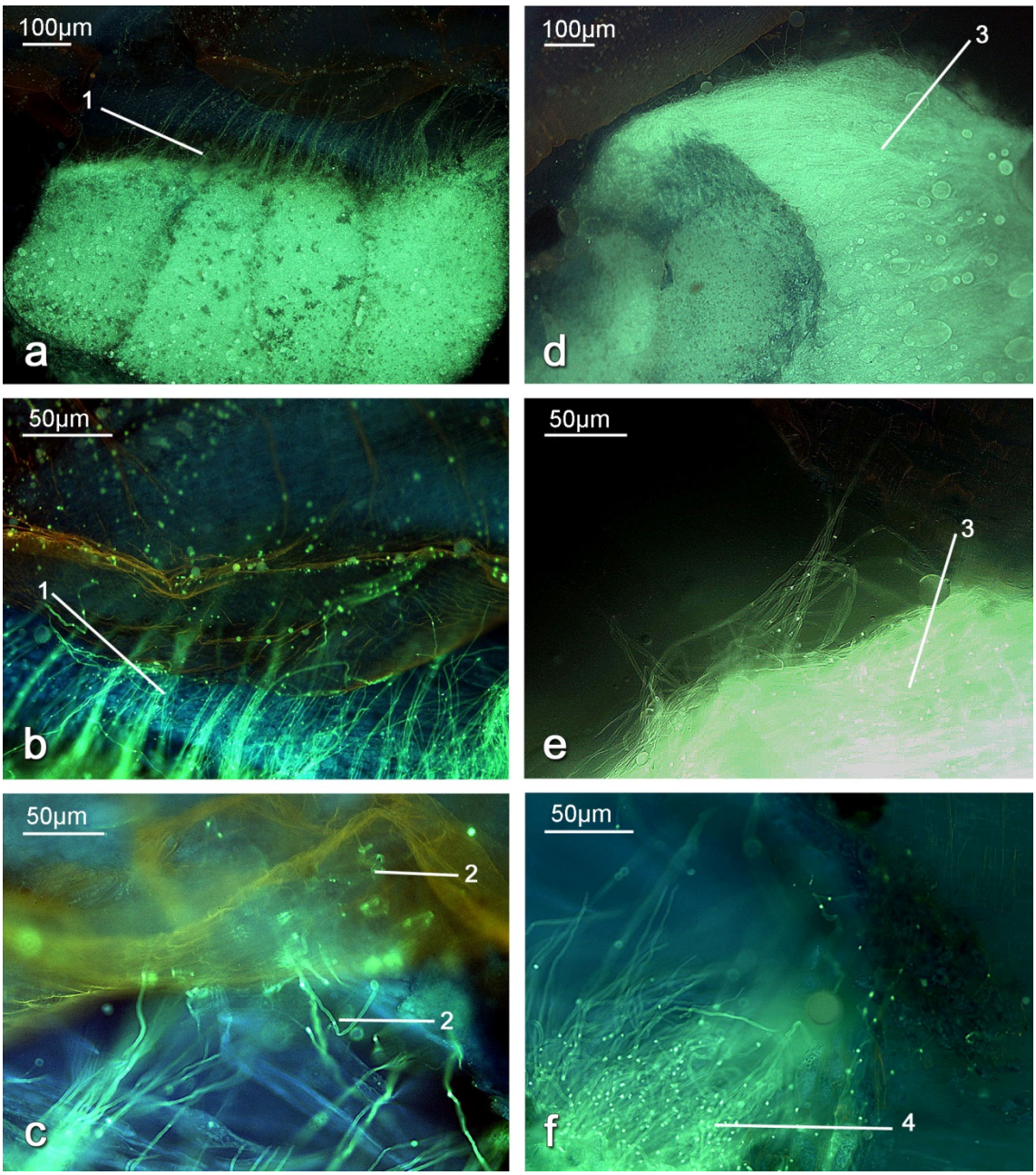


Plate 4-4: Pollen tube growth after 9 days, stylar tissue squash

(a), (b) and (c) *R. brachypus* × self; (a) Pollinia, germinating and elongating pollen tubes,

(b) and (c) details of pollen tube growth, showing haphazard pattern (c).

(d), (e) and (f) *R. brachypus* × *R. purpurea*; (d) pollinium and mass of pollen tubes,

(e) individual pollen tubes difficult to see, due to intense fluorescence from the numerous closely packed pollen tubes.

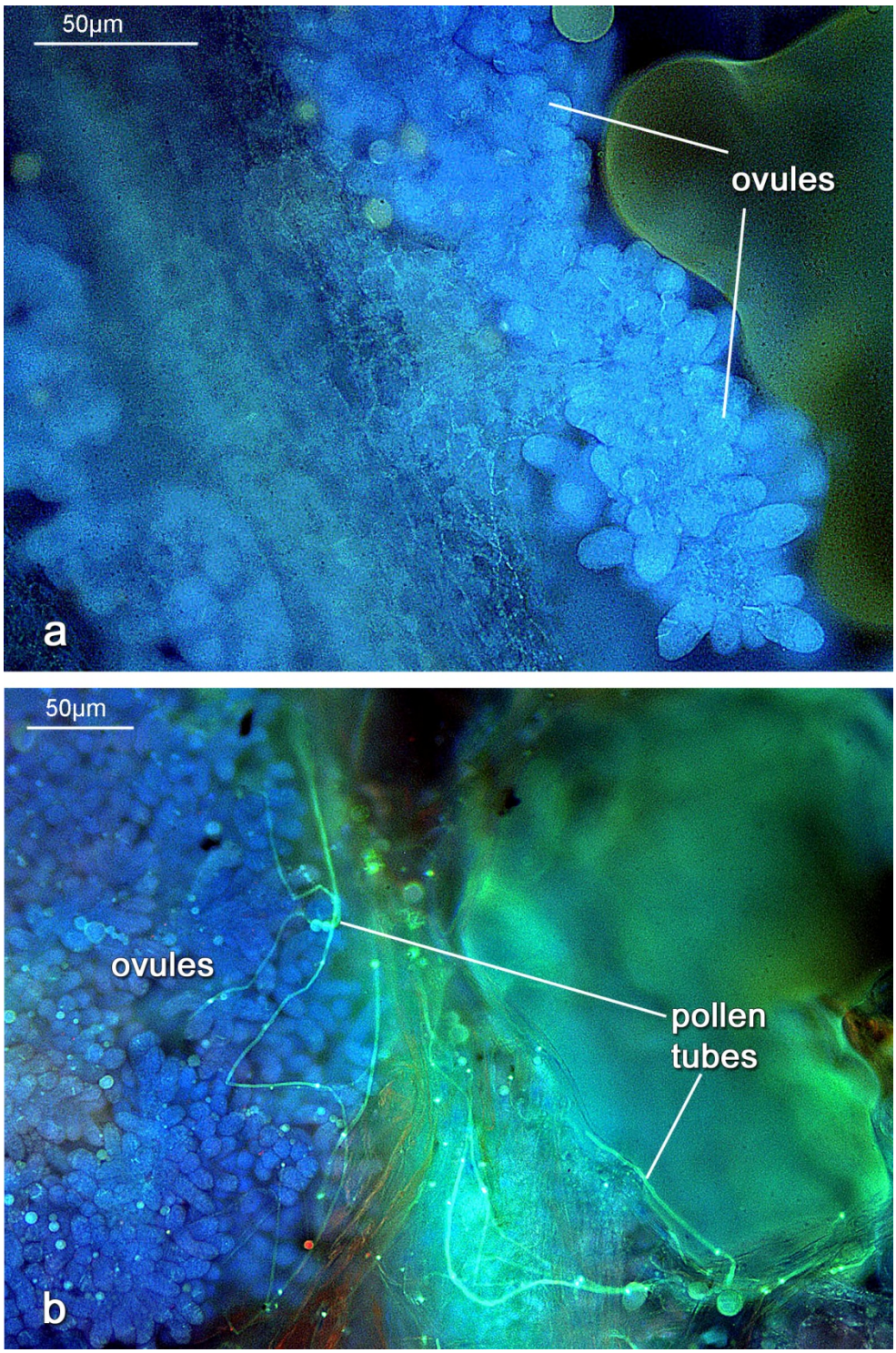


Plate 4-5: Pollen tube growth after 9 days, ovary tissue squash

(a) *R. brachypus* × self. Ovules can be seen in blue, either side of the central tissue. There is no pollen tube growth in the ovary.

(b) *R. brachypus* × *R. purpurea.* Pollen tubes can be seen just beginning to enter the mass of ovules on the left.

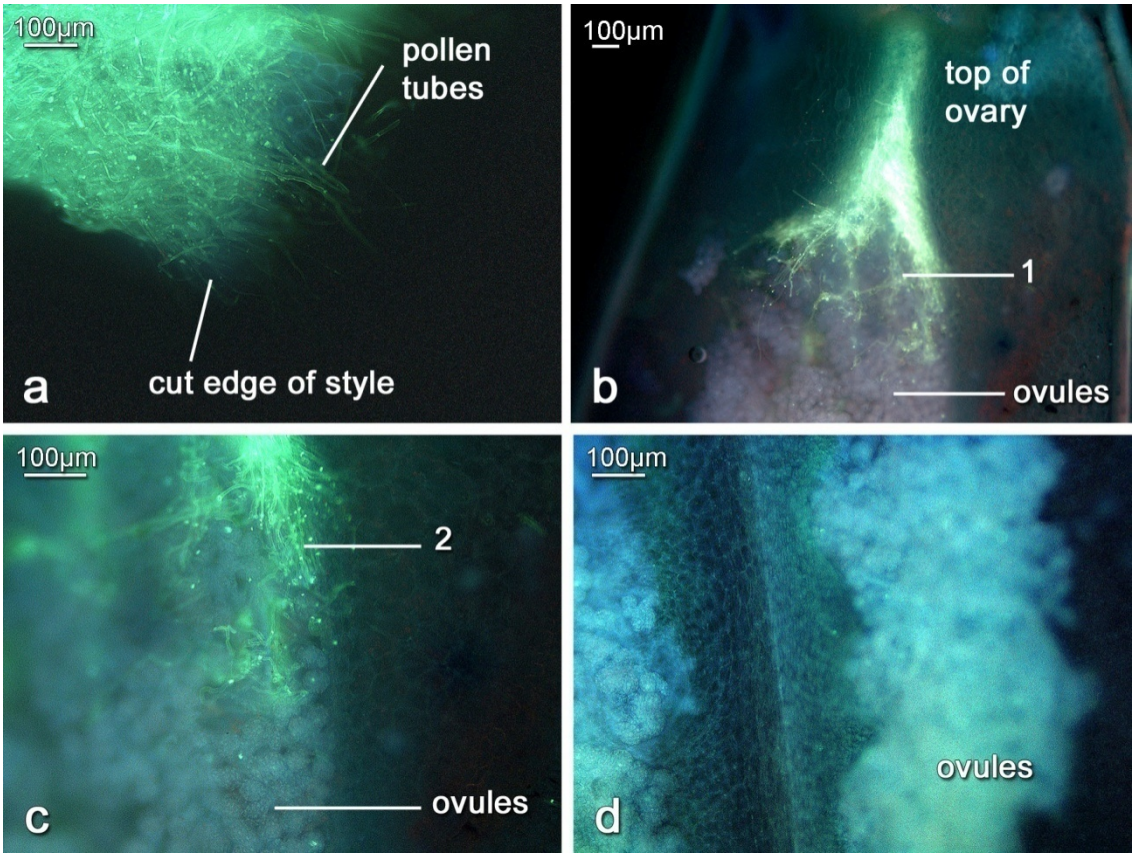


Plate 4-6: Pollen tube growth 3 weeks after self-pollination, ovary tissue squash.

(a) to (d) *R. brachypus* × self

(a) Cut edge of stylar tissue, pollen tubes can be seen protruding, indicating that pollen tubes have grown into the ovary. (b) Pollen tube growth arrested in the top third of the ovary.

(c) Detail of pollen tube growth, showing irregular growth. (d) Absence of pollen tube growth in the lower two thirds of the ovary.

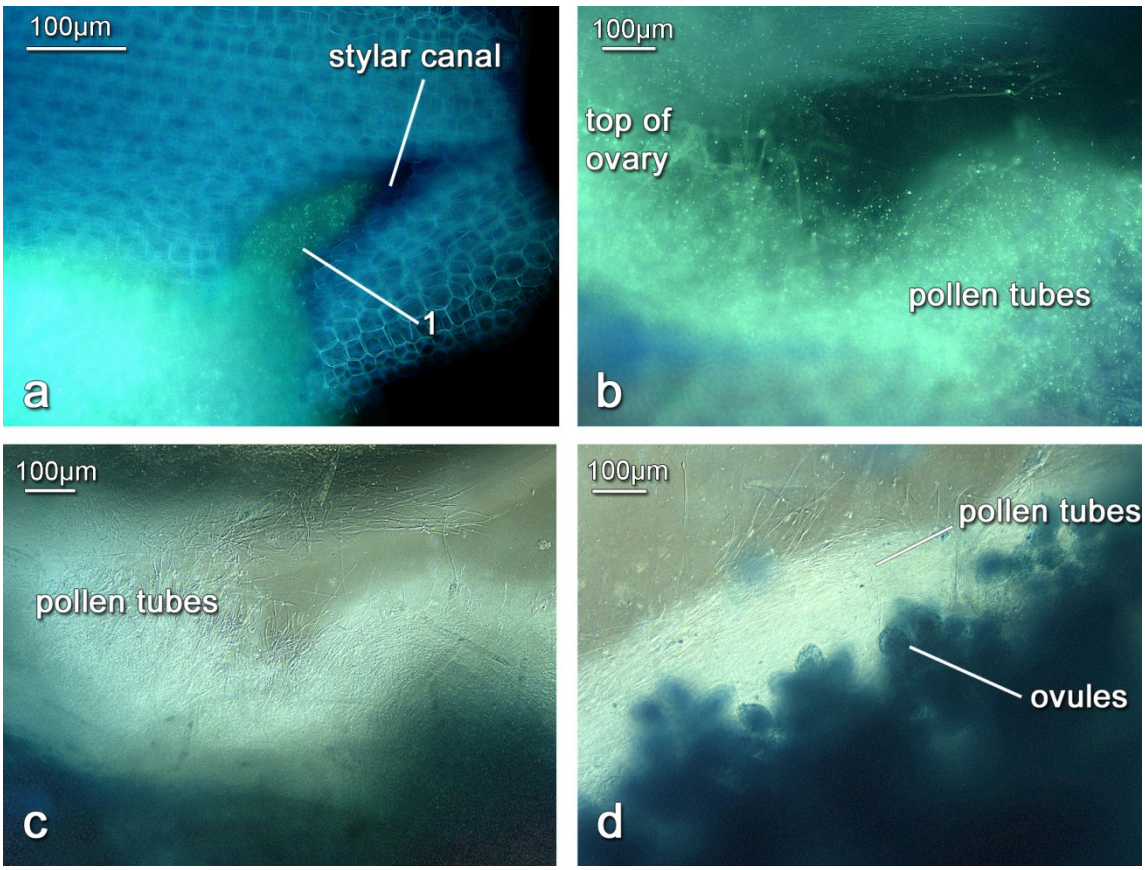


Plate 4-7: Pollen tube growth 3 weeks after cross-pollination, ovary tissue squash.

(a) to (d) *R. brachypus* × *R. purpurea*

(a) Highly fluorescent mass of pollen tubes entering the stylar canal, cut edge of style on right. (b) and (c) mass of pollen tubes in the top third of the ovary.

(d) Pollen tubes growing among the ovules in the lower ovary.

(c) and (d) differential Interference Contrast (DIC) has been used in addition to fluorescence to produce a 3D effect which shows the pollen tubes more clearly.

4.4 Discussion

The initial findings from the investigation described in this chapter may be summarised as follows: there is higher percentage of seed filling following cross-pollination (either intra- or inter-specific) compared to self-pollination; a comparison of the developmental stages that the resultant seedlings achieve shows that cross-pollinations produce offspring exhibiting heterosis. During the course of the investigation no seedling resulting from a self-pollination was raised beyond the *in vitro* stage, indicating high ID in these progeny. In contrast to the failure of self-pollinated seedlings to grow *ex vitro*, the new inter-specific hybrids (primary hybrids) produced have proved to be vigorous and many of these have now flowered.

The preliminary conclusion from these data is that of the species studied, with the exception of *R. aberrans*, none was able to produce viable offspring by self-pollination. These results represent ~50% of species in the genus. Although the findings themselves are clear, the explanation for them is not. While it is possible and consistent with other research to attribute loss of fitness as measured by fitness-related traits (here, developmental criteria) to ID, the identification of SI is more involved.

SI operates prior to fertilisation and is usually quantified by estimates of fruit set (Richards, 1997; Mena-Ali and Stephenson, 2007). In the current study, neither fruit set nor capsule formation were investigated, as it was considered to be an unreliable indicator for reasons previously outlined. The exception is *R. chocoënsis* which failed to set capsules by self-pollination on all occasions. *In lieu* of capsule set, seed filling or embryo formation was the measure chosen. The important question is whether or not seed filling can be used to assess SI. If empty seeds are attributable to early embryo abortion following fertilization, then SI did not operate. If fertilization did not take

place, and pollen tubes did not grow down into the ovary and fertilize the ovules, then SI can be said to have taken place.

There is indirect evidence for the possible operation of SI, in that it is well known for an orchid plant to produce a seed capsule that contains either no seeds or empty testae (as in the current study) following self-pollination (Warren, 2010; Seaton, 2010). It appears that the germination of the pollen grains acts as a biochemical trigger for capsule growth, 'seed' formation and flower senescence. If fertilization does not occur this represents a waste of the plant's resources, so this provides a further explanation for the many mechanisms to prevent self-pollination in this genus.

The study of pollen tube growth and development provided evidence to help answer this question. A marked difference was found between the growth of pollen tubes following self- and cross-pollination. After self-pollination, pollen tube development was slowed on the stigmatic surface and arrested in the upper third of the ovary. The pollen tubes were fewer in number and exhibited an irregular growth pattern when compared to that observed in cross-pollinations. This irregular growth habit has been described previously in *Pleurothallis* species (Borba and Semir, 2001) and provides supporting evidence for SI in this genus. It demonstrates that the empty seeds formed after self-pollinations are a result of pollen tube inhibition, operating pre-fertilization, and not ID or late acting SI, both of which operate post-zygote formation. A few pollen tubes may penetrate the top of the ovary which helps to explain why there are varying percentages of 'filled seeds' formed after self-pollinations.

The pollen tube observations also revealed that the time between pollination and fertilization for *Restrepia* is ~9 days. This was the earliest point in the timed series of self- and cross-pollinations at which pollen tubes were observed in the ovary of cross-pollinated flowers. The time between pollination and fertilization for orchid species in other orchid genera has been previously published (Arditti, 1992), but this is the first

time that this had been observed and recorded for any species in *Restrepia.* These data, in conjunction with evidence cited by Richards (1997), suggest that *Restrepia* exhibits a bifactorial GSI system. In addition, *R. chocoënsis* may exhibit sporophytic (SSI) incompatibility and *R. aberrans* exhibits neither. However, these hypotheses require further research for confirmation.

The majority of *Restrepia* species occur as narrow endemics (Luer, 1996a). The limited populations of many such narrow endemics result in depleted genetic resources and the link between SI and ID in such populations was established by Glémin *et al.* (2001). They found that, contrary to previous work by Bataillon and Kirkpatrick (2000), small populations that express GSI can maintain strong ID, but sufficient numbers of loci must be linked to the S locus. Deleterious alleles linked to the S locus strengthen ID in small populations. ID is an essential factor in the evolution of SI systems, SI being a widespread mechanism that prevents inbreeding in flowering plants (Glémin *et al.,* 2001).

Myophily is the second most frequent pollination syndrome in the Orchidaceae, occurring in nearly 25% of species (Christensen, 1994). The Pleurothallidinae, with 4000+ species, is the largest myophilous grouping within the Orchidaceae (van der Pijl and Dodson, 1966). Recent studies have identified SI within several Pleurothallid genera - *Stelis* (Christensen, 1992), *Lepanthes* (Tremblay and Ackerman, 2007), *Octomeria* (Barbosa *et al*., 2009), *Acianthera* and *Pleurothallis* (Borba *et al.,* 2001a). In addition, *Masdevallia* and *Dracula* are known by specialist growers not to set viable seed by self-pollination, suggesting the operation of SI in these genera (Barrow, 2006; Buckingham, 2008). Although cases of complete incompatibility are rare, SI has been linked to myophily within these genera, because SI is common in species pollinated by flies whose behaviour facilitates self-pollination (Borba *et al.,* 2001b; Borba *et al.,* 2002; Barbosa *et al.,* 2009). Indeed, SI and myophily may be regarded as biological

synapomorphies within the Pleurothallidinae (Barbosa *et al.,* 2009). This is in marked contrast to other literature which considers SI to be rare in the Orchidaceae, with most species being self-compatible and 'avoiding' self-pollination by other means (Borba and Semir 1999; Dressler 1990, 1993; Ingrouille and Eddie 2006; Singer and Cocucci 1999; van der Pijl and Dodson 1966). The genus *Restrepia* is thought to be myophilous (Luer, 1996a; Pridgeon and Stern, 1983), but this hypothesis has never been confirmed in the wild (Luer, 1996a).

Previous studies of SI in the Pleurothallidinae have only included a small number of species; in this investigation, however, we have demonstrated that 24 out of the 26 *Restrepia* species studied (Table 4-3) exhibited some degree of SI. The first exception to this 'rule' is *R. aberrans*, which is indigenous to Panama (Luer 1996a, b) and is known to be self-compatible and to set seed via self-pollination. The floral morphology of this species is significantly different to the rest of the genus and it exhibited no traits associated with either SI or ID in this investigation, having a cumulative score of 11 (Table 4-3). The second exception is *R. schizosepala* (Luer, 1996a, b) which had a cumulative score of 8 (Table 4-3), a lower score than *R. aberrans*. This species produced high germination rates from self-pollination (Table 4-3, score = 4, germination >20%), but the resulting protocorms failed to thrive; an effect attributed to ID and not SI.

SI has often been considered to be a qualitative trait of the breeding system (Richards, 1997). Species with a functional SI system are therefore obligate outbreeders and self-pollination is not possible (Mena-Ali and Stephenson, 2007). However, some plants that have functional GSI systems are capable of producing self-set seed (Travers *et al.*, 2004) and natural populations often exhibit marked phenotypic variation among individuals in the strength of SI (Stephenson *et al.,* 2000, Stone *et al.,* 2006). A degree of plasticity in the strength of SI (Travers *et al.,* 2004) would explain the different

results observed for seed filling (Table 4-4). Genetic and environmental factors that may induce variation in the strength of SI, include the condition of the stigmatic surface due to humidity and temperature, the age of the flower and mutation (Travers *et al.,* 2004). These factors provide an explanation for the some of the variation found in the results obtained.

Three of the species used in this investigation, i.e. *R. dodsonii, R. muscifera* and *R. trichoglossa* (Luer, 1996a), had cumulative scores of 6 or more with corresponding germination rates of 1 - 30% (Table 4-3) suggesting that they exhibit 'weak' SI with correspondingly reduced ID. These species are more widely distributed throughout the geographic range of the genus (Luer, 1996a). Widely distributed species would be most likely to exhibit less ID since there would be greater genetic variation remaining within the species.

The remainder of the *Restrepia* species studied are narrow endemics, all of which may be considered to exhibit SI from their cumulative scores (Tables 4-2 and 4-3). In these species, SI may be acting to prevent self-pollination in dwindling populations. As obligate outbreeding species, many of their pollination syndromes become counter-productive (Borba *et al.,* 2002) and populations may no longer be self-sustaining**.**

As these populations decline, the incidence of self-pollination will correspondingly increase: however, few of these self-pollinations will produce viable seed (Table 4-3). This situation is further exacerbated by the effects of ID. As shown for *R. brachypus* (Table 4-7) high seed filling is not sufficient to guarantee germination, the embryos within such seeds must also be viable. Inter-clonal crosses or out-crosses may produce high seed filling, but the seeds so formed may not be viable. Even if successful germination occurs, the resultant plantlets may only grow slowly and may not flower, both of which are consequences of ID.

These effects become of critical importance within small, wild populations. In populations such as these with increased inbreeding and hence increased homozygosity, out-crossing with plants with a sufficiently different genotype to produce viable offspring becomes markedly reduced. Such populations could potentially become unsustainable via seed production. Vegetative reproduction might persist for some time, but the population would be functionally extinct. The IUCN Red List of endangered species (IUCN, 2013a) does not currently include a category for functional extinction and species no longer viable or able to sustain themselves are classified as 'critically endangered' or 'extinct in the wild', if specimens remain in cultivation but wild specimens no longer exist.

Analytical methods for determining the current status of an orchid species or its individual populations have been reviewed by Tremblay and Hutchings (2003), who stated that a deeper understanding of the behaviour of populations of rare orchid species will permit better predictions of their future fates. Further studies are therefore required to establish the reproductive status of the remaining wild orchid populations that are currently distributed in small, hyper-dispersed populations (Tremblay, 1997; Ackerman, 1998) and those that are narrow endemics.

The main application of the data presented, is that they provide a practical solution for seed production in the genus by suitable inter-clonal cross-pollinations of individual *Restrepia* species. This has the benefit of removing the effects of SI and reducing CI and ID in the resulting F_1 generation. These offspring are useful for both *in situ* and *ex situ* conservation strategies, such as re-introduction and habitat restoration, conservation programmes. As a result of this study several species which have been propagated by seed in this manner are *R. brachypus, R. aristulifera, R. antennifera and R. guttulata.* This illustrates how *ex situ* populations of orchid genera can be managed via outbreeding through hand pollinations and not, as commonly practised, by self-

pollinations of 'choice' clones. Furthermore, these data can also be used to inform future seed banking initiatives in *Restrepia* where it is vital to ensure production of viable seed prior to storage.

By using *ex situ* populations to study aspects of reproduction in *Restrepia* this study has demonstrated the existence of obligate outbreeding in the genus by identifying the operation of SI in 53% of species, thus providing a 'relatively' robust description of SI and ID in the genus.

The incidence of SI in many out-crossing orchid genera is either not known or poorly understood, and the findings regarding *Restrepia* species may prove to be indicative of other obligate outbreeding genera, which also contain narrow endemic species. Previous low estimates of SI in the Orchidaceae may well prove to be inaccurate since research has highlighted SI as widespread in both New and Old World orchid genera (Charanasri and Kamemoto, 1977; Stort and de Lima Galdino, 1984; Agnew, 1986; Johansen, 1990; Christenen, 1992; Borba *et al.,* 2002; Blanco and Barbosa, 2005; Tremblay and Ackerman, 2007; Barbosa *et al.,* 2009; Cheng *et al.*, 2009; Gontijo *et al.,* 2010).

Any obligate outbreeding, self-incompatible species may therefore be in danger not only from loss of habitat, but also from the inability to set viable seed by self-pollination. The logical consequence of this, would be eventual functional extinction, should cross-pollination become impossible. With world-wide loss of orchid habitats, many more orchid species may also prove to be in jeopardy from habitat loss and failure to set seed. As such, the data presented provide vital information for the future conservation of *Restrepia* and other genera.

Inbreeding may be unavoidable in such small, isolated, narrow endemic populations causing substantial fitness reductions compared to out-bred populations. Small

populations have been shown to experience high levels of ID (Bataillon and Kirkpatrick, 2000). This loss of fitness in small populations has been predicted to elevate extinction risk giving it substantial conservation significance (Wright *et al.,* 2008). The increased extinction risk of small, inbred populations has been illustrated by a growing number of studies (Frankham 1995; Bijlsma *et al.,* 1999, 2000; Reed *et al.,* 2002, 2003). Population size is influenced by both natural selection and genetic drift. These processes influence reproductive systems, genetic architecture, allele frequencies and diversity which, in turn, influence ID and evolutionary potential (Tremblay and Otero, 2009).

The reduced genetic diversity resulting from inbreeding may mean a species cannot adapt to changes in environmental conditions. When a species becomes endangered, the population may fall below a minimum whereby the forced interbreeding between the remaining individuals will result in extinction.

The minimum viable population of a species is the smallest possible size at which the population can survive in the wild without facing extinction from natural disasters or demographic, environmental, or genetic stochasticity. Minimum Viable Population is usually estimated as the population size necessary to ensure between 90 and 95 per cent probability of survival between 100 to 1,000 years into the future. This term is generally applied to animal populations, but is an equally important concept for populations of plant species. However, there is currently very little data available for accurate estimates to be made. Gilpin and Soule (1986) proposed the concept of an 'extinction vortex' to describe how a reduction in population size may influence extinction risk. As population size decreases, then the probability of inbreeding increases and this will reduce fitness in the remaining population. Inbreeding now increases in the remaining smaller population and still further reduces fitness. Dropping

below the population size threshold into this feed-back loop was termed an 'extinction vortex'.

This investigation has identified *Restrepia* species as being obligate, self-incompatible outbreeders. These findings, together with issues related to habitat loss outlined previously, raise important questions regarding wild populations of *Restrepia* species. Namely, have populations become endangered from habitat loss, population decline and reduced fitness due to inbreeding? Secondly, have populations dropped below the theoretical threshold and into an 'extinction vortex'? These are not easy questions to answer. They involve comparing current and historical distribution and occurrence data, identifying the conservation status of *Restrepia* species, and establishing their current Global and National Red List status. The following chapter attempts to address some of these issues via a detailed analysis of the Red List status of *Restrepia* species.

Chapter Five:

A Red List Assessment of *Restrepia* species using GeoCAT and GBIF

Access roads, 2007

Undisturbed forest in Costa Rica, 2006

Photograph H. Millner

'So bleak is the picture…that the bulldozer …may turn out to be the most destructive invention of the 20th century."

Philip Shabecoff (1978)

5.1 Introduction and background

5.1.1 Growth of online resources since 2003

Over the past decade, the availability of online resources *and* databases concerning orchid species has increased considerably, the consequence of which is that accurate and detailed studies of present and past species distribution patterns, which were not previously possible, may now be undertaken with relative ease.

In recent years, the importance of *ex situ* conservation of plant species has become widely accepted (Maunder *et al*., 1997; Fay and Krauss, 2003; BGCI, 2012) and it is essential for researchers undertaking such projects to be able to perform conservation assessments from which to formulate their conservation strategies. The data from specimen records have now been published online by many herbaria, thus making them freely available and facilitating conservation assessments worldwide. The following sections present a brief review of the most important online resources currently available for conservation research.

5.1.1.1 Tropicos

Tropicos is the world's largest database of plant information and contains fully searchable online records of over 1.2 million plant names and nearly 4 million specimens. Originally created for internal research at Missouri Botanical Garden (MGB), all of the nomenclatural, bibliographic, and specimen data accumulated in MBG's electronic databases over the past 25 years are now freely available to the world's scientific community and publicly available via this website (Tropicos, 2013).

5.1.1.2 Kew Herbarium

The Herbarium at the Royal Botanic Gardens, Kew, houses approximately 7 million specimens collected from around the world, including approximately 350,000 type

specimens, either pressed and dried or preserved in spirit. Kew is committed to making this collection accessible to botanists worldwide, particularly those concerned with biodiversity, conservation, sustainable development and systematics (RBG Kew, 2013b). Access to the specimen records and images in this digital catalogue is now freely available online through the digitised Kew Herbarium Catalogue.

5.1.1.3 The Global Biodiversity Information Facility (GBIF)

The Global Biodiversity Information Facility (GBIF) was established by various governments in 2001, in order to encourage free and open access to biodiversity data, via the internet. The GBIF portal, which is searchable for species data, may be accessed on line. GBIF promotes and facilitates the mobilization, access, discovery and use of information about the occurrence of organisms over time and across the planet through a global network of countries and organizations (GBIF, 2013). Data discovered and accessed through GBIF web platforms are being used in a variety of scientific applications, many of which have direct relevance to key policy issues related to biodiversity. Since 2008, more than 500 peer-reviewed publications have cited use of GBIF-mediated data, 204 of which were published in 2011 (GBIF, 2013).

In addition, a large number of other herbaria now publish data of their specimen collections online. The accessibility of such specimen records has markedly increased over the past decade as more herbaria records are digitised. Many of these herbaria also publish their records through the GBIF portal, which currently boasts 83,027,468 indexed records from 456 such publishers. Examples of some of these publishers are shown in Table 5-1.

5.1.1.4 International Union for Conservation of Nature (IUCN) Red List of Threatened Species

Since 2001, the 'IUCN Red List of Threatened Species' (IUCN, 2013a) has been published solely online (IUCN, 2013a). The IUCN Species Programme has been working with the IUCN Species Survival Commission (SSC) for more than 40 years to assess the conservation status of species worldwide, in order to highlight taxa threatened with extinction, and thereby promote their conservation. Their aim is to make objective, scientifically-based information on the current status of globally threatened biodiversity readily available through the internet (IUCN, 2013a). Information on the conservation status and distribution of plants and animals assessed for the IUCN Red List provides the basis for making informed decisions about conserving biodiversity at both local and global levels (IUCN, 2013a).

5.1.1.4.1 Red List Categories

The IUCN Red List of Threatened Species (IUCN 2013a) provides taxonomic, conservation status and distribution data on plants and animals that have been globally evaluated using the IUCN Red List Categories and Criteria (IUCN, 2012a). This system was designed to determine the relative risk of extinction of species and genera and its main purpose is to catalogue and highlight those taxa facing a risk of global extinction (categorised as Critically Endangered, Endangered and Vulnerable) and taxa close to these thresholds (Near Threatened).The IUCN Red List also includes information on taxa that are considered Extinct, or Extinct in the Wild; and on taxa that cannot be evaluated due to insufficient information (Data Deficient): this is summarised in Figure 5-1 and a brief version of the formal definitions is given in Table 5-2.

Before 2003, Least Concern (LC) assessments did not appear on IUCN Red Lists. After this date, however, taxa with a low extinction risk were classified accordingly. Many species had been assessed to be of Least Concern before this, but because this

information was never formally recorded, they do not appear on the Red List. Hence, the list of Least Concern species on the IUCN Red List is not comprehensive. To date, only a small number of the world's plant and animal taxa (15,497 species currently listed on the IUCN Red List of Threatened Species) have been assessed (IUCN, 2013a).

Currently, there are no *Restrepia* species on the Global Red Lists (IUCN, 2013a), which suggests that this genus is not Globally Endangered. However, the most recently published National Red Lists for Ecuador (Léon-Yánez *et al.,* 2011) and Colombia (Calderón-Sáenz, 2007) both list *Restrepia* species, indicating these species to be Endangered in both these countries. Endemic *Restrepia* species, in either Ecuador or Colombia, categorised as Nationally Endangered, should therefore, also be listed as Globally Endangered, but currently are not.

5.1.1.4.2 Red List Criteria

Red List assessment involves evaluating data regarding a taxon against five criteria (A-E) any one of which may be used to identify the threat to the taxon (as not all of the criteria are applicable or suitable for all species or genera). The criteria may be summarised as:

- Criterion A- population reduction,
- Criterion B - geographic range including Extent Of Occupancy (EOO) and Area Of Occupancy (AOO).
- Criterion C - population decline,
- Criterion D - small or restricted populations
- Criterion E - quantitative analysis of extinction probability.

The formal summary of the criteria and their sub-categories, (IUCN, 2013C), is shown in Table 5-3.

Table 5-1:Data publishers with *Restrepia*[1] occurrence records published online via GBIF data portal (GBIF, 2013)

Data publishers with *Restrepia* occurrence records:
Berkeley Natural History Museums
California Academy of Sciences
Comisión nacional para el conocimiento y uso do la biodiversidad
Field Museum
GBIF - Spain
GBIF - Sweden
Harvard University Herbaria
Herbarium Hamburgense
Herbarium of the University of Aarhus
Instituto de Ciencias Naturales
Instituto de Investigación de Recursos Biológicos Alexander von Humboldt
Instituto Nacional de Biodiversidad (INBio), Costa Rica
Jardin Botanique de Montréal
Missouri Botanical Garden
MNHN - Museum national d'Histoire naturelle
Museo Nacional de Costa Rica
National Museum of Natural History, Smithsonian Institution
Natural History Musem, Vienna - Herbarium W
Organisation for Tropical Studies
Royal Botanic Garden, Edinburgh
Royal Botanic Garden, Kew
SysTax
UNIBIO, IBUNAM
University of California, Davis

Notes:

[1] The data set of *Restrepia* species (see Method and Materials, 5.2) was made up from the records from these data publishers via the GBIF data portal

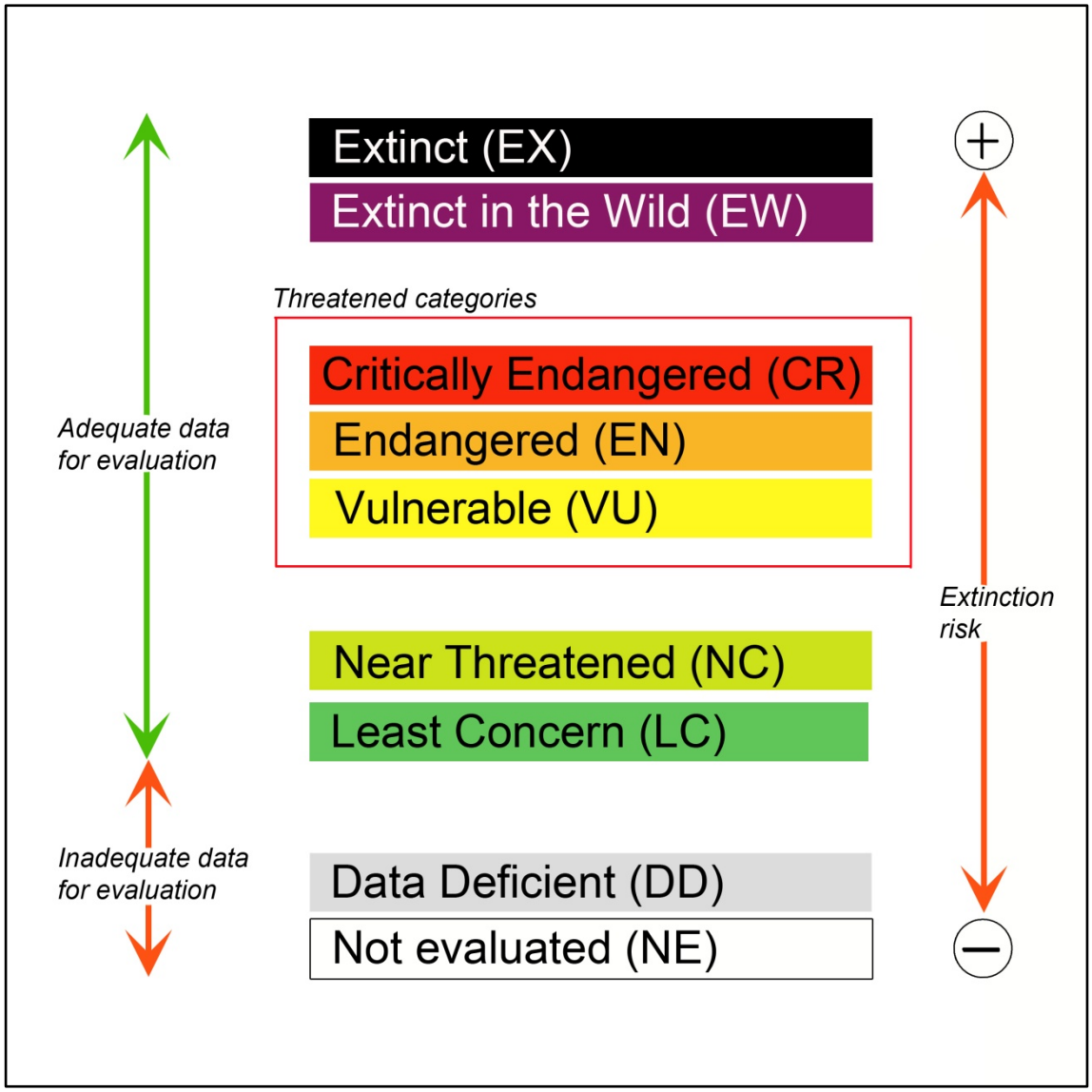


Figure 5-1: Relationship between the IUCN Red List Categories, extinction risk and availability of data, adapted from IUCN (2012a).

The Red List Category may be written out in full or abbreviated as follows (IUCN, 2000):

Extinct, EX
Extinct in the Wild, EW
Critically Endangered, CR
Endangered, EN
Vulnerable, VU
Near Threatened, NT
Least Concern, LC
Data Deficient, DD
Not Evaluated, NE

Table 5-2: IUCN CATEGORIES OF RISK Version 3.1 (IUCN 2012a)

CATEGORY	Abbreviated formal IUCN Definition
Extinct (EX)	A taxon is extinct when there is no reasonable doubt that the last individual has died
Extinct in the Wild (EW)	A taxon is extinct in the wild when it is known only to survive in cultivation, in captivity or as a naturalized population (or populations) well outside the past range
Critically Endangered (CR)	A taxon is critically endangered when the best available evidence indicates that it meets any of the criteria A to E for Critically Endangered and therefore faces an extremely high risk of extinction in the wild.
Endangered (EN)	A taxon is endangered when the best available evidence indicates that it meets any of the criteria A to E for Endangered and therefore faces a very high risk of extinction in the wild.
Vulnerable (VU)	A taxon is vulnerable when the best available evidence indicates that it meets any of the criteria A to E for Vulnerable (see Section V), and therefore faces a high risk of extinction in the wild.
Near Threatened (NT)	A taxon is near threatened when it has been evaluated against the criteria but does not qualify for Critically Endangered, Endangered or Vulnerable now, but is close to qualifying for or is likely to qualify for a threatened category in the near future.
Least Concern (LC)	A taxon is least concern when it has been evaluated against the criteria and does not qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened.
Data Deficient (DD)	A taxon is data deficient when there is inadequate information to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status. A taxon in this category may be well studied, and its biology well known, but appropriate data on abundance and/or distribution are lacking. Data Deficient is therefore not a category of threat but indicates that more information is required.
Not Evaluated (NE)	A taxon is not evaluated when it is has not yet been evaluated against the criteria.

Table 5-3: Summary of the five criteria (A-E) used to evaluate if a taxon belongs in a Threatened category (Critically Endangered, Endangered or Vulnerable) [1](IUCN, 2013c).

Table removed

[1]a full explanations of terms and concepts used here are found in IUCN Red List Categories and Criteria, (IUCN, 2012a) and Guidelines for Using the IUCN Red List Categories and Criteria, (IUCN, 2013b).

5.1.1.5 The Geospatial Conservation Assessment Tool (GeoCAT)

Applying the Red List criteria to plants has proven difficult because the kind of data required for Red List assessments e.g. population size and dynamics, is not often collected for plants (RHS, 2013a). However, aspects such as geographic range size can often be determined from herbarium records (RHS, 2013a).

Until 2010 there were few effective tools that took such primary biodiversity data and used them to perform analyses of the geographic range of a particular species range (Bachman *et al.,* 2011). GeoCAT was developed to fill this gap and harnesses primary biodiversity data for semi-automated IUCN Red List assessment and analysis. This tool, although currently still in beta, has been made available through the internet to give conservationists easy access to a fast, quantifiable and reliable species conservation assessment tool, (RBG Kew, 2013a).

GeoCAT is an open source, browser based tool that performs rapid geospatial analysis for the process of Red Listing taxa. It was developed to use spatially referenced primary occurrence data, and analyses performed focus on two aspects of the geographic range of a taxon: the extent of occurrence (EOO) and the area of occupancy (AOO) (Bachman et al., 2011). To calculate the EOO and AOO, GeoCAT presently uses two algorithms (Bachman *et al.,* 2011). EOO is a measure of the geographic range size of a species and may be calculated by a convex hull. This is defined as the smallest polygon that contains all sites of occurrence (Figure 5-2) and in which no internal angle exceeds 180˚ (Bachman *et al.,* 2011). AOO, in contrast, is a measure of the area in which a species occurs. One way to measure this is by calculating the sum of the area of square grids or cells the species occupies (Bachman *et al.,* 2011). The choice of scale and cell size influences the size of AOO (Figure 5-3). The most appropriate scale depends on the taxon, the origin and the comprehensiveness of its distribution data (IUCN, 2013b). Within GeoCAT, the default is 2km cell width, as recommended in the IUCN guidelines

(IUCN, 2013b). At present, this tool can only produce a preliminary assessment based on EOO and AOO, and does not give a full assessment of Criterion B, for which addition data is required (Table 5-3).

It is also possible to make an assessment of Red List Criterion A that deals with 'reduction' or decline in population size by examining occurrence through time. Within GeoCAT historical specimens can be removed or 'hidden' (this is discussed later in Materials and Methods and Figure 5-6) when they occur in areas that are known to have been subject to habitat loss. Reductions in EOO and AOO can then be recorded and applied to Criterion A (Bachman *et al.,* 2011). At present, assessments can only be carried out one at a time. In order to speed up this process a batch option is required so that a single file of occurrence data for multiple species can be uploaded and processed.

5.1.2 Problems with data quality and uncertainty

The data used to evaluate taxa against the criteria often contain considerable uncertainty. Historic data from herbarium specimens, in particular, often contain inaccurate geographical data lacking geospatial coordinates (Wieczorek *et al.,* 2004). This makes applying the Red List Criteria difficult, as they are quantitative in nature. In general, uncertainty in data can arise from three factors: natural variation, semantic vagueness in terms and definitions, and measurement error (IUCN, 2013b). Measurement error is often the largest source of uncertainty; this may be due to inaccuracies in estimating values or a lack of knowledge. Measurement error may be reduced by acquiring additional data (Akçakaya et al., 2000; Burgman et al., 1999) but this alone will not always reduce some types of error (such as those found in historic herbarium specimen records). It is therefore recommended that great care is taken to identify the most likely, plausible range of values and that extreme or unlikely values are excluded from the data, as this will reduce uncertainty (IUCN, 2013b).

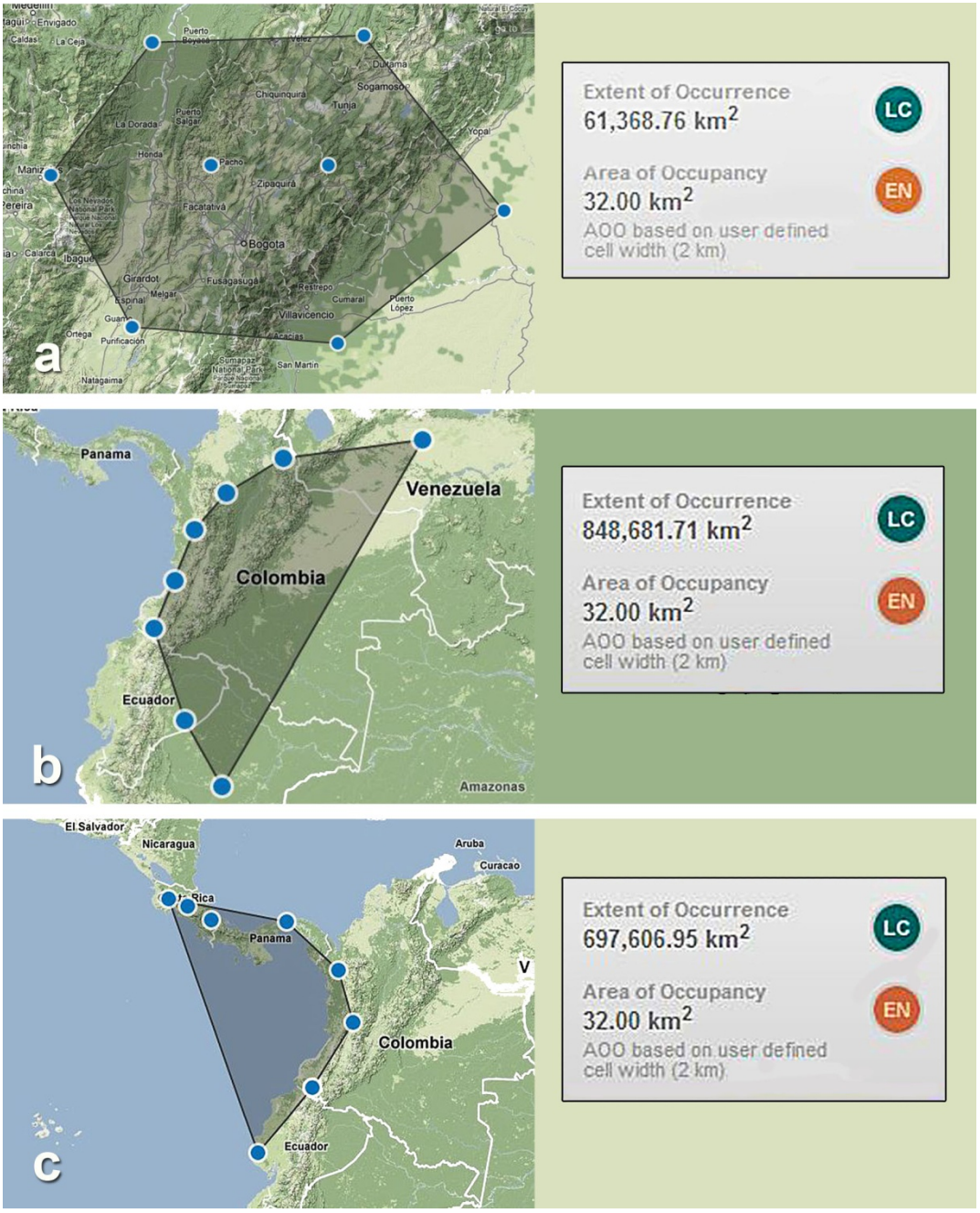


Figure 5-2: Extent Of Occupancy (EOO), (IUCN, 2012b; 2013b)
These examples have been constructed using 'model' screenshots from GeoCAT designed to show the relationship between EOO and AOO – see also Figures 5-3 and 5-4

In examples (a-c) each distribution contains eight 'locations', which gives the same AOO for each example (i.e. 8 × 4 = 32km^2), where the AOO is based on the default, recommended cell width of 2km.

(a) The EOO shows an area enclosed by all the 'points'; (b) the enclosed area includes a large area in Colombia in which no points occur; (c) the enclosed area includes an expanse of sea.

In these examples the EOO was measured using the convex hull, in which no external angle is >180º. Although (b) and (c) would seem a substantial overestimate of the range, this method has been shown to be unlikely to bias the assessment of EOO thresholds under criterion B, even for irregularly shaped ranges (Ostro et al., 1999; IUCN, 2013b). As the EOO is assessed as LC in (a-c) above, this is substantiated.

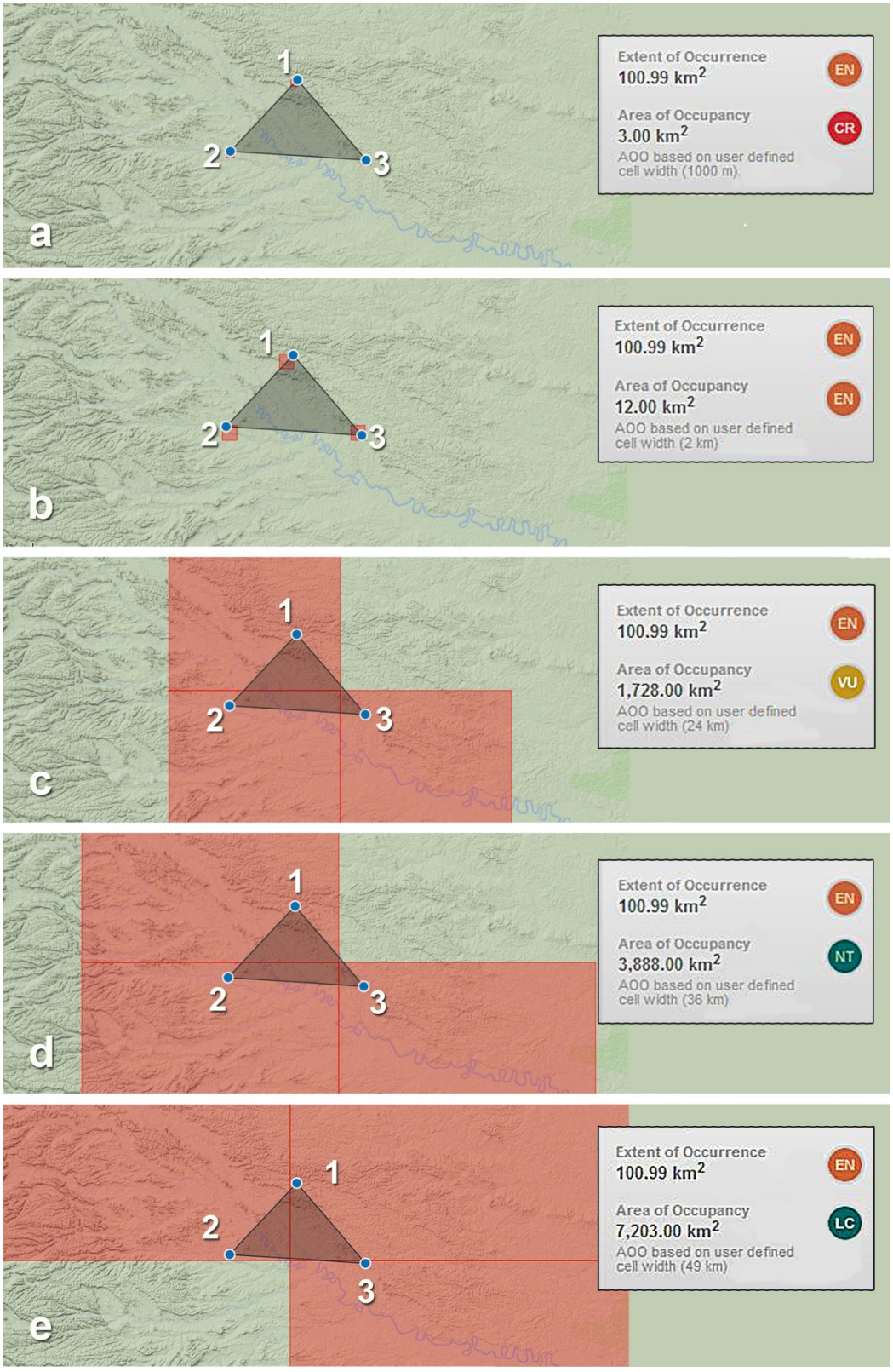


Figure 5-3: Area OF Occupancy, (AOO), (IUCC, 2012b; 2013b)

These examples have been constructed using 'model' screenshots from GeoCAT designed to show the relationship between EOO and AOO - see also Figures 5-2 and 5-4.

In (a) - (e) the EOO has the same value (100.99km^2), the triangular area enclosed by the 'points' 1, 2 and 3 is the same. In (a) - (e) the cell width is made progressively larger, resulting in a corresponding increase in AOO values. (b) shows the recommended cell width of 2 km.

The effect of altering the cell width changes the Red List Category assigned from CR to LC for AOO, but the EOO remains unchanged as EN.

It is also recommended that the absence of high-quality data should not deter attempts at applying the criteria, as methods involving estimation, inference and projection are acceptable (IUCN, 2013b). Another potential problem is that of insufficient data with which to carry out an assessment. Some taxa may have only a few records and the question therefore arises as to how many records are needed for a reliable assessment. When data are very uncertain or missing, the category of 'Data Deficient' may be assigned. Even poorly documented taxa can often be assigned a threat category by using background information concerning the deterioration of their habitat; for this reason the 'liberal use' of 'Data Deficient' is discouraged (IUCN, 2013b).

5.1.3 The use of these resources with regard to *Restrepia*

Potentially all of these resources could enable a qualitative assessment of the 'threat' to *Restrepia* species, to be performed by researchers not based in the countries of origin, i.e. South America. The extensive locally collected species distribution data produced in countries such as Ecuador are otherwise unavailable, even if such data exist.

The genus *Restrepia* has members ranging from those comparatively well represented in herbaria databases to those with only one or two records and to some with no records at all. The question of the minimum number of records required for an assessment is therefore of particular relevance with regard to some of these species. This range, or disparity, between the more common and rarer members of the genus might in theory hinder the full use of these resources to assess the current conservation status of *Restrepia* species.

The following questions were identified regarding the extent to which these resources could be applied in order to further the present understanding of the current conservation status for of this genus.

1. Can online resources be used to produce useful evaluations for all *Restrepia* species in line with the Red List categories and criteria?

2. Is it possible using online resources to produce an assessment of the current threats to *Restrepia* species in the wild?

3. What improvements could be made to these resources to improve the results obtained? Is the quality of the available data adequate to give useful results?

In order to try and answer some of these questions, the following aims and objectives were formulated -

5.1.4 Chapter aims

1. To produce a Red List assessment of all *Restrepia* species so that the direct threats to each species can be identified.

2. To evaluate the use of current online resources in assessing the threatened status of *Restrepia* species.

5.1.5 Chapter objectives

1. To search available online resources for data on *Restrepia* distribution and occurrence.

2. To use GeoCAT to perform calculations of EOO, and AOO and produce a Red List value for each species.

3. By comparing past and present locations in Google Earth to establish habitat/sub-population loss within the genus.

4. By comparison of the data produced, using the above to modify the Red List values for each *Restrepia* species and produce a final assessment.

5.2 Materials and Methods

5.2.1 Definitions of terms used in the Red List Criteria

The following terms have acquired different meanings when used in the context of the Red List Criteria:

5.2.1.1 Population: The term 'population' is used in a specific sense different to its common biological usage. It is defined as the total number of individuals of a taxon. A species population in this investigation therefore refers to the individuals comprising the complete (global) range of the taxon in question (IUCN, 2012a).

5.2.1.2 Sub-population*:* Sub-populations are defined as geographically or otherwise distinct groups in the population between which there is little genetic exchange (IUCN, 2012a). In this investigation a sub-population may be considered to occur at each recorded location. The total number of sub-populations of a species within a country comprises the national range of the taxon.

5.2.1.3 Location*:* Location defines a geographically distinct area containing all or part of a sub-population of the taxon, and is a small proportion of the taxon's total distribution (IUCN, 2012a). For the purposes of this investigation, each recorded location was considered to contain a different sub-population.

5.2.2 Distribution data and assembling the data set

An online search was performed through the GBIF data portal for all recorded occurrences of *Restrepia* species. The search results were downloaded as an Excel file. This data set of 753 entries contained many synonyms, duplicated entries, false entries (other genera with similar names) and entries with insufficient information for the purpose of this investigation (i.e. no collection and location data). Only those entries that contained adequate location data to enable later entry into GeoCAT were retained. In addition, data fields not required for this investigation were deleted.

This initial database search produced no records for some species and a subsequent online search of the New York Botanic Gardens Herbarium (C.V. Starr Virtual Herbarium, 2013) produced no additional data. Online searches of Tropicos and the Kew Herbarium for data regarding the missing species also yielded limited results. Details of the geographic distribution as described by Luer (1996a) were the only other data available and therefore were included in the data set for these species.

A complete list of the online herbaria searched (via GBIF) and other reference resources is shown in Table 5-1. A list of the species and their corresponding data sources is shown in Table 5-4. The resulting data set (Set A) contained the following fields: species name, collection year, location data (descriptions only) and altitude. Not all fields were complete for every species. The descriptive location data were later used for entering species details into GeoCAT.

5.2.3 Using GeoCAT

5.2.3.1 Entering data

The initial search produced latitude and longitude coordinates for very few of the species. When these were entered into GeoCAT they were found to be very inaccurate and could not be used. This meant that one very powerful feature of GeoCAT, i.e. importing spatially referenced primary occurrence data quickly and accurately could not be implemented. As a result each location point had to be entered individually. This process involved reference to location descriptions and altitude details from the data set, together with other distribution details (Luer, 1996a). The location descriptions for some points were not very accurate, but as many as possible from the data available were entered. Once entered, latitude and longitude details for each of these entries were subsequently calculated by GeoCAT.

Table 5:4 Sources of occurrence data used in the final data set for each species

	Sources of data used in the final data set		
Restrepia species	Global Biodiversity Information Facility (GBIF)	Luer (1996a)	other
R. aberrans	GBIF	Luer (1996a,b)	
R. antennifera	GBIF	Luer (1996a)	
R. aristulifera	GBIF		
R. aspasiensis	GBIF		
R. brachypus	GBIF	Luer (1996a)	
R. chameleon	GBIF		
R. chocoensis	GBIF	Luer (1996a)	S Manning[1]
R. citrina	GBIF		
R. condorensis	GBIF		
R. contorta	GBIF		
R. cuprea	GBIF		
R. cymbula	GBIF		
R. dodsonii	GBIF		
R. echo	GBIF		
R. elegans	GBIF		
R. ephippium	GBIF		
R. falkenbergii	GBIF		
R. flosculata	GBIF		
R. guttulata	GBIF		
R. iris	GBIF		
R. lansbergii	GBIF		
R. mohrii	GBIF		
R. muscifera	GBIF	Luer (1996a)	
R. nittiorhyncha	GBIF		
R. pandurata	GBIF		
R. pelyx	GBIF		
R. purpurea	GBIF		
R. roseola	GBIF		
R. sanguinea	GBIF		
R. schizosepala	GBIF		
R. teaguei	GBIF		
R. trichoglossa	GBIF	Luer (1996a)	
R. tsubotae	GBIF		
R. vasquezii	GBIF		
R. chrysoglossa		Luer (1996a)	
R. cloesii		Luer (1996a)	
R. echinata		Luer (1996a)	
R. escobarina		Luer (1996a)	
R. jesupiana		Luer (1996a)	

	Sources of data used in the final data set		
Restrepia species	Global Biodiversity Information Facility (GBIF)	Luer (1996a)	other
R. limbata		Luer (1996a)	
R. mendozae		Luer (1996a)	
R. metae		Luer (1996a)	
R. radulifera		Luer (1996a)	
R. renzii		Luer (1996a)	
R. seketii		Luer (1996a)	
R. tabeae		Luer (1996a)	
R. wageneri	GBIF - no location data	Luer (1996a)	
*R. fritillina**			Tropicos
*R. howeii**			Tropicos; C. Howe[2]
*R. persiciana**			Tropicos
*R. piperitosa**	GBIF - Peru, no location data		Tropicos
*R. portillae**	GBIF		Tropicos; D and M Smallman[3]

Notes:

* Species discovered after 1996. Their distribution/location data is very limited, with no collection details available for *R. piperitosa* and *R. howei*

[1], [2] and [3] members of the Pleurothallid Alliance UK.

5.2.3.2 Calculations in GeoCAT

When all available location entries for a species had been entered into GeoCAT, the programme was used to calculate the EOO and AOO values, plus the Red List status for each species' overall geographic range and for each of its countries of origin.

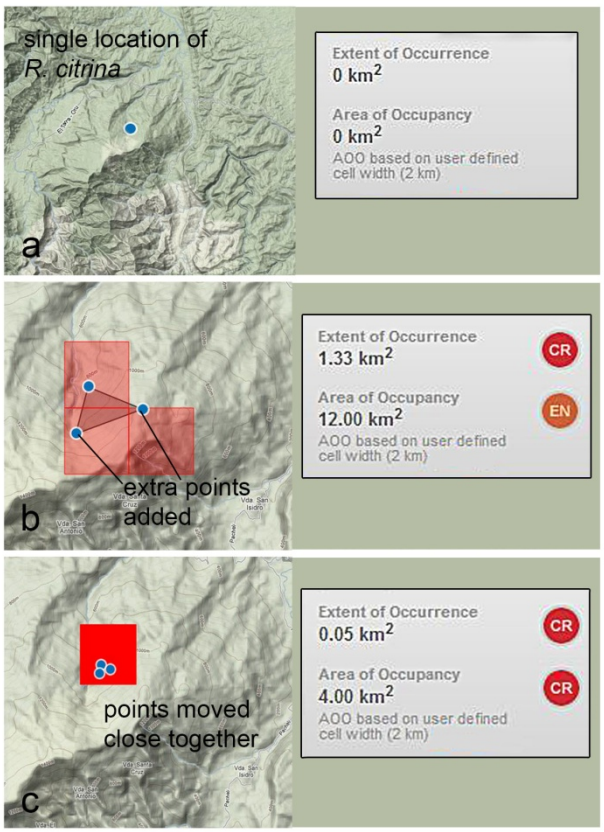
Data entry presented a particular difficulty when there was only one recorded location for a species; the programme could not calculate the EOO and AOO values from such limited data. The solution was to enter 2 extra notional points as illustrated in Figure 5-4. This method produced a value for AOO and a 'notional' one for EOO. When the EOO was less than AOO, the EOO 'notional' values were changed to make them equal to AOO, thus ensuring consistency with the definition of AOO as an area within EOO (IUCN, 2013b). From these data it now became possible to construct tables of the EOO and AOO values together with the Red List status for the complete range and each country of origin for each species (Results: Table 5-5, Section 1).

5.2.3.3 Google Earth and statistical analysis

The geographical details (latitude and longitude coordinates) recorded for each species overall range were exported from GeoCAT as XML files. These were then imported into Google Earth[1] and each position examined to establish if there was still suitable forest habitat at that location. This involved over 400 locations, many of which were difficult to interpret as details were not always clearly visible. Incidences of the type of habitat loss found are included in the Results (Figures 5-7 to 5-12) using *R. antennifera, R. trichoglossa* and *R. roseola* as exemplars.

Details of the locations where forest habitat had been lost were entered into the

[1] It should be noted here that the GeoCAT programme is currently in beta form and still under development. One such development is that it is now possible to export data directly from GeoCAT into Google Earth without going through this intermediate stage. This had been required previously and was used in this investigation.



Single location entry for *Restrepia citrina.*

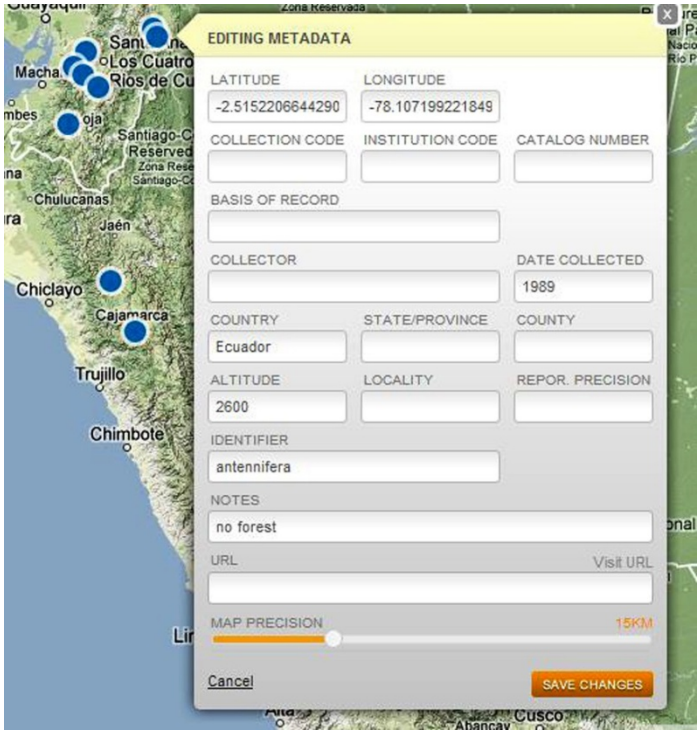
For both single and double locations, EOO and AOO are not calculated by the programme.

By adding two extra points, EOO and AOO may be calculated. However this produces values which are too high.
AOO for a single point is based on a cell width of 2cm, giving a value of 4 km^2

If the points are moved closer together, this reduces the estimates of EOO and AOO

When close enough, the AOO will be calculated as 4.00 km^2, which is the correct value for one point location.

Figure 5-4: Entering species with only one or two recorded locations in GeoCAT. These examples have been constructed using 'model' screenshots from GeoCAT.



Meta data file for one location of *R. antennifera,* GeoCAT

This record shows that this location for *R. antennifera* is: in Ecuador, 2600m altitude, was collected in 1989 and the original forest has been lost.

The details have to be entered for each separate point. The latitude and longitude coordinates are calculated by GeoCAT and then saved automatically.

Figure 5-5: Entering meta data for each location point in GeoCAT
Metadata provides information about an item's content. In this example the meta data provides further information about each of the location points in the distribution map. The details were derived from the information in the data set (Set B) in which details of forest/habitat loss had been identified from Google Earth images.

corresponding meta data file for each location in GeoCAT, an example of which is shown (Figure 5-5). These meta data were then exported as a Comma Separated Values or CSV (.csv) file which could be imported into Excel. This final data set (Set B)[2] now contained the following fields: species name, collection year, altitude, location data (latitude and longitude) and any habitat loss/sub-population loss. Not all fields were complete for every location; for example, the collection year and/or altitude might be missing. From these data, the correlations between collection year, altitude and habitat loss were calculated for the complete *Restrepia* range and each of its native countries. The altitudinal ranges at which the majority of collections/discoveries have been made were shown in the form of a histogram. Further distribution graphs were then made for a few representative species.

5.2.3.4 Correction for habitat loss

In GeoCat it is possible to 'hide' locations within a distribution and thereby exclude them from calculations. All locations that had undergone habitat loss were treated in this way. Screenshots of the distribution, country by country, for every species with 'hidden' or 'greyed' out points for habitat loss were saved. When the locations were clustered together, then larger scale screenshots of the distribution were saved. This was because GeoCAT generates relatively large points on a smaller scale map and these can often overlap, making details difficult to determine. Figure 5-6 illustrates part of the range for *R. contorta* and how these 'hidden' points may be utilised.

New EOO and AOO values were then calculated following this correction for habitat loss. A second table was then constructed of the new EOO and AOO values, plus the corresponding Red List status for the complete range and country of origin for each species, (Results: Table 5-7, Section 2).

[2] This is the data set referred to as Set B in the notes to Table 5-6 and Table 5-7, Section 1.

Figure 5-6: 'Hidden' points in GeoCAT screenshot

Part of the range for *R. contorta* in Colombia and Ecuador is shown. Each point represents the estimated location of a sub-population of *R. contorta.* Some overlapping points have been moved slightly for clarity on this representation.

(a) 'Hidden' points shown in pale blue, indicating locations where habitat has been lost;
(b) Normal point shown in dark blue, indicating locations where there is still suitable habitat remaining.

The example above contains 16 locations with remaining habitat and 8 location where habitat has been lost, a habitat loss of 33% in the sample area shown.

5.2.4 Preliminary Red List Status

Although GeoCAT produces Red List values from the calculated EOO and AOO values, this in itself does not complete a Red List assessment under criterion B (geographic range), which requires additional sub-criteria to be met.

Red List assessment involves evaluating data regarding a taxon against five criteria (A-E), any one of which may be used to identify the threat to the taxon, since not all of these criteria are applicable to or suitable for all species or genera. The criteria may be summarised as: criterion A- population reduction, criterion B - geographic range, including EOO and AOO, criterion C - population decline, criterion D - small or restricted populations and criterion E - quantitative analysis of extinction probability. The formal summary of the criteria and their sub-categories, (IUCN, 2013c), is shown in Table 5-3.

5.2.4.1 Analysing the five criteria (A–E) to identify the criteria and categories suitable for evaluating the category of threat for **Restrepia** *species.*

With regard to *Restrepia* species and the data available from this investigation, only three of the criteria could be used to assess their level of threat: population reduction (criterion A), geographic range (criterion B) and very small or restricted population (criterion D). Criterion C, small population size and decline, could not be evaluated from the available data as it involved estimating the number of mature individuals over time. The locations recorded in the current data do not indicate sub-population size, but only where such have been discovered or subsequently recorded. Criterion E, quantitative analysis to estimate the probability of extinction in the wild, had not been carried out.

5.2.4.1.1 Criterion A: Population reduction

Under this Criterion, the decline in population should be measured over 10 years or 3 generations whichever is the longer. The quantitative requirements for Criterion A are shown in Table 5-5a. The reduction in population may be identified in various ways by either -

(a) direct observation

(b) an index of abundance appropriate to the taxon

(c) a decline in AOO, EOO and/or habitat quality

(d) actual or potential levels of exploitation

(e) effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.

Since each of the sub-categories (A1, A2, A3 and A4) may be met by specifying any of the above points, not all the points (a-e) are needed to assess threat under this criterion. In this investigation (a) and (b) were not possible and (d) and (e) could not be estimated. This left only (c) that could be assessed under criterion A. The data used in this investigation include records made over the past 50 years and thus fulfill the requirement that the population decline should have been measured over 10 years. The monograph by Luer was published in 1996, which means that data included from this source are nearly 20 years old. Calculating the reduction in sub-populations gave an estimate for both the decline in sub-populations in each country of origin and the decline in population for the entire range of the species.

The difference between the four sub-categories is based on whether the reduction can be regarded as past, present, future, ceased or continuing (Table 5-3, A1, A2, A3 and A4). From the data available no assumption could be made as to whether any reduction had ceased, or was reversible (A1); future reduction could not be projected

(A3 and A4) which left A2c as the most easily most quantifiable sub-criterion. A2c, indicates a past reduction that has not ceased, as shown by a decline in AOO, EOO and/or habitat quality. This sub-criterion used for the assessment of *Restrepia* species.

5.2.4.1.2 Criterion B: Geographic range

The geographic range for a species may be recorded as EOO or AOO. The numerical limits used to categorise the level of threat are shown in Table 5-5b. If either the EOO or AOO meets these quantitative requirements, then in order to fulfil this criterion, two out of the additional requirements (Table 5-5c) must also be met.

In the current investigation, EOO and AOO were calculated using the GeoCat tool and the numbers of locations were determined from records. The continuing decline, b (i, ii and iv) was calculated by comparing present EOO and AOO values calculated from current locations with previous values. Direct assessments of b (iii) and b (v) were not carried out using the available data. Extreme fluctuations, c, were difficult to assess and were excluded from the assessment. This left a and b, which were used in the assessment of this criterion (Table 5-5d).

In order to make a full assessment of Criterion B: Geographic Range, B1 and 2 (EOO and AOO), a (number of locations), and b (i,ii,iv) (continuing decline in any of EOO, AOO or number of locations) were used.

5.2.4.1.3 Criterion D: Very small or restricted populations

Many *Restrepia* species are only known from a few locations and some may have been collected only once. These locations do not give any indication of the sub-population size and so cannot be used to assess D1, which requires the number of mature individuals to be known. However, the data collected can be used to assess D2, in the VU category only; this is based solely on the size of AOO and/or number of locations. The numerical limits for Criterion D are shown in Table 5-5e. This left the following

Table 5-5a: Quantitative requirements for criterion A[1] (IUCN, 2013c)

	Critically Endangered	Endangered	Vulnerable
A1	≥ 90%	≥ 70%	≥ 50%
A2, A3 & A4	≥ 80%	≥ 50%	≥ 30%

Table 5-5b: Quantitative requirements for criterion B[1] (IUCN, 2013c)

	Critically Endangered	Endangered	Vulnerable
B1. Extent of occurrence (EOO)	< 100 km²	< 5,000 km²	< 20,000 km²
B2. Area of occupancy (AOO)	< 10 km²	< 500 km²	< 2,000 km²

Table 5-5c: Additional requirements for criterion B[1] (IUCN, 2013c)

	Critically Endangered	Endangered	Vulnerable
a. Number of locations	= 1	≤ 5	≤ 10
b.Continuing decline in any of:	(i) extent of occurrence; (ii) area of occupancy; (iii) area, extent and/or quality of habitat; - not assessed (iv) number of locations or sub-populations; (v) number of mature individuals – not assessed		
c.Extreme fluctuations in any of:	**(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** number of locations or subs; **(iv)** number of mature individuals.		

Notes:

[1]Details are taken from the Red List Summary Sheet (IUCN, 2013c).

Table 5-5d: Additional requirements for criterion B[1] (IUCN, 2013c)

	Critically Endangered	Endangered	Vulnerable
a. Number of locations	= 1	≤ 5	≤ 10
b. Continuing decline in any of:	(i) extent of occurrence; (ii) area of occupancy; (iv) number of locations or sub-populations;		

Table 5-5e: Quantitative requirements for criterion D[1] (IUCN, 2013c)

	Critically Endangered	Endangered	Vulnerable
D1. Number of mature individuals	< 50	< 250	< 1,000
D2. Restricted AOO or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.			AOO < 20 km² Number of locations ≤ 5

Table 5-5f: Additional requirements for criterion D[1] (IUCN, 2013c)

	Vulnerable
D2. Restricted AOO or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.	AOO < 20 km² Number of locations ≤ 5

Notes:

[1]Details are taken from the Red List Summary Sheet (IUCN, 2013c).

sub-criteria with which it was possible to assess criterion D using data from this investigation.

5.2.5 Calculations and assessments

Using the EOO and AOO values produced after correction for habitat loss together with the remaining recorded locations following habitat loss (Table 5-7, Section 2), the percentage declines in EOO, AOO and location numbers were calculated (Table 5-8, columns (b), (c) and (d)). Each species was subsequently assessed to determine which of the above quantitative criteria (A, B and C) were satisfied (Tables 5-9 and 5-10). In Table 5-10, the summary of the assessed Red List criteria follows the hierarchical alphanumeric numbering system of criteria and sub-criteria from the IUCN guidelines (IUCN, 2012a).

5.2.5.1 Amending the preliminary Red List calculations

The values produced in Table 5-10 represent every quantitative value that each species has met for the criteria and categories. There are strict guidelines (IUCN, 2012b; 2013b) for assigning the final category of risk for a species and these purely quantitative figures represent the initial stage in this process. In particular, a species can only be assessed as at risk under Criterion B if sub-categories B1 and/or B2 together with (a) and (b) are also met (Table 5-5c; see also Table 5-3).

In addition, other factors need to be taken into consideration, before a final category of risk can be assigned; for example, the type of risk a species is facing. Loss of habitat may occur through natural causes, as well as through human activity. Conversely, a sub-population may persist in a protected habitat and not face immediate habitat loss, despite being depleted.

5.2.5.2 Establishing patterns of habitat loss

The saved screenshots of species distributions with grey points for lost sub-populations, i.e. positions where suitable habitat had been lost (e.g. Figure 5-6), were superimposed onto previously created maps of each individual country using Adobe Photoshop CS6. Care was taken to ensure that the alignment was accurate. The position of each point could then be transferred onto a new layer in the Photoshop map. This had the following layers –outline map, physical features, major road(s), main towns and the locations of national parks, reserves or private reserves. The colour code for the position of sub-populations was: red to indicate positions where sub-populations had been lost; blue to indicate positions where sub-populations still existed, but the species had lost sub-populations elsewhere and purple to indicate positions of species that had lost no sub-populations. Each of these was created on a separate layer of the map that could be manipulated individually.

Once the positions of the sub-populations for each country's indigenous *Restrepia* species had been positioned it was possible to identify patterns in sub-population decline. The positions of the remaining sub-populations were compared to establish if they occurred in conservation areas such as nature reserves or national parks and were therefore less threatened. The Red List assessment could be adjusted if any species currently persisted in a 'safe' location and the risk was clearly reduced. This affects Criterion A2, in which the threat to a taxon is considered to be ongoing. For sub-populations in protected locations, there is no immediate threat of loss of habitat. This means that such sub-populations cannot be considered threatened using Criterion A2. This exercise produced a final Red List assessment for each species (Tables 5-11 to 5-16). A summary of habitat loss in each country and a table of each species and their simplified Red List criteria are presented in Figure 5-20 and Table 5-17.

5.3 Results

5.3.1 Google Earth imagery

Some of the 'typical' types of habitat loss and historical imagery recorded from Google Earth[1] are presented in Figures 5-7 to 5-11. An example of the historical imagery available is presented in Figure 5-7 (Google Earth, 2013a). This sequence, taken from Western Ecuador, shows: (a) virtually intact forest cover in 1970; (b) slight fragmentation in 2004 and (c) near complete deforestation five years later in 2009. During the time period 2004 – 2009, land use changed primarily to farming with only scattered patches of the original forest remaining. This example illustrates the rapid and extensive rate of deforestation and changes in land use that has occurred in recent years. This pattern of habitat loss was observed for many of the 'lost' locations for *Restrepia* species, some further examples of which are shown in Figures 5-8 to 5-11.

Similar patterns of habitat loss were also observed in Central America, an example of which is shown in Figure 5-8 (Google Earth, 2013b) to illustrate the loss of habitat for *R. trichoglossa.* In this screenshot from Google Earth, the land is primarily farm land and no longer forest. This is evident from the pattern of fields and roads, while a few fragmented patches of forest still remain on the left hand side of the image. However, 38 out of the 55 original recorded locations for *R. trichoglossa* still remain elsewhere.

The only recorded location for *R. roseola* is shown in Figure 5-9 (Google Earth, 2013c), in which the forest cover has been replaced by urban development and road building. The small fragmented patches of forest that remain may also have been lost by now. *R. roseola* is probably extinct in this location in Venezuela, but other unrecorded sub-populations may persist elsewhere.

[1]By default, the clearest available imagery is always displayed in Google Earth. It is possible to view any historical imagery available, using the time slider to observe changes over time

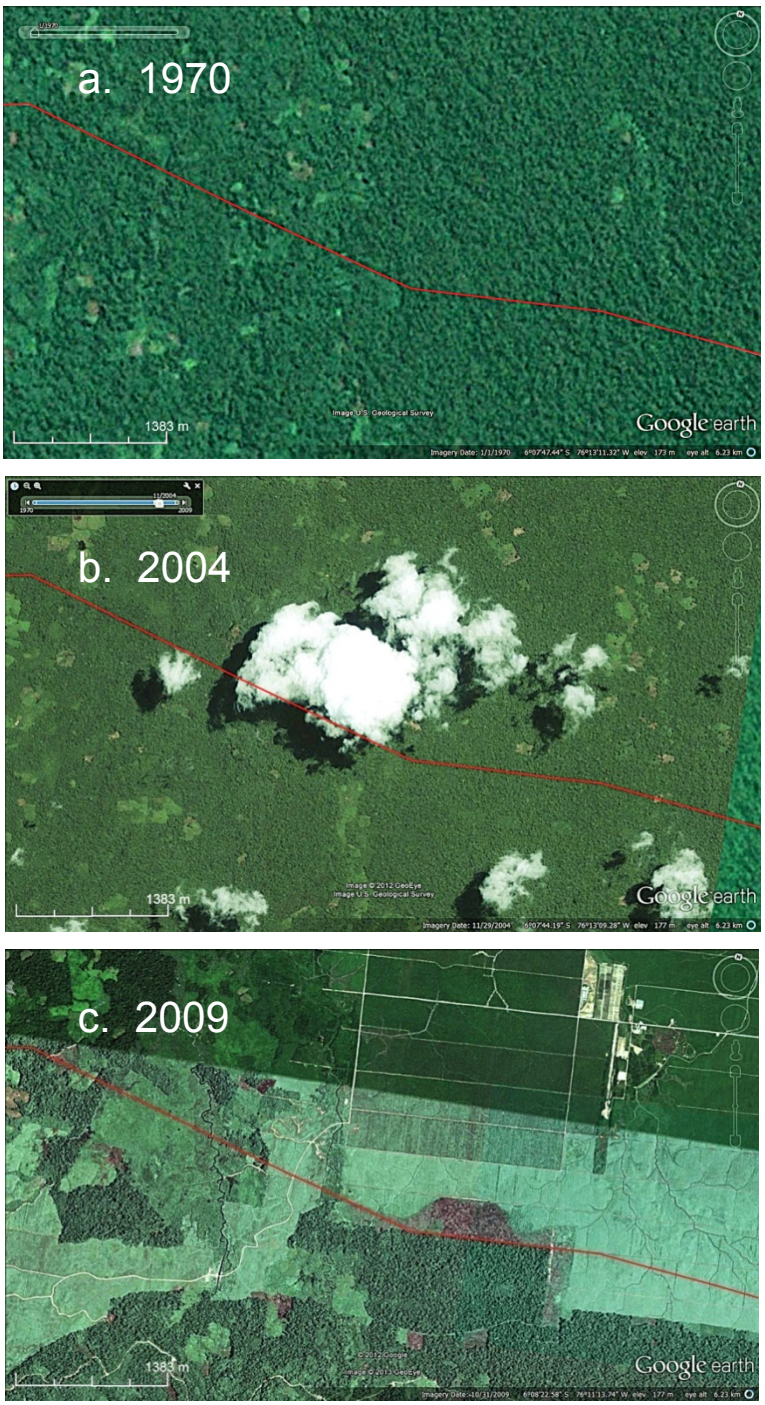


Figure 5-7: Stages of habitat loss, Western Colombia (Google Earth, 2013a).

Regional boundary is shown in red for clarity on each map.

(a) Intact forest cover - 1970
(b) Thirty years later (2004) slight degradation of cover
(c) Five years later (2009) evidence of changes in land use and most of the forest cover has now been lost.

Figure 5-8: Habitat loss, *R. trichoglossa,* Central America (Google Earth, 2013b).
Yellow (Google Earth) marker indicates the recorded location of *R. trichoglossa*. Land use has changed to mainly farming with a few very fragmented patches of forest (left hand side). However, 38 out of 55 original recorded locations remain elsewhere.

Figure 5-9: Habitat loss, *R. Roseola,* Venezuela (Google Earth, 2013c).
This shows the only recorded location for *R. roseola.* Very small fragmented patches of forest remain; *R. roseola* is probably extinct in this location but may persist elsewhere.

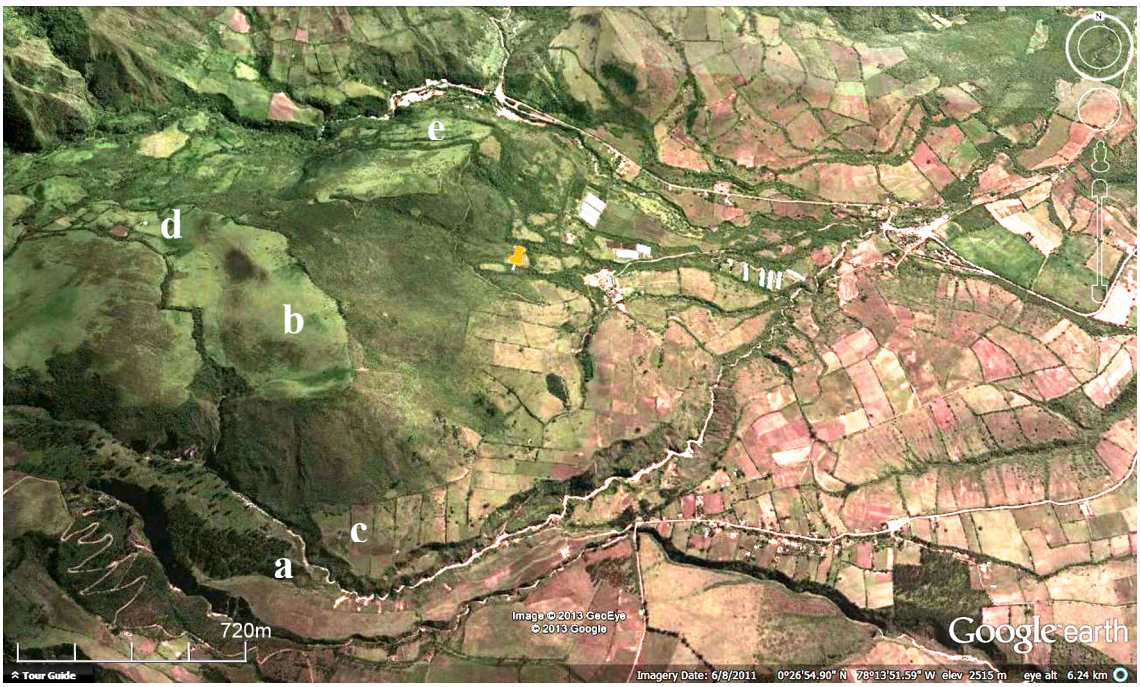


Figure 5-10: Habitat loss, *R. antennifera,* Colombia (Google Earth, 2013d).
Yellow (Google Earth) marker indicates the recorded location of *R. antennifera*. (a) One fragmented patch of forest remains; (b) forest has been cleared; (c), (d) and (e) fields replace the original forest cover.

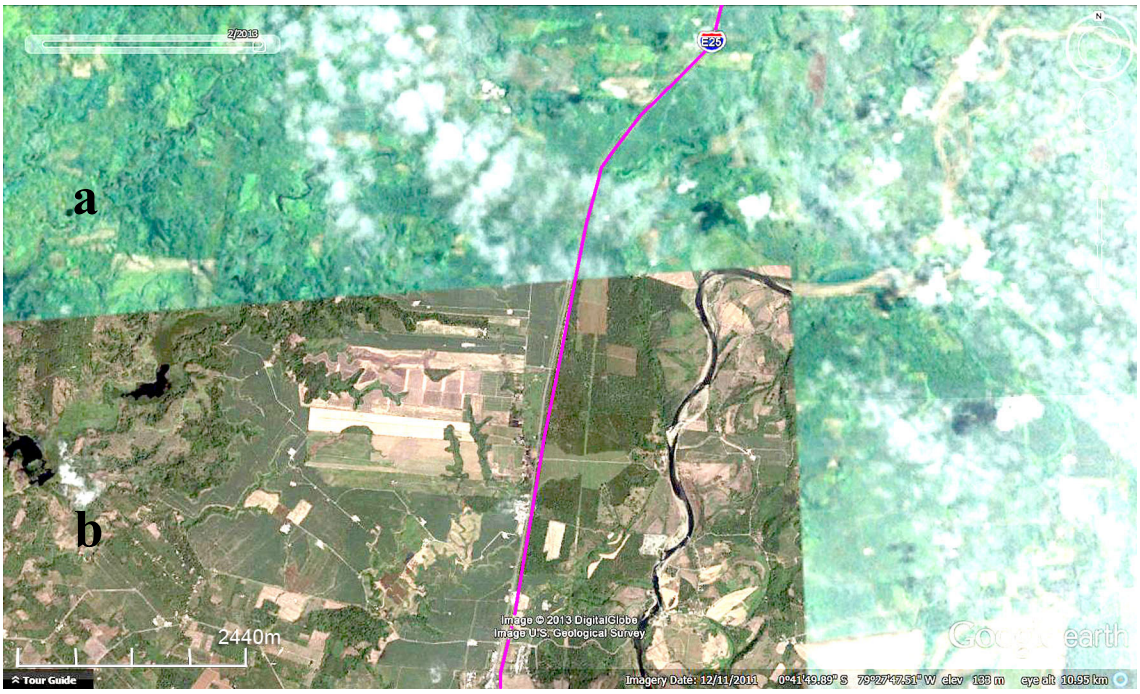


Figure 5-11: Example of habitat loss occurring along the Pan-American Highway, Colombia (Google Earth, 2013e).
Pan-American Highway is highlighted in purple; (a) historic satellite imagery, 1970, which shows the original forest cover, and (b) current satellite imagery, 2011. The changes in land use that have occurred since 1970 may be seen by comparing (a) and (b).

Further habitat loss is illustrated in Figure 5-10 (Google Earth, 2013d) which shows one of the recorded locations for *R. antennifera.* In this example, much of the land is now agricultural and many roads have been built in the area. There is one fragmented patch of forest left on the left hand side of the picture (a), there are indications that this is being increasingly used for farm land as fields can be observed adjacent to it. Part of the forest has been cleared (b) and fields appear to be encroaching on the rest (c, d and e). *R. antennifera* is a common species and there are still 19 out of the original 37 recorded locations remaining elsewhere.

A section of the Pan-American Highway in Colombia is illustrated in Figure 5-11 (Google Earth, 2013e). Historical imagery (1970) is displayed (a) together with the satellite imagery for 2011 (b).These two contrasting views show the increase in environmental change that has occurred in this area since the highway was completed. This is typical of the habitat loss that was observed (using Google Earth) along the length of this highway throughout Central and South America.

5.3.2 Statistical evaluation of data set

The narrow altitudinal band in which most *Restrepia* species naturally occur is illustrated in Figure 5-12. This figure shows the collection frequency and altitude at which the species were discovered and shows that the highest collection frequencies have been between 1500 and 2000 metres, with very few collected below 1000 metres or above 3000 metres.

The correlation results are presented in Table 5-6. No strong correlation was found between either collection year and habitat loss, collection year and altitude or forest loss and altitude. This was true for the complete *Restrepia* range and in each of its native countries, as confirmed by the correlation coefficients ($r < 0.6$ in all instances). In Peru, the correlation between collection year and altitude and between forest loss and altitude

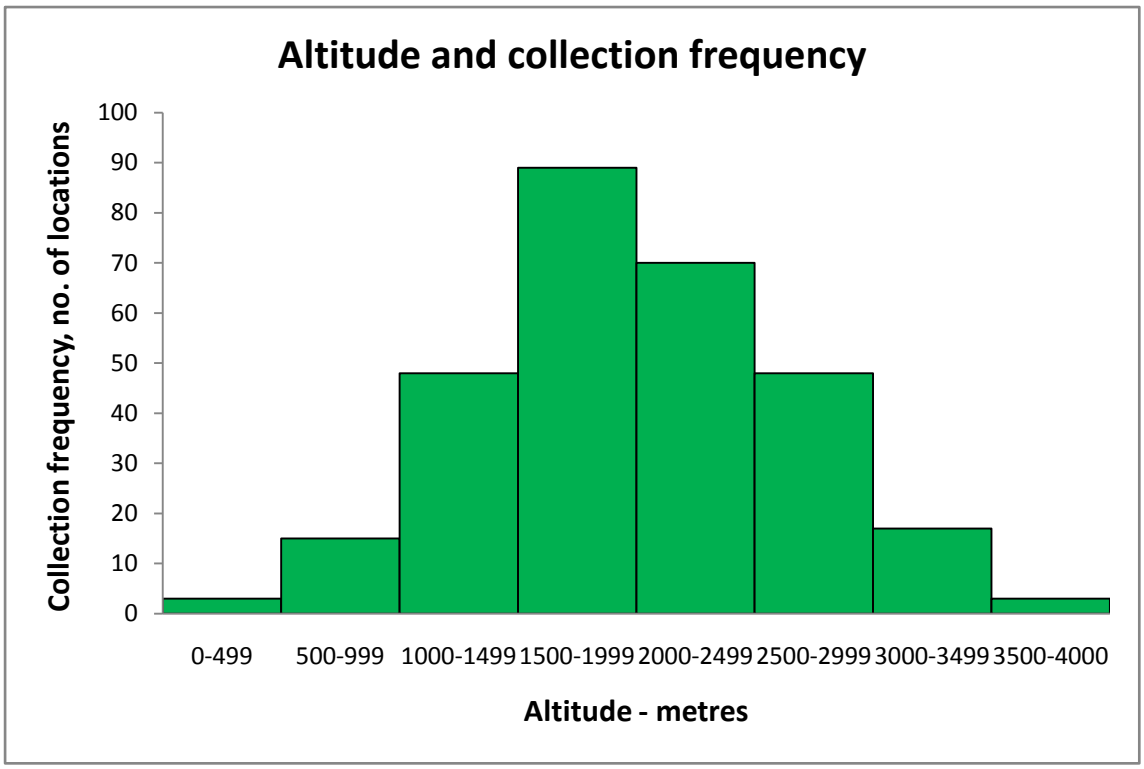


Figure 5-12: Relationship between collection frequency and altitude. The majority of collections have been between 1500 and 2000 metres, with correspondingly few below 1000 metres and above 3000 metres. See footnote [1]

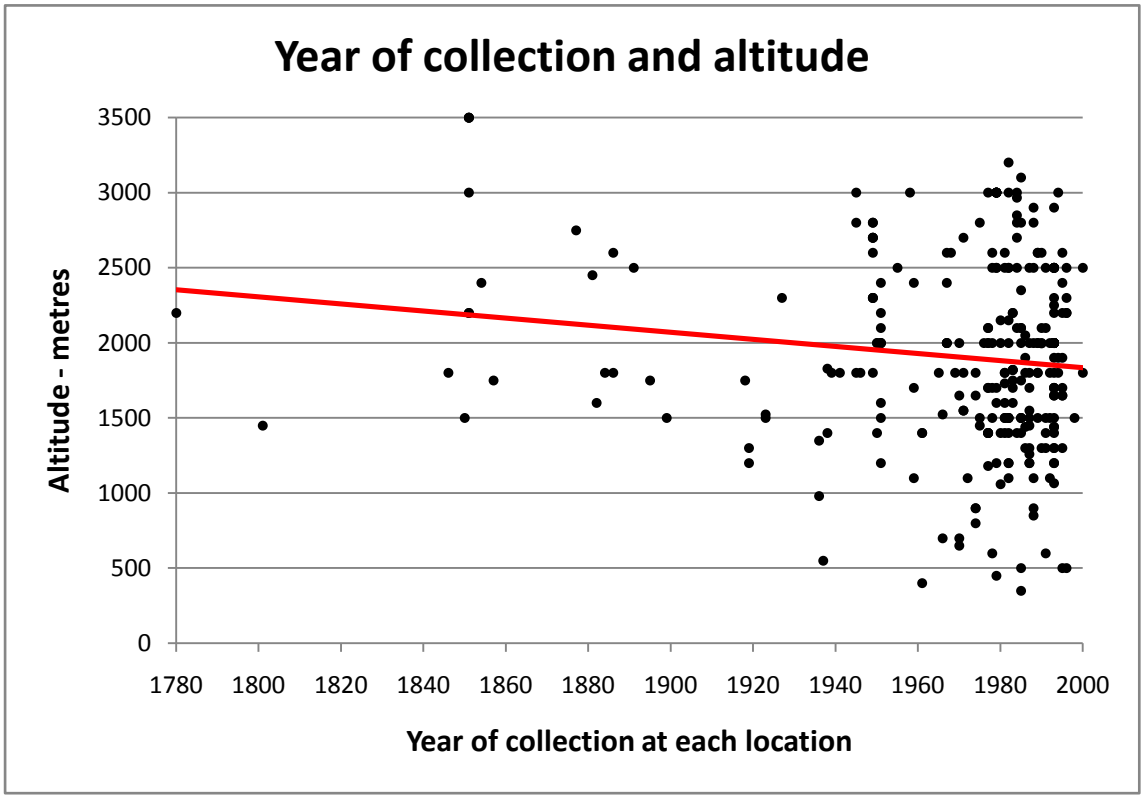


Figure 5-13: The relationship between year of collection and altitude. The linear trend line ($r = -0.136$; $n = 289$; $p < 0.05$) indicating a very weak but nevertheless significant correlation. See footnote [1]

[1] Figures 5-12 and 5-13 were produced from data in Set B, see section *5.2.3.3*

Table 5-6: Summary of correlations between collection year, forest loss and altitude for the complete *Restrepia* range and also in individual countries, using data from Set B [1]

		Coll. year/forest loss		Coll. year/altitude		Forest loss/altitude	
	n	r	p	r	p	r	p
Complete Range	289	0.106	0.070	-0.136	0.02*	-0.049	0.410
Colombia	94	0.105	0.310	-0.243	0.01**	-0.049	0.630
Ecuador	81	0.036	0.750	-0.117	0.290	-0.171	0.126
Central America	57	-0.090	0.505	0.036	0.790	0.038	0.778
Venezuela	38	0.280	0.090	0.181	0.270	-0.035	0.830
Peru	10	0.365	0.299	-0.496	0.144	-0.464	0.176
Bolivia	9	-0.098	0.801	-0.565	0.112	0.584	0.098

Notes:

[1]final data set (Set B) – section *5.2.3.3*

A significant correlations is denoted by * ($p < 0.05$) and a very significant correlation by ** ($p < 0.01$).

The negative correlation between collection year and altitude is significant for the complete range of species in all countries ($r = -0.136$; $n = 289$; $p = 0.02$) and in Colombia alone ($r = -0.243$; $n = 94$; $p = 0.01$), although the effect is very weak. There is no significant effect for any other country.

was stronger than elsewhere (r=-0.496 and -0.464 respectively) but this was not significant because of the much smaller sample size (n=10). A similar situation was found in Bolivia for the correlation between collection year and altitude and between forest loss and altitude (r=-0.565 and 0.584 respectively; n = 9). There was, however, a significant but weak negative correlation between collection year and altitude for Columbia ($r = -0.243$; $n = 94$; $p < 0.01$) and also for the complete range of countries ($r = -0.136$; $n = 289$; $p < 0.05$). Data for collection year and altitude for all the countries are presented in more detail in Figure 5-13. The vast majority of points occur after 1960, with most occurring after 1980. The linear trend line illustrates how the later collections have tended to be at lower altitudes as mentioned in the previous paragraph.

Distribution graphs for a representative sample of the species are shown in Figure 5-14. *R. antennifera* and *R. contorta,* both examples of common and widespread species, have undergone substantial loss throughout their range. There are no remaining recorded locations for *R. antennifera* in Peru and *R. contorta* has only one remaining location in Venezuela. In contrast, *R. muscifera* has lost many locations in Central America and very few in Colombia and Ecuador. *R. pelyx*, a less common species, has fewer recorded locations (5) but has lost few of these. However, it is worth noting that with few recorded locations, a loss of only one or two represents a large percentage loss. This also applies to *R. muscifera* in Colombia and Ecuador. The final two species, *R. citrina* and *R. purpurea,* have very few recorded locations and have not lost any of them. Their global range is the same as their national range as they have only been recorded in one country.

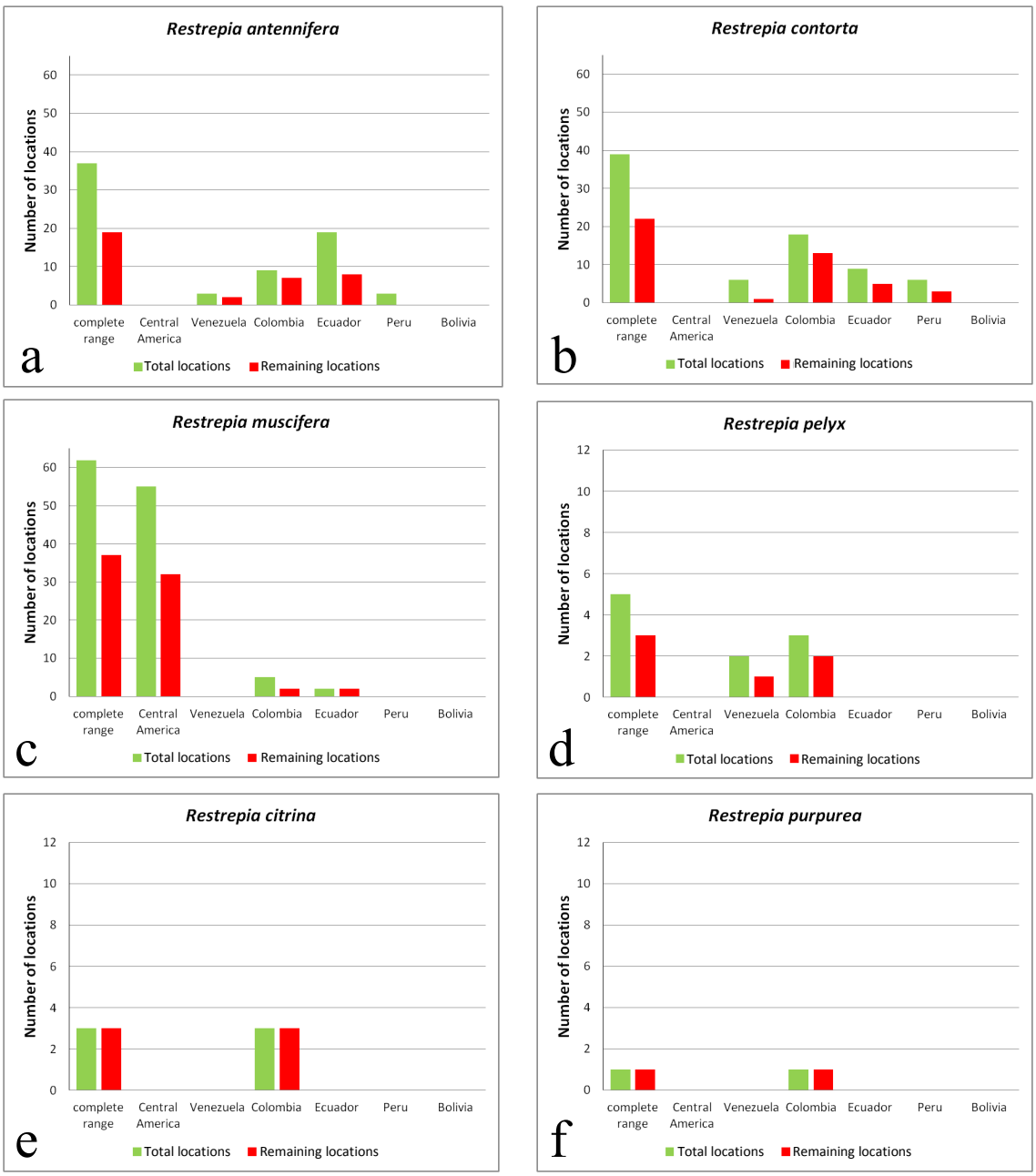


Figure 5-14: Distribution graphs of some *Restrepia* species[1]

(a) *R. antennifera* and (b) *R. contorta* are both common species with wide distributions in S. America. Although many of their locations remain, (19/37, *R. antennifera* and 22/39 *R. contorta*) they have undergone substantial location losses of 48.6% and 43.6% respectively throughout their ranges.

(c) *R. muscifera* is common in Central America where 32/55 locations remain, a substantial loss of approximately 40%. It is less common in Colombia and Ecuador where losses have been less severe.

(d) *R. pelyx* is a less common species; 3/5 locations remain but this still represents a substantial 40% loss.

(e) and (f) *R. citrina* and *R. purpurea* are narrow endemic species with few recorded locations from one country of origin. In contrast, such species have not undergone habitat loss, one explanation being that these locations were discovered within protected areas, such as nature reserves.

[1] all the graphs were produced using the final data set (Set B) – section *5.2.3.3*

5.3.3 GeoCat results

EOO and AOO values as calculated by GeoCAT using all the recorded locations are presented in Table 5-7, Section1. The EOO and AOO values as calculated by GeoCAT after allowing for habitat loss (i.e. loss of locations) are presented in Table 5-7, Section 2. After adjusting for habitat loss, the Red List status for AOO was found to be either EN or CR. The Red List status changed from EN to CR if the number of remaining locations fell below three, as with *R. aspasiensis* and *R. cuprea.* The Red List status values for EOO values ranged from LC to CR, e.g. *R. trichoglossa,* LC and *R. pandurata,* CR.

Data in Table 5-7, Section 2, were used to calculate the values presented in Table 5-8: the number of locations remaining after habitat loss; percentage loss of habitat (= percentage loss of locations); percentage change in EOO and percentage change in AOO. The figures in Table 5-8 were used to produce the preliminary Red List assessments presented in Table 5-10 and Table 16, Stage 1.

Table 5-7: EOO and AOO values and their accompanying Red List status as calculated by GeoCAT

		Section 1: EOO, AOO and Red List status calculated by GeoCat after entering all recorded locations for each species from data Set B[1]					Section 2: EOO, AOO and Red List status calculated by GeoCAT using remaining locations after allowing for habitat loss, as identified in Goggle Earth imagery.				
		EOO km^2	STATUS	AOO km^2	STATUS	locations	EOO km^2	STATUS	AOO km^2	STATUS	remaining locations
R. aberrans	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Central America	4	CR	4	CR	1	4	CR	4	CR	1
R. antennifera	Complete range	1998657.2	LC	148	EN	37	1796828.21	LC	76	EN	19
	Venezuela	73.31	CR	12	EN	3	16.7	CR	8	EN	2
	Colombia	99027.64	LC	36	EN	9	97471.15	LC	28	EN	7
	Ecuador	37765.49	NT	76	EN	19	31637.26	NT	32	EN	8
	Peru	372.84	EN	12	EN	3	94.43	CR	8	EN	2
R. aristulifera	Complete range	7205.9	VU	36	EN	9	3409.36	EN	20	EN	5
	Venezuela	2834.98	EN	24	EN	6	771.84	EN	12	EN	3
	Colombia	23.72	CR	12	EN	3	8	CR	8	CR	2
R. aspasiensis	Complete range	885.24	EN	12	EN	3	4	CR	4	CR	1
	Venezuela	4	CR	4	EN	1					0
	Colombia	8	CR	8	EN	2	4	CR	4	CR	1
R. brachypus	Complete range	1781104	LC	164	EN	41	1270565	LC	96	EN	25
	Colombia	90887.36	LC	92	EN	23	63302.99	LC	64	EN	16
	Ecuador	48664.2	LC	60	EN	15	36065.79	NT	32	EN	8
	Peru	4	CR	4	CR	1					0
	Bolivia	8	CR	8	EN	2	4	CR	4	CR	1

Table 5-7: continued

		E00 km^2	STATUS	AOO km^2	STATUS	locations	E00 km^2	STATUS	AOO km^2	STATUS	locations
R. chameleon	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. chocoensis	Complete range	25.87	CR	12	EN	3	25.87	CR	12	EN	3
	Colombia	25.87	CR	12	EN	3	25.87	CR	12	EN	3
R. chrysoglossa	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. citrina	Complete range	12	CR	12	EN	3	12	CR	12	EN	3
	Colombia	12	CR	12	EN	3	12	CR	12	EN	3
R. cloesii	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Peru	4	CR	4	CR	1	4	CR	4	CR	1
R. condorensis	Complete range	14.08	CR	12	EN	3	14.08	CR	12	EN	3
	Ecuador	14.08	CR	12	EN	3	14.08	CR	12	EN	3
R. contorta	Complete range	1266506	LC	156	EN	39	1002340	LC	88	EN	22
	Venezuela	36028.85	NT	24	EN	6	4	CR	4	CR	1
	Colombia	228075.27	LC	72	EN	18	215752	LC	52	EN	13
	Ecuador	36150.87	NT	36	EN	9	3032	EN	20	EN	5
	Peru	18458.6	VU	24	EN	6	2972	EN	12	EN	3
R. cuprea	Complete range	4954.95	EN	16	EN	4	18.06	CR	8	CR	2
	Colombia	4954.95	EN	16	EN	4	18.06	CR	8	CR	2
R. cymbula	Complete range	4	CR	4	EN	1	4	CR	4	EN	1
	Ecuador	4	CR	4	EN	1	4	CR	4	EN	1
R. dodsonii	Complete range	4413.57	EN	44	EN	11	1904.23	EN	20	EN	5
	Ecuador	4413.57	EN	44	EN	11	1904.23	EN	20	EN	5

Table 5-7: continued

		E00 km^2	STATUS	AOO km^2	STATUS	locations	E00 km^2	STATUS	AOO km^2	STATUS	locations
R. echinata	Complete range	95,313.95	LC	12	EN	3	95,313.95	LC	12	EN	3
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
	Peru	11.28	CR	8	CR	2	11.28	CR	8	CR	2
	Ecuador	1351.63	EN	20	EN	5	586.53	EN	16	EN	4
R. jesupiana	Complete range	2897.1	EN	16	EN	4	43.16	CR	8	CR	2
	Venezuela	2897.1	EN	16	EN	4	43.16	CR	8	CR	2
	Ecuador	1197.1	EN	12	EN	3	8	CR	8	EN	2
	Peru	4	CR	4	CR	1	4	CRR	4	CR	1
R. limbata	Complete range	8	CR	8	CR	2	8	CR	8	CR	2
	Colombia	8	CR	8	CR	2	8	CR	8	CR	2
R. mendozae	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Ecuador	4	CR	4	CR	1	4	CR	4	CR	1
R. metae	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. mohrii	Complete range	9871.56	VU	12	EN	3	139.27	EN	8	EN	2
	Peru	9871.56	VU	12	EN	3	139.27	EN	8	EN	2
R. muscifera	Complete range	1705831.83	LC	248	EN	62	1682108.03	LC	144	EN	37
	Central America	539444.66	LC	220	EN	55	534506.46	LC	128	EN	32
	Colombia	7346.63	VU	20	EN	5	11.28	CR	8	CR	2
	Ecuador	23.42	CR	8	CR	2	8	CR	8	CR	2
R. nittiorhyncha	Complete range	3562.11	EN	12	EN	3	4	CR	4	CR	1
	Colombia	3562.11	EN	12	EN	3	4	CR	4	CR	1
R. pandurata	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1

Table 5-7: continued

		E00 km^2	STATUS	AOO km^2	STATUS	locations	E00 km^2	STATUS	AOO km^2	STATUS	locations
R. pelyx	Complete range	22081	NT	24	EN	5	6448.79	VU	12	EN	3
	Venezuela	8	CR	8	EN	2	4	CR	4	CR	1
	Colombia	4857	ENN	12	EN	3	62.36	CR	8	EN	2
R. purpurea	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. radulifera	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Venezuela	4	CR	4	CR	1	4	CR	4	CR	1
R. renzii	Complete range	8	CR	8	CR	2	4	CR	4	CR	1
	Venezuela	8	CR	8	CR	2	4	CR	4	CR	1
R. roseola	Complete range	4	CR	4	CR	1					0
	Venezuela	4	CR	4	CR	1					0
R. sanguinea	Complete range	109740.03	LC	36	EN	9	105669.42	LC	24	EN	6
	Venezuela	1191.53	EN	12	EN	3	1191.54	EN	12	EN	3
	Colombia	14796.69	VU	24	EN	6	10726.08	VU	12	EN	3
R. schizosepala	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Ecuador	4	CR	4	CR	1	4	CR	4	CR	1
R. seketii	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. tabeae	Complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. teaguei	Complete range	11.93	CR	8	CR	2	11.93	CR	CR	8	CR
	Ecuadpr	11.93	CR	8	CR	2	11.93	CR	8	CR	2

Table 5-7 continued

		E00 km^2	STATUS	AOO km^2	STATUS	locations	E00 km^2	STATUS	AOO km^2	STATUS	locations
R. trichoglossa	complete range	2030007.2	LC	220	EN	55	1129207.21	LC	152	EN	38
	Central America	190560.95	LC	136	EN	34	16862.29	VU	76	EN	19
	Venezuela	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	38595.46	NT	48	EN	12	28807.86	NT	40	EN	10
	Ecuador	36986.12	NT	32	EN	8	36986.12	NT	32	EN	8
R. tsubotae	complete range	4	CR	4	CR	1	4	CR	4	CR	1
	Colombia	4	CR	4	CR	1	4	CR	4	CR	1
R. vasquezii	complete range	19894.43	VU	16	EN	4	19894.43	VU	16	EN	4
	Bolivia	19894.43	VU	16	EN	4	19894.43	VU	16	EN	4
R. wagnerii	complete range	179.85	EN	8	EN	2	179.85	EN	8	EN	2
	Venezuela	179.85	EN	8	EN	2	179.85	EN	8	EN	2
R. piperitosa*	Peru					1					DD
R. portillae*	Ecuador	4	CR	4	CR	1	4	CR	4	CR	1
R. howeii*	Ecuador					1					DD
R. persiciana*	Ecuador	4	CR	4	CR	1	4	CR	4	CR	1
R. fritillina*	Colombia					1					DD

Notes:

[1] Date set B - see section *5.2.3.3* for explanation.

Pink shading represents the entire range for a species - Global values. Grey shading represents each native country for a species - National values

The species marked * have very little collection data, having been discovered since 2000; their national distributions are the same as their global distributions.

Table 5-8: Number of remaining locations following habitat loss and % loss of habitat[1], and % change of EOO and AOO values throughout complete range, Colombia, Ecuador, Venezuela, Peru, Bolivia and Central America[2].

Restrepia species	Complete range				Colombia				Ecuador				Venezuela				Peru				Bolivia				Central America			
	a[3]	b[4]	c[5]	d[6]	a[3]	b[4]	c[5]	d[6]	a[3]	b[4]	c[5]	d[6]	a[3]	b[4]	c[5]	d[6]	a[3]	b[4]	c[5]	d[6]	a[3]	b[4]	c[5]	d[6]	a[3]	b[4]	c[5]	d[6]
R. aberrans	1	0	0	0																					1	0	0	0
R. antennifera	19	49	10	49	7	22	2	22	8	58	16	58	2	33	77	33	0	100	100	100	2	33	75	33				
R. aristulifera	5	44	53	44	2	33	78	33					3	50	73	50												
R. aspasiensis	1	67	100	67	1	50	96	67					0	100	100	100												
R. brachypus	25	39	29	41	16	30	30	30	8	47	26	47									1	50	94	67				
R. chameleon	1	0	0	0	1	0	0	0																				
R. chocoensis	3	0	0	0	3	0	0	0																				
R. chrysoglossa	1	0	0	0	1	0	0	0																				
R. citrina	3	0	0	0	3	0	0	0																				
R. cloesii	1	0	0	0													1	0	0	0								
R. condorensis	3	0	0	0					3	0	0	0																
R. contorta	22	44	21	44	13	28	5	28	5	44	92	44	1	83	100	83	3	50	84	50								
R. cuprea	2	50	100	50	2	50	100	50																				
R. cymbula	1	0	0	0					1	0	0	0																
R. dodsonii	5	55	57	55					5	55	57	55																
R. echinata	3	0	0	0	1	0	0	0									2	0	0	0								
R. echo	8	20	5	20	8	20	5	20																				
R. elegans	3	40	31	40									3	40	31	40												
R. ephippium	2	33	99	33					2	33	99	33																
R. escobarina	1	0	0	0	1	0	0	0																				
R. falkenbergii	3	0	0	0	3	0	0	0																				
R. flosculata	4	20	31	43	1	0	57	67	3	25	48	25																
R. guttulata	16	41	51	43	9	44	67	50	6	33	4	33	0	100	100	100	1	0	0	0								
R. iris	4	20	57	20					4	20	57	20																
R. jesupiana	2	50	99	50									2	50	99	50												

Table 5-8 continued

R. lansbergii	5	17	12	0					2	33	100	33	2	0	69	33	1	0	60	50				
R. limbata	2	0	0	0	2	0	0	0																
R. mendozae	1	0	0	0					1	0	0	0												
R. metae	1	0	0	0	1	0	0	0																
R. mohrii	2	33	99	33													2	33	99	33				
R. muscifera	37	40	1	42	2	60	100	60	2	0	96	0											32	42	1	42
R. nittiorhyncha	1	67	100	67	1	67	100	67																
R. pandurata	1	0	0	0	1	0	0	0																
R. pelyx	3	40	71	50	2	33	99	33					1	50	80	50								
R. purpurea	1	0	0	0	1	0	0	0																
R. radulifera	1	0	0	0									1	0	0	0								
R. renzii	1	50	95	50													1	50	95	50				
R. roseola	0	100	100	100									0	100	100	100								
R. sanguinea	6	33	4	33	3	50	28	50					3	0	0	0								
R. schizosepala	1	0	0	0					1	0	0	0												
R. seketii	1	0	0	0	1	0	0	0																
R. tabeae	1	0	0	0	1	0	0	0																
R. teaguei	2	0	0	0					2	0	0	0												
R. trichoglossa	38	31	44	31	10	17	25	17	8	0	0	0	1	0	0	0							19	44	91	44
R. tsubotae	1	0	0	0	1	0	0	0																
R. vasquezii	4	0	0	0																	4	0	0	0
R. wagnerii	2	0	0	0									2	0	0	0								
R. fritillina*	DD				DD																			
R. howeii*	DD								DD															
R. persiciana*	1	0	0	0					1	0	0	0												
R. piperitosa*	DD																DD							
R. portillae*	1	0	0	0					1	0	0	0												

Notes:

[1] Number of remaining locations – Table 5-7, Section 2; % loss of habitat was calculated from data presented in Table 5-7, Sections 1 and 2.

[2] % change in EOO and AOO values were calculated from the values in Table 5-7, Sections 1 and 2.

a[3] number of locations remaining after habitat loss; b[4] % loss of habitat; c[5] % change in EOO and d[6] % change in AOO values.

Purple shading represents values over the complete range for a species – Global values; grey shading represents values in each country for a species – National values.

Table 5-9: Recording Red List Criteria assessment for a single species – exemplar *R. brachypus*[1]

A. Population reduction		A2c past reduction, not ceased		
		CR	EN	VU
		≥ 80%	≥ 50%	≥ 30%
Decline in AOO, EOO and/or habitat quality	Complete range			VU
	Venezuela			
	Colombia			VU
	Ecuador			VU
	Bolivia	CR		

Key to Red List Categories:

LC	Least Concern
VU	Vulnerable
EN	Endangered
CR	Critically Endangered

B. Geographic range		B1 (EOO)			B2 (AOO)			(a) number of locations			(b) decline in:		
		CR	EN	VU	CR	EN	VU	CR	EN	VU	(i)	(ii)	(iv)
		< 100 km²	< 5,000 km²	< 20,000 km²	< 10 km²	< 500 km²	< 2,000 km²	=1	≤ 5	≤ 10	EOO	AOO	locations
	Complete range			LC		EN					i	ii	iv
	Venezuela												
	Colombia			LC		EN							
	Ecuador			NT		EN				a	i	Ii	iv
	Bolivia	CR			CR			a			i	Ii	iv

D. Very small or restricted population		D2		Criteria met:	*R. brachypus*
		VU	VU		
		AOO < 20 km²	Number of locations ≤ 5		
Restricted AOO or number of locations, with plausible future threat	Complete range			Complete range	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Venezuela			Venezuela	
	Colombia			Colombia	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Ecuador			Ecuador	VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)
	Bolivia	VU	VU	Bolivia	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2

Notes:

[1] The figures from Table 5-8 together with the Red List values calculated by GeoCAT for criteria B1 and B2 (Table 5-7, Section 2) were used to produce the preliminary Red List Assessment shown in Table 5-9 above for *R. brachypus;* see also Table 5-10 which shows the details for all the species

Table 5-10: Preliminary Global[1] and National[2] Red List Assessment for *Restrepia* species[3]

	CRITERIA:	A. Population reduction	B. Geographic range						D. Very small or restricted population		Preliminary Red List Assessment
		A2 past reduction, not ceased	B1 (EOO)	B2 (AOO)	(a) loc-ations	(b) a decline in:			D2. restricted AOO or number or number of locations		i.e. a summary of all the sub-criteria satisfied in Criteria A, B and D (columns A, B and D, left)
		(c) decline in AOO, EOO and/or habitat quality				(i) EOO	(ii) AOO	(iv) locs	AOO < 20 km²	locations ≤ 5	
R. aberrans	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Central America		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. antennifera	Complete range	VU	LC	EN		i	ii	iv			VU A2c LC B1b(i,ii,iv) EN B2b(i,ii,iv)
	Venezuela	EN	CR	EN	a	i	ii	iv	VU	VU	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
	Colombia		LC	EN	a	i	ii	iv			EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)
	Ecuador	EN	NT	EN	a	i	ii	iv			EN A2c; B2ab(i,ii,iv) NT B1ab(i,ii,iv)
	Peru	EN	CR	EN	a	i	ii	iv	VU	VU	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU DU
R. aristulifera	Complete range	VU	EN	EN	a	i	ii	iv	VU	VU	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	EN	EN	EN	a	i	ii	iv	VU	VU	EN A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	EN	CR	CR	a	i	ii	iv	VU	VU	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. aspasiensis	Complete range	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	CR			?	i	ii	iv			CR A2c
	Colombia	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv)

Table 5-10 continued

R. brachypus	Complete range	VU	LC	EN		i	ii	iv			VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Colombia	VU	LC	EN		i	ii	iv			VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Ecuador	VU	NT	EN	a	i	ii	iv			VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)
	Peru	CR	CR	CR		i	ii	iv	VU	VU	CR A2c; B1b(i,ii,iv)+B2b(i,ii,iv)
	Bolivia	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. chameleon	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. chocoensis	Complete range		CR	EN	a				VU	VU	CR B1a EN B2a VU D2
	Colombia		CR	EN	a				VU	VU	CR B1a EN B2a VU D2
R. chrysoglossa	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. citrina	Complete range		CR	EN	a				VU	VU	CR B1a EN B2a VU D2
	Colombia		CR	EN	a				VU	VU	CR B1a EN B2a VU D2
R. cloesii	Complete range		CR	CR	a				VU		CR B1a+2a VU D2
	Peru		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. condorensis	Complete range		CR	EN	a				VU	VU	CR B1a EN B2a VU D2
	Ecuador		CR	EN	a				VU	VU	CR B1a EN B2a VU D2
R. contorta	Complete range	VU	LC	EN		i	ii	iv			VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Venezuela	CR	CR	CR		i	ii	iv	VU	VU	CR A2c; B1b(i,ii,iv)+B2b(i,ii,iv) VU D2
	Colombia		LC	EN		i	ii	iv			EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Ecuador	CR	EN	EN	a	i	ii	iv	VU	VU	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Peru	CR	EN	EN	a	i	ii	iv	VU	VU	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2

Table 5-10 continued

R. cuprea	Complete range	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. cymbula	Complete range		CR	EN	a				VU	VU	CR B1a EN B2a VUD2
	Ecuador		CR	EN	a				VU	VU	CR B1a EN B2a VUD2
R. dodsonii	Complete range	EN	EN	EN	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Ecuador	EN	EN	EN	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. echinata	Complete range		LC	EN	a				VU	VU	EN B2a LC B1a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Peru		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. echo	Complete range		VU	EN	a	i	ii	iv			VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
	Colombia		VU	EN	a	i	ii	iv			VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
R. elegans	Complete range		VU	EN	a	i	ii	iv	VU	VU	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
	Venezuela		VU	EN	a	i	ii	iv	VU	VU	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
R. ephippium	Complete range	EN	CR	CR	a	i	ii	iv	VU	VU	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU
	Ecuador	EN	CR	CR	a	i	ii	iv	VU	VU	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU
R. escobarina	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. falkenbergii	Complete range		EN	EN	a				VU	VU	EN B1a+2a VUD2
	Colombia		EN	EN	a				VU	VU	EN B1a+2a VUD2
R. flosculata	Complete range	VU	EN	EN	a	i	ii	iv	VU	VU	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	EN	CR	CR	a	i	ii		VU	VU	EN A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2
	Ecuador	VU	EN	EN	a	i	ii	iv	VU	VU	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2

Table 5-10 continued

R. guttulata	Complete range	EN	LC	EN		i	ii	iv			EN A2c; B2b(i,ii,iv); LC B1b(i,ii,iv)
	Venezuela	CR			?	i	ii	iv			CR A2c , (b)(i),(ii),(iv)
	Colombia	EN	LC	EN	a	i	ii	iv			EN A2c; B2ab(i,ii,iv) LC B1ab(i,ii,iv)
	Ecuador	VU	VU	EN	a	i	ii	iv			VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv)
	Peru		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. iris	Complete range	VU	EN	EN	a	i	ii	iv	VU	VU	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Ecuador	VU	EN	EN	a	i	ii	iv	VU	VU	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. jesupiana	Complete range	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. lansbergii	Complete range		LC	EN	a	i		iv			EN B2ab(i,iv) LC B1ab(i,iv)
	Venezuela	VU	CR	CR	a	i	ii		VU	VU	VU A2c CR B1ab(i,ii,)+B2ab(i,ii,) VU D2
	Ecuador	EN	CR	EN	a	i	ii	iv	VU	VU	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
	Peru	EN	CR	CR	a				VU	VU	EN A2c CR B1a+B2a VU D2
R. limbata	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. mendozae	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Ecuador		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. metae	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. mohrii	Complete range	EN	EN	EN	a	i	ii	iv	VU	VU	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2
	Peru	EN	EN	EN	a	i	ii	iv	VU	VU	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2
R. muscifera	Complete range	VU	LC	EN		i	ii	iv			VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Central America	VU	LC	EN		i	ii	iv			VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Colombia	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Ecuador	EN	CR	CR	a				VU	VU	EN A2c CR B1a+B2a VU D2

Table 5-10 continued

R. nittiorhyncha	Complete range	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. pandurata	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. pelyx	Complete range	EN	VU	EN	a	i	ii	iv	VU	VU	EN A2c; B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
	Venezuela	EN	CR	CR	a	i	ii	iv	VU	VU	EN A2c; CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	EN	CR	EN	a	i	ii	iv	VU	VU	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
R. purpurea	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. radulifera	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Venezuela		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. renzii	Complete range	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	CR	CR	CR	a	i	ii	iv	VU	VU	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. roseola	Complete range	CR/EX			?	i	ii	iv			CR/EX A2c B2b(i,ii,iv)
	Venezuela	CR/EX			?	i	ii	iv			CR/EX A2c B2b(i,ii,iv)
R. sanguinea	Complete range	VU	LC	EN	a	i	ii	iv			VU A2c EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)
	Venezuela		EN	EN	a				VU	VU	EN B1a+B2a VU D2
	Colombia	VU	VU	EN	a	i	ii	iv	VU	VU	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv) VU D2
R. schizosepala	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Ecuador		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. seketii	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. tabeae	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2

Table 5-10 continued

R. teaguei	Complete range		CR	CR	a				VU	VU	CR B1a+B2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+B2a VU D2
R. trichoglossa	Complete range	VU	LC	EN		i	ii	iv			VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)
	Central America	EN	VU	EN		i	ii	iv			EN A2c; B2b(i,ii,iv) VU B1b(i,ii,iv)
	Venezuela		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		NT	EN	a	i	ii	iv			EN B2a(i,ii,iv) NT B1a(I,ii,iv)
	Ecuador		NT	EN	a						EN B2a NT B1a
R. tsubotae	Complete range		CR	CR	a				VU	VU	CR B1a+2a VU D2
	Colombia		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. vasquezii	Complete range		VU	EN	a				VU	VU	VU B1 EN B2a VU D2
	Bolivia		VU	EN	a				VU	VU	VU B1 EN B2a VU D2
R. wagnerii	Complete range		EN	EN	a				VU	VU	EN B1a+2a VU D2
	Venezuela		EN	EN	a				VU	VU	EN B1a+2a VU D2
R. piperitosa*	Peru										DD
R. portillae*	Ecuador		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. howeii*	Ecuador										DD
R. persiciana*	Ecuador		CR	CR	a				VU	VU	CR B1a+2a VU D2
R. fritillina*	Colombia										DD

Notes:

[1]Global assessments shown in pink; [2]National assessments shown in grey.

[3]The figures from Table 5-8 together with the Red List values calculated by GeoCAT for criteria B1 and B2 (Table 5-7, Section 2) were used to produce the preliminary Red List Assessment presented in Table 5-10 above, see also Table 5-9 which shows the details for exemplar *R. brachypus.*

5.3.4 Maps and accompanying tables

The maps (Figures 5-15 to 5-19) show the distribution of species in relation to national parks or nature reserves, main towns and the Pan-American Highway. In the accompanying table for each map (Tables), details are shown of the sub-populations/locations that were recorded, either still remaining or lost in each country (Table 5-7, Sections 1 and 2). The Tables (5-11 to 5-15) also include the Red List assessment from Table 5-10 that has been amended for Criterion B, together with final amendments to the Red List Assessment after adjustment to Criterion A2 made for those species remaining within a protected habitat (see also Table 5-16 for a comparison of these stages in the Red List assessment). A map of Bolivia was not included, as the only endemic species in this country is *R. vasquezii*, with four recorded locations, details of which are included in Table 5-15.

A map showing the distribution of the two *Restrepia* species native to Central America is presented in Figure 5-19 and the accompanying table showing the details for each species is presented in Table 5-15. Map (a) shows the geographic range throughout Central America and maps (b) and (c) display details of the distributions in Costa Rica and Panama, where the recorded locations of *R. muscifera* and *R. trichoglossa* are more numerous. From the colour coding of the points, it is possible to determine that there has been a greater loss of habitat in Central America (Mexico to Nicaragua) compared with Costa Rica and Panama.

The Venezuelan, Colombian, Ecuadoran and Central American maps show that most 'lost' locations (shown in red) coincide with the route of the Pan American Highway, major towns and industrial areas. The remaining locations (shown in blue and purple) occur further away from the main route. Purple points (indicating species that have not lost any recorded locations) occur typically furthest away from the highway. These

points generally indicate species that have only one or two recorded locations in total. Venezuela has 22/40 locations remaining, all currently in protected areas such as national parks. This represents a loss of 45% of recorded locations (since approximately 1961 when the Pan American Highway was opened) with 55% remaining for 15 species (Figure 5-20). In Colombia and Ecuador, which have more *Restrepia* species (30 and 18 respectively), the overall loss of locations is less. Colombia has 100/138 locations remaining, with 46 of these in protected areas, which represents a loss of 28% of recorded locations for 30 species. Ecuador has 61/96 locations remaining, with 36 in protected areas, representing a loss of 36% for 18 species (Figure 5-20). In both Colombia and Ecuador these losses have occurred since the building of the Pan American Highway (1961).

In Peru, the loss of locations does not coincide with the Pan-American Highway, as the road does not follow the Andes but was built along the coast (Figure 5-18). There are fewer endemic species occurring in this country (8 species) and 10/17 locations remain, with six of these in protected areas. This represents a loss of 41% for these species (Figure 5-20) but it is harder to establish when losses occurred from the map evidence due to the fact that deforestation and degradation have occurred as the result of a variety of human activities over time.

Central America can be divided into two areas for comparison - Mexico to Nicaragua and Costa Rica plus Panama. In Mexico to Nicaragua, there are fewer recorded locations for the *Restrepia* species *R. trichoglossa* and *R. muscifera* than in Panama and Costa Rica, but the loss of habitat has been substantial. Only 4/21 of the recorded locations remain, with 2 of these in protected locations; this represents a loss of 81% (Figures 5-19(a) and 5-20). By contrast, in Costa Rica and Panama, 47/68 recorded locations remain, with 22 of these in protected areas, which represents a loss of 32%. The greatest loss occurs in one area in central Costa Rica and coincides with several

towns that are connected by the Pan-American Highway (Figure 5-19 (b) and (c)) which opened around 1961. In Panama, the greatest loss has occurred around Panama City and the industrial area around the Panama Canal (Figure 5-19(c)), which was completed in 1914.

5.3.5 Summary tables

A summary showing all the stages in the Red List assessment process is presented in Table 5-16. Global and national assessments are indicated by different colours.

Summary charts of habitat/location loss in each country are shown in Figure 5-20. A much simplified summary of the Red List assessment in shown in Table 5-17. An 'average' value of the criteria met is shown in B. For example CR B1ab(i,ii,iv) + B2ab(i,ii,iv) is summarised as CR, and EN B1ab(i,ii,iv) + CR B2ab(i,ii,iv) is summarised as EN. This is intended to serve merely as a guide to the level of assessed threat for each species in their respective countries of origin; whereas the full Red List Assessment is presented in Table 5-16.

Table 5-11: Venezuela - remaining species locations, Red List assessment amended for Criterion B and final amendments to allow for the lower risk associated with protected habitat.

Venezuela

Species that have lost locations

species	Original number of locations[1]	Total number of locations remaining[2]	Locations remaining in protected areas[3]	**Red List assessment from Table 5-10 (final column) amended for Criterion B** Criterion B is only awarded if all the requirements are met (Tables 5-5b and 5-5c)	**Final amendments to Red List assessment allowing for protected habitat** Degree of threat is considered to be reduced for remaining locations in protected areas.
R. antennifera	3	2	2	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
R. aristulifera	6	3	3	EN A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. aspasiensis	1	0	0	CR A2c	EX
R. contorta	6	1	1	CR A2c VU D2	VU D2
R. elegans	5	3	3	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
R. guttulata	1	0	0	CR A2c	EX
R. jesupiana	4	2	2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. pelyx	2	1	1	EN A2c; CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. renzii	2	1	1	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. roseola	1	0	0	CR/EX A2c	EX

Species that have not lost locations

R. lansbergii	2	2	VU A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2	CR B1ab(i,ii)+B2ab(i,ii) VU D2
R. radulifera	1	1	VU D2	VU D2
R. sanguinea	3	3	VU D2	VU D2
R. trichoglossa	1	1	VU D2	VU D2
R. wagnerii	2	2	VU D2	VU D2

Notes: [1] and [2] data from Table 5-7, Sections 1 and 2; [3]As shown on map (b) in Figure 5-15.

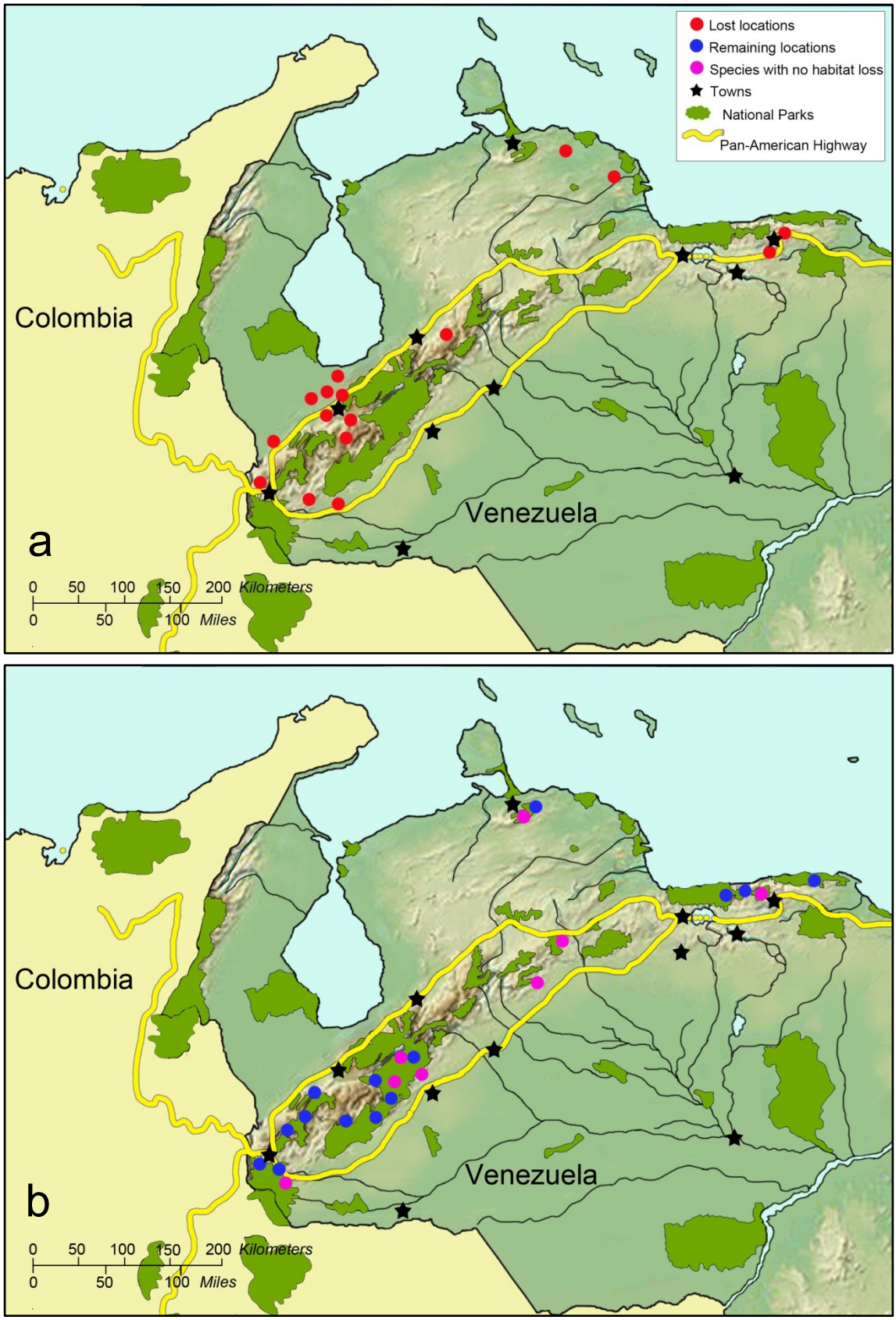


Figure 5-15: *Restrepia* species in Venezuela (a) Recorded locations for *Restrepia* species where habitat has been lost; (b) recorded locations where suitable habitat still remains. Blue points indicate existing locations for species with habitat loss elsewhere. Purple points indicate existing locations for species with no loss elsewhere. Forty five % of total recorded locations have been lost and few remain outside protected areas. (See also Table 5-11 and Figure 5-20). (Geographic details: Shadowxfox, 2008a; 2013; artwork: Millner, 2013).

Table 5-12: Colombia - remaining species locations, Red List assessment amended for Criterion B and final amendments to allow for the lower risk associated with protected habitat.

Colombia

Species that have lost locations

species	Original number of locations[1]	Total number of locations remaining[2]	Locations remaining in protected areas[3]	**Red List assessment from Table 5-10 (final column) amended for Criterion B** Criterion B is only awarded if all the requirements are met (Tables 5-5b and 5-5c)	**Final amendments to Red List assessment allowing for protected habitat** Degree of threat is considered to be reduced for remaining locations in protected areas.
R. antennifera	9	7	5	EN B2ab(i,ii,iv) VU LC B1ab(i,ii,iv)	EN B2ab(i,ii,iv) VU LC B1ab(i,ii,iv)
R. aristulifera	3	2	2	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. aspasiensis	2	1	1	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv)	CR B1ab(i,ii,iv)+B2ab(i,ii,iv)
R. brachypus	23	16	3	VU A2c	VU A2c
R. contorta	18	13	5	LC	LC
R. cuprea	4	2	1	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. echo	10	8	2	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
R. guttulata	16	9	5	EN A2c; B2ab(i,ii,iv) LC B1ab(i,ii,iv)	EN A2c; B2ab(i,ii,iv) LC B1ab(i,ii,iv)
R. muscifera	5	2	2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. nittiorhyncha	3	1	0	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. pelyx	3	2	1	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	VU A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
R. sanguinea	6	4	4	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv) VU D2	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv) VU D2
R. trichoglossa	12	10	5	LC	LC
R. teaguei	2	2	1	VU D2	VU D2

Table 5-12: Colombia, continued

Species that have not lost locations				
R. chameleon	1	1	VU D2	VU D2
R. chocoënsis	3	0	VU D2	VU D2
R. chrysoglossa	1	1	VU D2	VU D2
R. citrina	3	1	VU D2	VU D2
R. echinata	1	0	VU D2	VU D2
R. escobariana	1	0	VU D2	VU D2
R. falkenbergii	3	1	VUD2	VUD2
R. flosculata	1	0	EN A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2	EN A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2
R. limbata	2	2	VU D2	VU D2
R. metae	1	0	VU D2	VU D2
R. pandurata	1	0	VU D2	VU D2
R. purpurea	1	0	VU D2	VU D2
R. seketii	1	1	VU D2	VU D2
R. tabeae	1	1	VU D2	VU D2
R. tsubotae	1	1	VU D2	VU D2
*R. fritillina**			DD	DD

Notes: [1] and [2] data from Table 5-7, Sections 1 and 2; [3]As shown on map (b) in Figure 5-16.

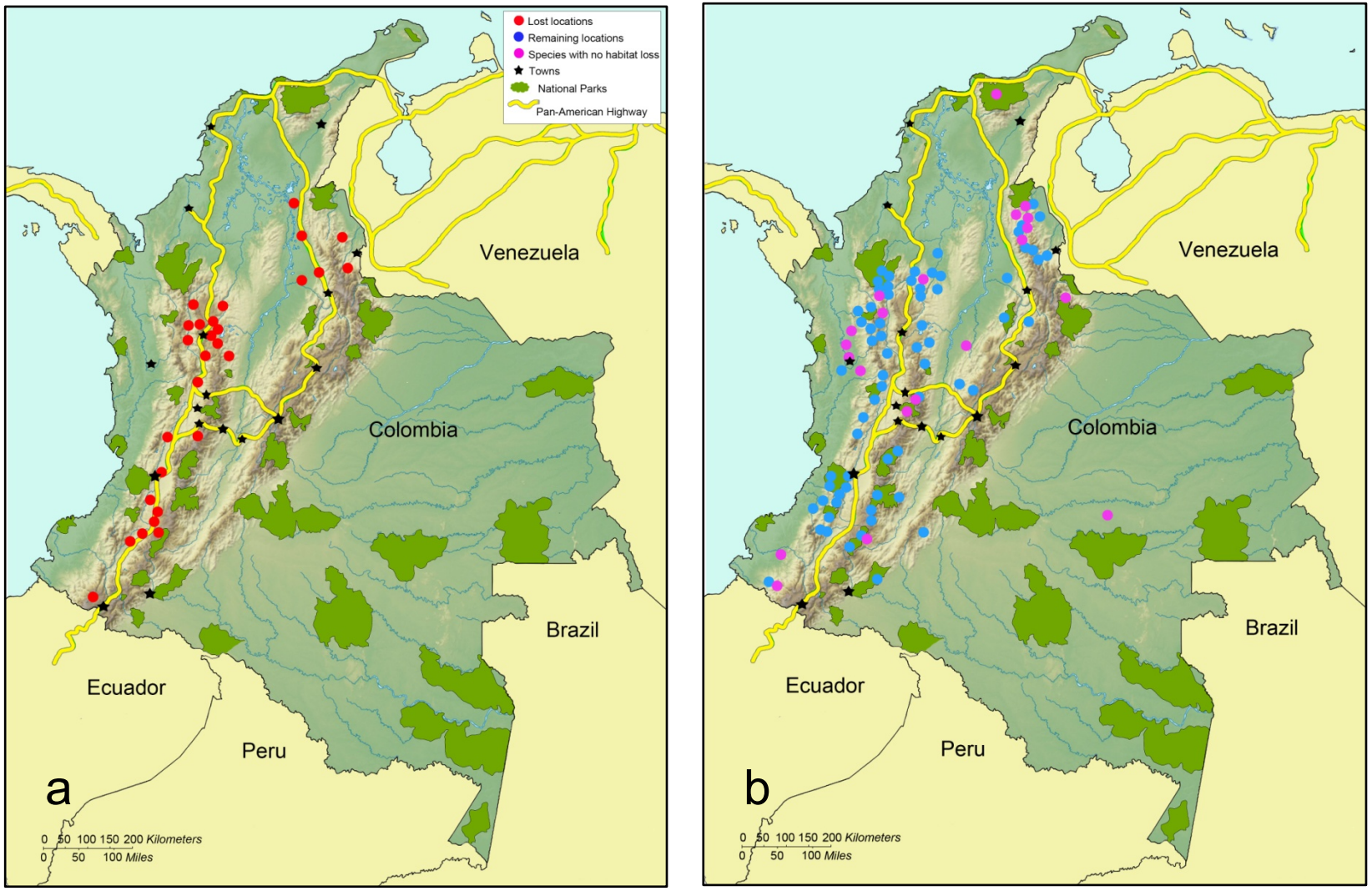


Figure 5-16: *Restrepia* species in Colombia. (a) Loss of recorded locations in relation to the Pan-American Highway; (b) remaining locations further away from the highway and towns. In total, 98/136 locations remain representing a loss of 28% (See also Table 5-12 and Figure 5-20). (Geographic details: Brains, 2012; artwork: Millner, 2013).

Table 5-13: Ecuador - remaining species locations, Red List assessment amended for Criterion B and final amendments to allow for the lower risk associated with protected habitat.

Ecuador

Species that have lost locations

species	Original number of locations[1]	Total number of locations remaining[2]	Locations remaining in protected areas[3]	**Red List assessment from Table 5-10 (final column) amended for Criterion B** Criterion B is only awarded if all the requirements are met (Tables 5-5b and 5-5c)	**Final amendments to Red List assessment allowing for protected habitat** Degree of threat is considered to be reduced for remaining locations in protected areas.
R. antennifera	19	8	5	EN A2c; B2ab(i,ii,iv) NT B1ab(i,ii,iv)	VU A2c; B2ab(i,ii,iv) NT B1ab(i,ii,iv)
R. brachypus	15	8	5	VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)	VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)
R. contorta	9	5	4	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. dodsonii	11	5	2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. ephippium	3	2	2	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU
R. flosculata	4	3	2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. guttulata	9	6	6	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
R. iris	5	4	1	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. lansbergii	3	2	1	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
Species that have not lost locations					
R. condorensis		3	1	VU D2	VU D2
R. cymbula		1	1	VU D2	VU D2
R. mendozae		1	0	VU D2	VU D2
R. muscifera		2	2	EN A2c VU D2	VU D2
R. persiciana		1	1	VU D2	VU D2
R. portillae		1	0	VU D2	VU D2
R. schizosepala		1	1	VU D2	VU D2
R. trichoglossa		8	2	LC	LC
*R. howei**				DD	DD

Notes: [1] and [2] data from Table 5-7, Sections 1 and 2; [3]As shown on map (b) in Figure 5-17.

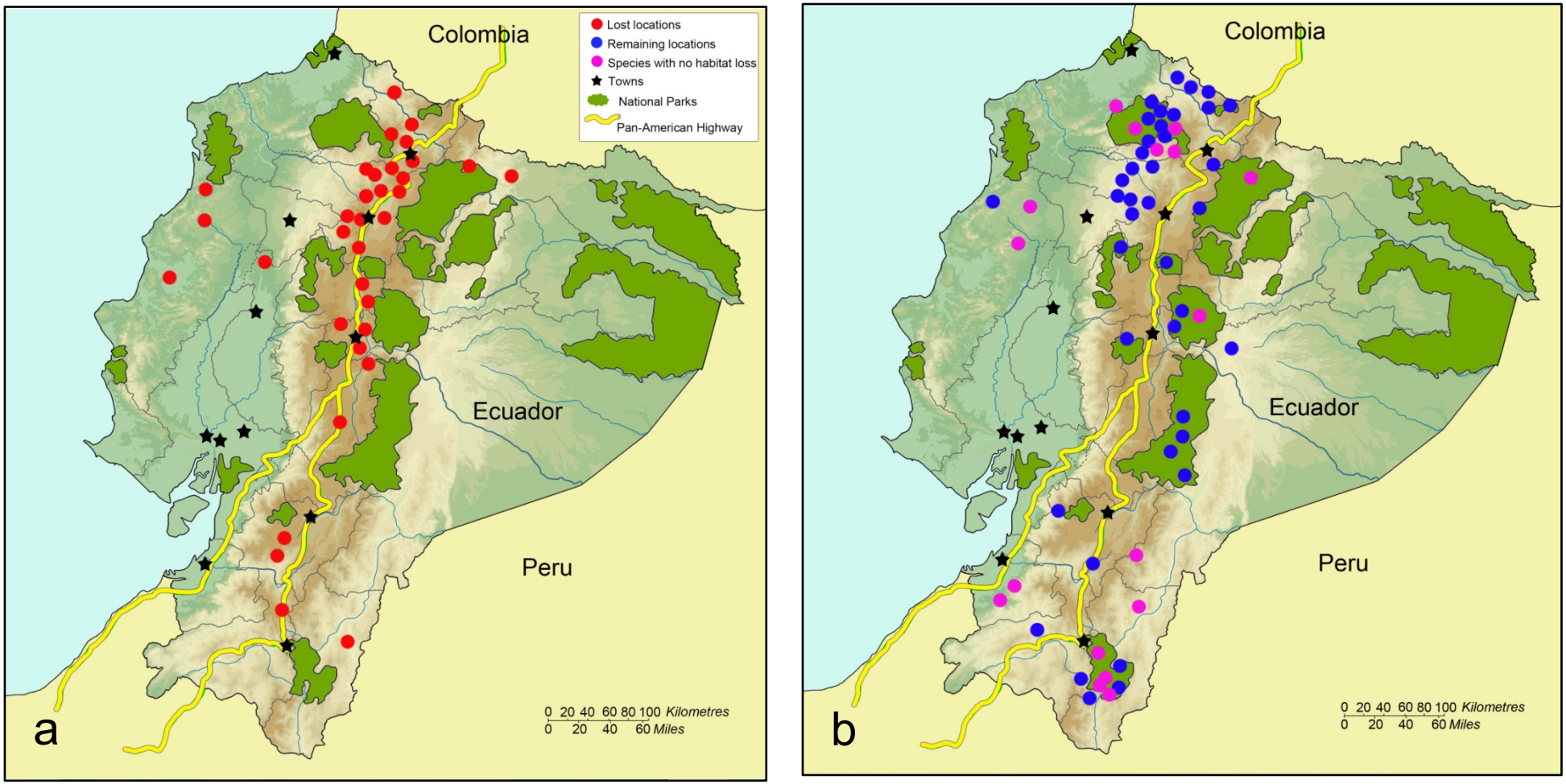


Figure 5-17: *Restrepia* species in Ecuador. (a) Loss of recorded locations in relation to the Pan-American Highway; (b) Remaining locations further away from the highway and towns. In total, 61/96 locations remain representing a loss of 36% (See also Table 5-13 and Figure 5-20). (Geographic details: Shadowxfox, 2012; Ministero del Ambienti, 2012; artwork: Millner, 2013).

Table 5-14: Peru - remaining species locations, Red List assessment amended for Criterion B and final amendments to allow for the lower risk associated with protected habitat.

Peru

Species that have lost locations

species	Original number of locations[1]	Total number of locations remaining[2]	Locations remaining in protected areas[3]	**Red List assessment from Table 5-10 (final column) amended for Criterion B** Criterion B is only awarded if all the requirements are met (Tables 5-5b and 5-5c)	**Final amendments to Red List assessment allowing for protected habitat** Degree of threat is considered to be reduced for remaining locations in protected areas.
R. antennifera	2	0	0	EX	EX
R. brachypus	1	0	0	EX	EX
R. contorta	6	3	1	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. mohrii	3	2	1	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2

Species that have not lost locations

R. cloesii	1	1	VU D2	VU D2
R. echinta	2	2	VU D2	VU D2
R. guttulata	1	1	VU D2	VU D2
R. lansbergii	1	1	EN A2c VU D2	VU D2
*R. piperitosa**			DD	DD

Notes: [1] and [2] data from Table 5-7, Sections 1 and 2; [3]As shown on map (b) in Figure 5-18.

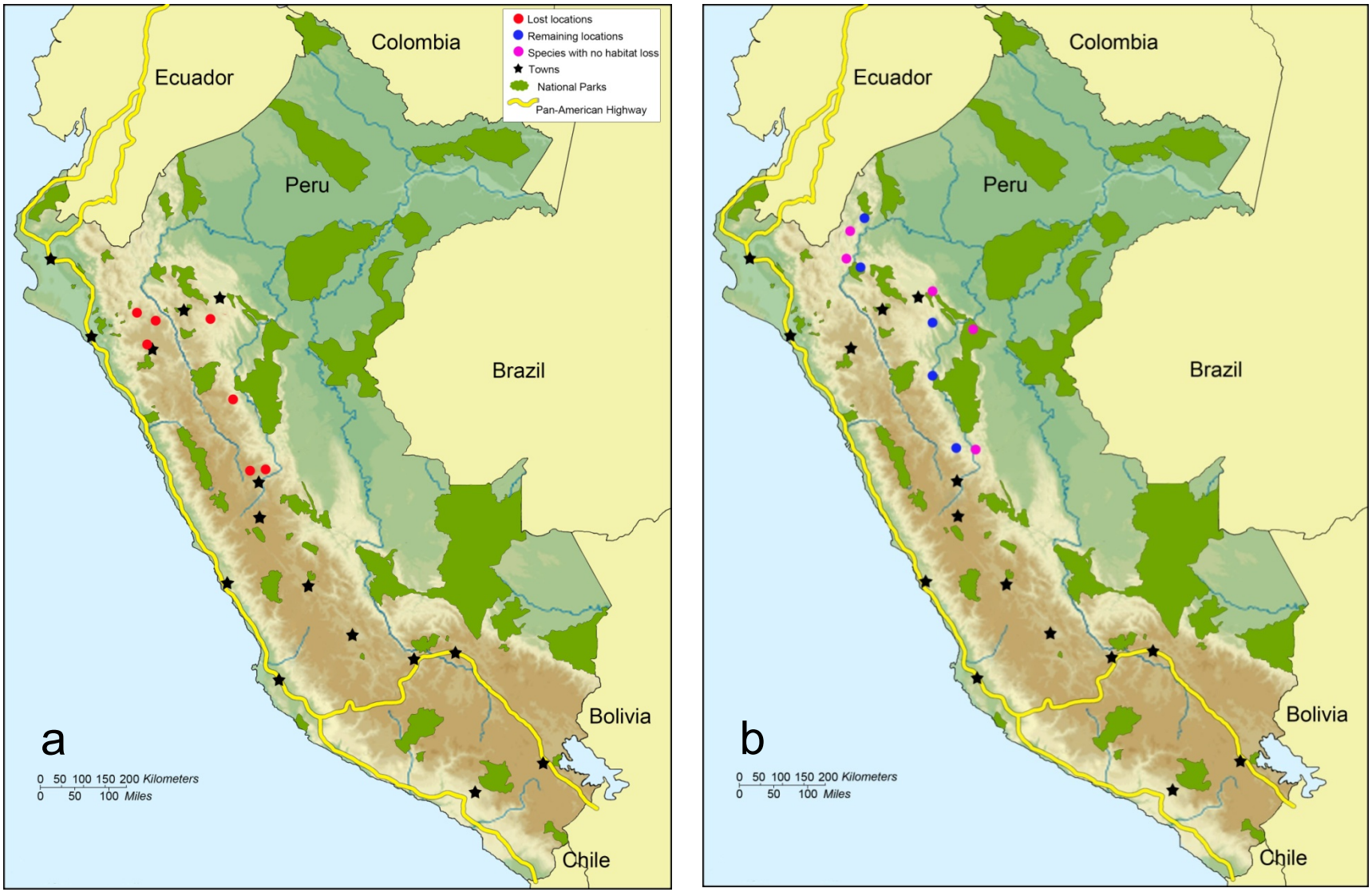


Figure 5-18: *Restrepia* species in Peru. (a) Lost locations; (b) remaining locations in protected areas. In total, 10/1 7 locations remain representing a loss of 41% (see also Table 5-14 and Figure 5-20). Overall, fewer species were found in this country. (Geographic details: Urutseg, 2011; Mnistero del Ambiente, Peru, 2008; artwork: Millner, 2013).

Table 5-15: Central America and Bolivia - remaining species locations, Red List assessment amended for Criterion B and final amendments to allow for the lower risk associated with protected habitat.

Central America

Species that have lost locations

species	Original number of locations[1]	Total number of locations remaining[2]	Locations remaining in protected areas[3]	**Red List assessment from Table 5-10 (final column) amended for Criterion B** Criterion B is only awarded if all the requirements are met (Tables 5-5b and 5-5c)	**Final amendments to Red List assessment allowing for protected habitat** Degree of threat is considered to be reduced for remaining locations in protected areas.
R. muscifera					
Complete range	55	32	12	VU A2c	VU A2c
Mex to Nic	19	4	2	EN A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
C Rica and Pan	36	28	10	LC	LC
R. trichoglossa					
Complete range	34	19	10	VU A2c	VU A2c
Mex to Nic	2	0	0	EX	EX
C Rica and Pan	32	19	12	VU A2c	VU A2c

Species that have not lost locations

R. aberrans	1	1	1	VU D2	VU D2

Bolivia

R. vasquezii	4	4	2	VU D2	VU D2
R. brachypus	2	1	0	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2

Notes: [1] and [2] data from Table 5-7, Sections 1 and 2; [3]As shown on map (b) in Figure 5-19 (Bolivia, not included in the maps).

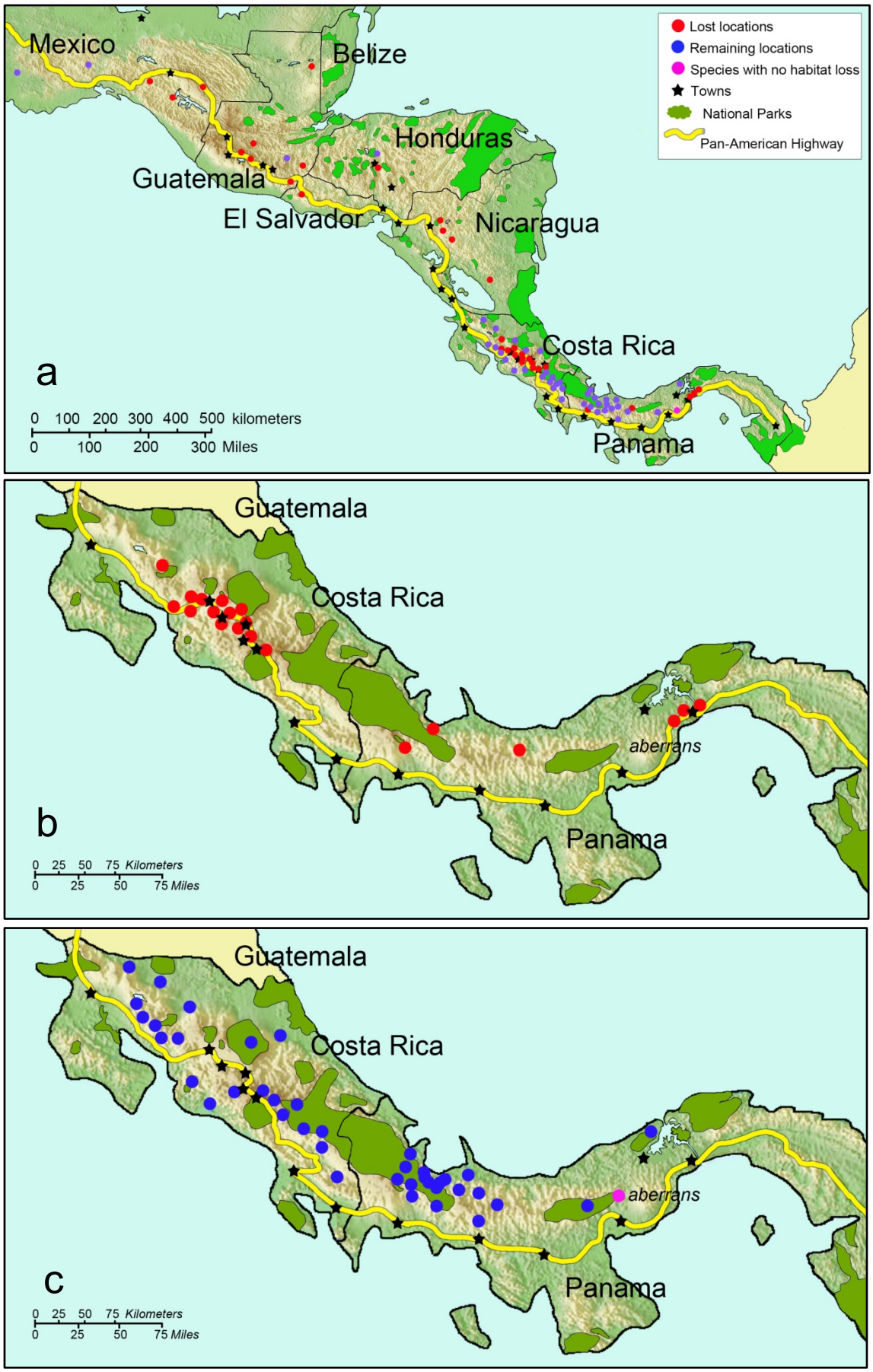


Figure 5-19: *Restrepia* species in Central America. (a) Locations lost along the Pan-American Highway and near towns; 4/21 remaining (81% loss) Mexico to Nicaragua; (b) and (c) lost and remaining locations (47/68, 32% loss) in Costa Rica and Panama. (See also Table 5-15 Figure 5-20). (Geographic details: CIA, 1987; Toucan Guide, 2006; Bnktcp, 2010; artwork: Millner, 2013).

Table 5-16: Stages in formulating the final Red List Assessment for *Restrepia* species.

		STAGE 1	STAGE 2	STAGE 3
		Preliminary Red List assessment[1]	Assessment amended for Criterion B[2]	Final assessment for safe habitat[3]
R.aberrans	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Central America	CR B1a+2a VU D2	VU D2	VU D2
R. antennifera	Complete range	VU A2c LC B1b(i,ii,iv) EN B2b(i,ii,iv)	VU A2c	VU A2c
	Venezuela	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
	Colombia	EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)	EN B2ab(i,ii,iv) VU LC B1ab(i,ii,iv)	EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)
	Ecuador	EN A2c; B2a(i,ii,iv) NT B1a(i,ii,iv)	EN A2c; B2ab(i,ii,iv) NT B1ab(i,ii,iv)	VU A2c; B2ab(i,ii,iv) NT B1ab(i,ii,iv)
	Peru	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU DU	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU DU	EX
R aristulifera	Complete range	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	EN A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. aspasiensis	Complete range	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	CR A2c	CR A2c	EX
	Colombia	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv)	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv)	CR B1ab(i,ii,iv)+B2ab(i,ii,iv)
R. brachypus	Complete range	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)	VU A2c	VU A2c
	Colombia	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)	VU A2c	VU A2c
	Ecuador	VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)	VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)	VU A2c EN B2ab(i,ii,iv) NT B1ab(i,ii,iv)
	Peru	CR A2c; B1b(i,ii,iv)+B2b(i,ii,iv)	CR A2c	EX
	Bolivia	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. chameleon	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2

Table 5-16: contined

		Preliminary Red List assessment[1]	Assessment amended for Criterion B[2]	Final assessment for safe habitat[3]
R. chocoensis	Complete range	CR B1a EN B2a VU D2	VU D2	VU D2
	Colombia	CR B1a EN B2a VU D2	VU D2	VU D2
R. chrysoglossa	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. citrina	Complete range	CR B1a EN B2a VU D2	VU D2	VU D2
	Colombia	CR B1a EN B2a VU D2	VU D2	VU D2
R. cloesii	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Peru	CR B1a+2a VU D2	VU D2	VU D2
R. condorensis	Complete range	CR B1a EN B2a VU D2	VU D2	VU D2
	Ecuador	CR B1a EN B2a VU D2	VU D2	VU D2
R. contorta	Complete range	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)	VU A2c	VU A2c
	Venezuela	CR A2c; B1b(i,ii,iv)+B2b(i,ii,iv) VU D2	CR A2c VU D2	VU D2
	Colombia	EN B2b(i,ii,iv) LC B1b(i,ii,iv)	LC	LC
	Ecuador	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Peru	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. cuprea	Complete range	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. cymbula	Complete range	CR B1a EN B2a VUD2	VU D2	VU D2
	Ecuador	CR B1a EN B2a VUD2	VU D2	VU D2
R. dodsonii	Complete range	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Ecuador	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. echinata	Complete range	EN B2a LC B1a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
	Peru	CR B1a+2a VU D2	VU D2	VU D2

Table 5-16: continued

		Preliminary Red List assessment[1]	Assessment amended for Criterion B[2]	Final assessment for safe habitat[3]
R. echo	Complete range	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
	Colombia	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
R. elegans	Complete range	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
	Venezuela	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
R. ephippium	Complete range	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU
	Ecuador	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU	EN A2c CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU DU
R. escobarina	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. falkenbergii	Complete range	EN B1a+2a VUD2	VU D2	VU D2
	Colombia	EN B1a+2a VUD2	VU D2	VU D2
R. flosculata	Complete range	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	EN A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2	EN A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2	EN A2c CR B1ab(i,ii)+B2ab(i,ii) VU D2
	Ecuador	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. guttulata	Complete range	EN A2c; B2b(i,ii,iv); LC B1b(i,ii,iv)	EN A2c	EN A2c
	Venezuela	CR A2c , (b)(i),(ii),(iv)	CR A2c	EX
	Colombia	EN A2c; B2ab(i,ii,iv) LC B1ab(i,ii,iv)	EN A2c; B2ab(i,ii,iv) LC B1ab(i,ii,iv)	EN A2c; B2ab(i,ii,iv) LC B1ab(i,ii,iv)
	Ecuador	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv)	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv)
	Peru	CR B1a+2a VU D2	VU D2	VU D2
R. iris	Complete range	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Ecuador	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	VU A2c EN B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. jesupiana	Complete range	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2

Table 5-16: continued

		Preliminary Red List assessment[1]	Assessment amended for Criterion B[2]	Final assessment for safe habitat[3]
R. lansbergii	Complete range	EN B2ab(i,iv) LC B1ab(i,iv)	EN B2ab(i,iv) LC B1ab(i,iv)	EN B2ab(i,iv) LC B1ab(i,iv
	Venezuela	VU A2c CR B1ab(i,ii,)+B2ab(i,ii,) VU D2	VU A2c CR B1ab(i,ii,)+B2ab(i,ii,) VU D2	CR B1ab(i,ii)+B2ab(i,ii) VU D2
	Ecuador	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2
	Peru	EN A2c CR B1a+B2a VU D2	EN A2c VU D2	EN A2c VU D2
R. limbata	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. mendozae	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Ecuador	CR B1a+2a VU D2	VU D2	VU D2
R. metae	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. mohrii	Complete range	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2
	Peru	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2	EN A2c; B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2	EN B1ab(i,ii,iv)+B2b(i,ii,iv) VU D2
R. muscifera	Complete range	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)	VU A2c	VU A2c
	Central America	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)	VU A2c	VU A2c
	Colombia	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Ecuador	EN A2c CR B1a+B2a VU D2	EN A2c VU D2	VU D2
R. nittiorhyncha	Complete range	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. pandurata	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. pelyx	Complete range	EN A2c; B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN A2c; B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2	EN A2c; B2ab(i,ii,iv) VU B1ab(i,ii,iv) VU D2
	Venezuela	EN A2c; CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	EN A2c; CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Colombia	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	EN A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2	VU A2c; B2ab(i,ii,iv) CR B1ab(i,ii,iv) VU D2

Table 5-16: continued

		Preliminary Red List assessment[1]	Assessment amended for Criterion B[2]	Final assessment for safe habitat[3]
R. purpurea	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. radulifera	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Venezuela	CR B1a+2a VU D2	VU D2	VU D2
R. renzii	Complete range	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
	Venezuela	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR A2c; B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2	CR B1ab(i,ii,iv)+B2ab(i,ii,iv) VU D2
R. roseola	Complete range	CR/EX A2c B2b(i,ii,iv)	CR/EX A2c	EX
	Venezuela	CR/EX A2c B2b(i,ii,iv)	CR/EX A2c	EX
R. sanguinea	Complete range	VU A2c EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)	VU A2c EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)	VU A2c EN B2ab(i,ii,iv) LC B1ab(i,ii,iv)
	Venezuela	EN B1a+B2a VU D2	VU D2	VU D2
	Colombia	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv) VU D2	VU A2c; B1ab(i,ii,iv) EN B2ab(i,ii,iv) VU D2	VU B1ab(i,ii,iv) EN B2ab(i,ii,iv) VU D2
R. schizosepala	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Ecuador	CR B1a+2a VU D2	VU D2	VU D2
Dxsc ccccc	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. tabeae	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. teaguei	Complete range	CR B1a+B2a VU D2	VU D2	VU D2
	Colombia	CR B1a+B2a VU D2	VU D2	VU D2
R. trichoglossa	Complete range	VU A2c EN B2b(i,ii,iv) LC B1b(i,ii,iv)	VU A2c	VU A2c
	Central America	EN A2c; B2b(i,ii,iv) VU B1b(i,ii,iv)	EN A2c	EN A2c
	Venezuela	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	EN B2a(i,ii,iv) NT B1a(I,ii,iv)	LC	LC
	Ecuador	EN B2a NT B1a	LC	LC

Table 5-16: continued

		Preliminary Red List assessment[1]	Assessment amended for Criterion B[2]	Final assessment for safe habitat[3]
R. tsubotae	Complete range	CR B1a+2a VU D2	VU D2	VU D2
	Colombia	CR B1a+2a VU D2	VU D2	VU D2
R. vasquezii	Complete range	VU B1 EN B2a VU D2	VU D2	VU D2
	Bolivia	VU B1 EN B2a VU D2	VU D2	VU D2
R. wagnerii	Complete range	EN B1a+2a VU D2	VU D2	VU D2
	Venezuela	EN B1a+2a VU D2	VU D2	VU D2
R. piperitosa*	Peru	DD	DD	DD
R. portillae*	Ecuador	CR B1a+2a VU D2	VU D2	VU D2
R. howeii*	Ecuador	DD	DD	DD
R. persiciana*	Ecuador	CR B1a+2a VU D2	VU D2	VU D2
R. fritillina*	Colombia	DD	DD	DD

Notes:

[1] Preliminary assessment as shown in Table 5-10 (final column), representing all the criteria and sub-criteria that for each species has met.

[2] Preliminary Assessment (Stage 1) amended with regard to Criterion B, as shown in Tables 5-11 to 5-15. Criterion B was not assigned if the additional sub-criteria (a) and (b) were not also met (Tables 5-5b and 5-5c).

[3] Final assessment. Stage 2 amended with regard to 'safe' or 'protected' habitat. If a taxon was found to persist in 'safe' habitats, i.e. nature reserves or national parks, the overall level of threat was considered to be reduced.

Colour shading in table: pink - Global assessments; grey - National assessments.

Table 5-17: Colour coded comparison of simplified Red List Global and National Categories.

		Complete range			Colombia			Ecuador			Venezuela		
SPECIES	RANGE	**A**	**B**	**D**		**A**	**B**	**D**		**A**	**B**	**D**		**A**	**B**	**D**
R.aberrans	CA			VU	***R. antennifera***		NT		***R. antennifera***	EN			***R. antennifera***	EN	CR	VU
R. antennifera	C,E,V,P	VU			***R aristulifera***	EN	CR	VU	***R. brachypus***	VU	EN		***R aristulifera***	EN	EN	VU
R aristulifera	C,V	VU	EN	VU	***R. aspasiensis***	CR	CR		***R. condorensis***			VU	***R. aspasiensis***	CR		
R. aspasiensis	C,V	CR	CR	VU	***R. brachypus***	VU	NT		***R. contorta***	CR	EN	VU	***R. contorta***	CR		VU
R. brachypus	C,B,P	VU			***R. chameleon***			VU	***R. cymbula***			VU	***R. elegans***		EN	VU
R. chameleon	C			VU	***R. chocoensis***			VU	***R. dodsonii***	CR	CR	VU	***R. guttulata***	CR		
R. chocoensis	C			VU	***R. chrysoglossa***			VU	***R. ephippium***	EN	CR	VU	***R. jesupiana***	CR	CR	VU
R. chrysoglossa	C			VU	***R. citrina***			VU	***R. flosculata***	VU	EN	VU	***R. lansbergii***	VU	CR	VU
R. citrina	C			VU	***R. contorta***	LC	LC	LC	***R. guttulata***	VU	EN		***R. pelyx***	EN	CR	VU
R. cloesii	P			VU	***R. cuprea***	CR	CR	VU	***R. iris***	VU	EN	VU	***R. radulifera***			VU
R. condorensis	E			VU	***R. echinata***			VU	***R. lansbergii***	EN	CR	VU	***R. renzii***	CR	CR	VU
R. contorta	C,E,V,P	VU			***R. echo***		VU		***R. mendozae***			VU	***R. roseola***	CR/EX		
R. cuprea	C	CR	CR	VU	***R. escobarina***			VU	***R. muscifera***	EN		VU	***R. sanguinea***			VU
R. cymbula	E			VU	***R. falkenbergii***			VU	***R. schizosepala***			VU	***R. trichoglossa***			VU
R. dodsonii	E			VU	***R. flosculata***	EN	CR	VU	***R. trichoglossa***	LC	LC	LC	***R. wagnerii***			VU
R. echinata	C,P			VU	***R. guttulata***	EN	NT		***R. portillae****			VU				
R. echo	C		EN		***R. limbata***			VU	***R. howeii****	DD						
R. elegans	V		EN	VU	***R. metae***			VU	***R. persiciana****	.		VU				
R. ephippium	E	EN	CR	VU	***R. muscifera***	CR	CR	VU								
R. escobarina	C			VU	***R. nittioryncha***	CR	CR	VU								
R. falkenbergii	C			VU	***R. pandurata***			VU								
R. flosculata	C,E	VU	EN	VU	***R. pelyx***	EN	EN	VU								
R. guttulata	C,E,V	EN			***R. purpurea***			VU								
R. iris	E	VU	EN	VU	***R. sanguinea***	VU	VU	VU								
R. jesupiana	V	CR	CR	VU	***R. seketii***			VU								

Table 5-17 continued; Colour coded comparison of simplified Red List Global and National Categories.

		Complete range		
		A	B	D
R. lansbergii	E,V,P		EN	
R. limbata	C			VU
R. mendozae	E			VU
R. metae	C			VU
R. mohrii	P	EN	EN	VU
R. muscifera	C,E,CA	VU		
R. nittioryncha	C	CR	CR	VU
R. pandurata	C			VU
R. pelyx	C,V	EN	EN	VU
R. purpurea	C			VU
R. radulifera	V			VU
R. renzii	V	CR/EX	CR	VU
R. roseola	V	CR/EX		
R. sanguinea	C,V	VU	NT	
R. schizosepala	E,V			VU
R. seketii	C			VU
R. tabeae	C			VU
R. teaguei	C			VU
R. trichoglossa	C,E,CA	VU		
R. tsubotae	C			VU
R. vasquezii	B			VU
R. wagnerii	V			VU
*R. piperitosa**	P	DD	DD	DD
*R. portillae**	E			VU
*R. howeii**	E	DD	DD	DD
*R. persiciana**	E			VU
*R. fritillina**	C	DD	DD	DD

	Colombia cont'd		
	A	B	D
R. tabeae			VU
R. teaguei			VU
R. trichoglossa	LC	LC	LC
R. tsubotae			VU
*R. fritillina**	DD		

	Peru		
R. antennifera	EN	CR	VU
R. brachypus	CR		
R. cloesii		EN	VU
R. contorta	CR	EN	VU
R. echinata			VU
R. lansbergii	EN		VU
R. mohrii	EN	EN	VU
*R. piperitosa**	DD	DD	DD

	Bolivia		
R. brachypus	CR	CR	VU
R. vasquezii			VU

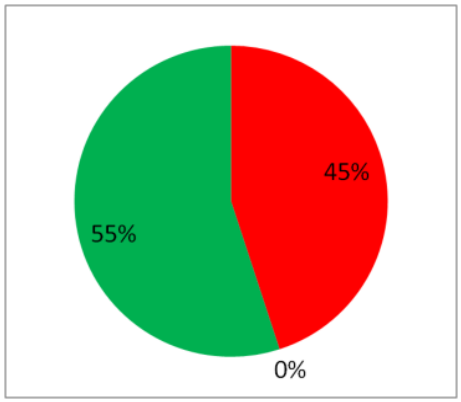
	Central America		
	A	B	D
R. aberrans (Pan)			VU
R. muscifera (CA)	VU		
(Mex-Nic)	EN	CR	VU
(CR and Pan)	LC	LC	LC
R. trichoglossa(CA)	VU		
(Mex-Nic)	EX		
(CR and Pan)	VU		

KEY:	
C	Colombia
E	Ecuador
V	Venezuela
P	Peru
CA	Central America
Mex-Nic	Mexico to Nicaragua
CR-Pan	Costa Rica and Panama

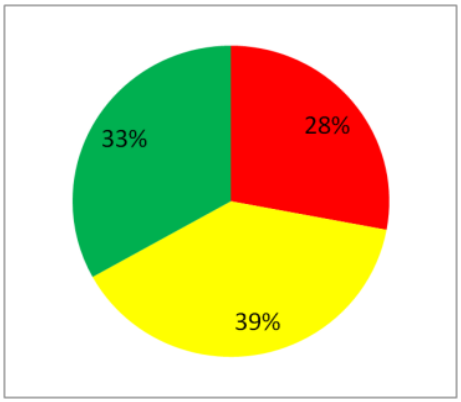
The countries comprising the range for a species are shown in column 2 (RANGE) after the species name.

The Red list values for each country within a range can then be found by looking up the species name in each country list and comparing the values.

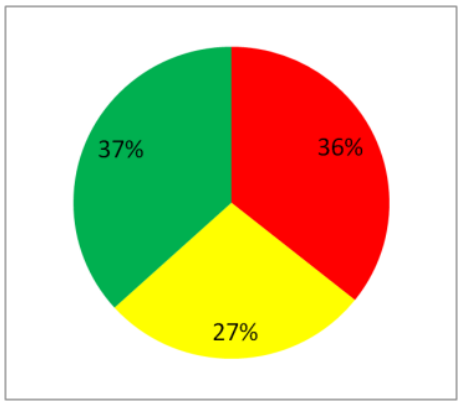
Figure 5-20: Summary charts illustrating lost and 'safe' locations for *Restrepia* species (using the data from Tables 5-11 to 5-15).



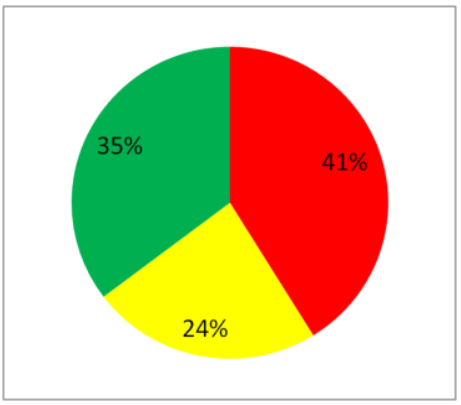
Venezuela (15 species)



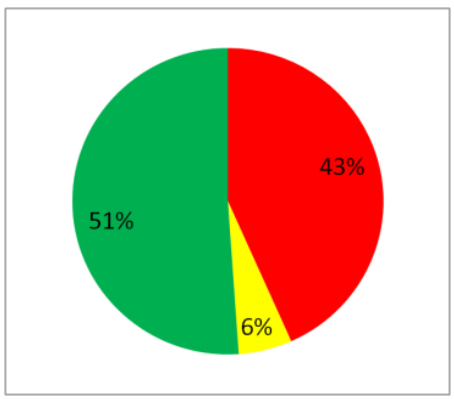
Colombia (30 species)



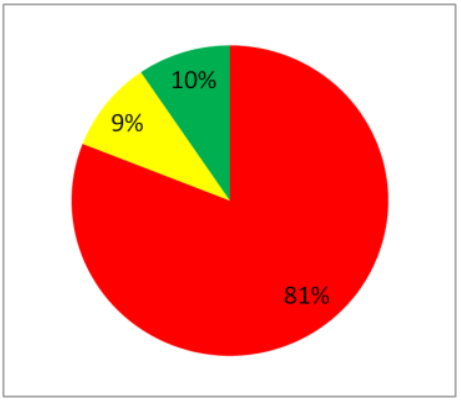
Ecuador (18 species)



Peru (8 species)



Central America (3 species)



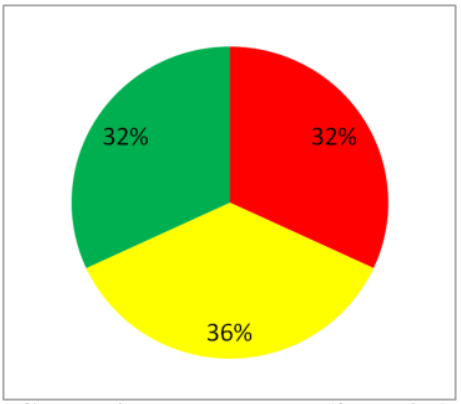
Mexico to Nicaragua (2 species)

Key:

Lost locations.

Vulnerable existing locations

Existing locations in protected habitats e.g. national parks.



Costa Rica and Panama (3 species)

5.4 Discussion

5.4.1 Analysis of the data set

Analysis of the original data set confirmed that the majority of species had been discovered since 1960 and at altitudes between 1000 and 3000 metres (Figure 5-12 and 5-13). Later collections were made at slightly lower altitudes on average, as indicated by the weak but significant negative correlation between altitude and collection year for all countries ($r = 0.136$; $n=289$; $p < 0.05$; Figure 5-13) and also for Columbia ($r = -0.243$; $n= 94$; $p < 0.01$) but not for any other individual country (Table 5-6). This was surprising as it was expected that earlier discoveries would have been at lower altitudes than later ones. It raises the question about what might have occurred after 1960 in the countries of origin and at altitudes between 1000 and 3000 metres to trigger the discovery of so many new *Restrepia* species

5.4.2 Species distributions and maps

The maps (Figures 5-15 to 5-19) showing the species locations for *Restrepia* provide a possible answer to this question. Initially, only locations where habitat had been lost were entered onto each map (red points). These locations closely coincide with the route of the Pan American Highway in Central America, Colombia, Ecuador and to a lesser extent in Venezuela. In these countries, the route of the highway is through the valleys of the high Andes and not at the highest altitudes. The exception is in Peru, where the highway route is along the coast where few *Restrepia* species have been found. It can therefore be postulated that the greatest losses of *Restrepia* habitat (red points) coincide with the route of the Pan American Highway in Central America, Venezuela, Colombia and Ecuador. Further support for this was provided when the positions for remaining locations were entered onto the maps (blue and purple points).

The blue points (locations for species with loss of habitat elsewhere) typically occurred further away from the highway, whilst the purple points (locations for species with no loss of habitat) occurred at the greatest distances from the highway. The dates at which the road was built correspond with the dates of discovery for *Restrepia* species; i.e. most sections of the highway in South American countries were built between 1960 and 1975.

The highway forms part of the Pan American Highway System, which was intended to link the mainland countries of North and South America. The only break that remains is the section of rainforest between Panama and the Colombian border called the Darién Gap, a United Nations Educational, Scientific and Cultural Organisation World Heritage Site for tropical forests (Hassig and Quek, 2007). Many groups are opposed to the completion of this portion of the highway. Their various reasons include protecting the rain forest, containing the spread of tropical disease, protecting the livelihood of indigenous peoples, preventing drug trafficking from Colombia, and preventing foot and mouth disease from entering North America. A previous extension of the highway as far as Yaviza in the Darién province of Panama had resulted in severe deforestation alongside the highway within a decade (Hassig and Quek, 2007).

Comparable deforestation and habitat loss can be observed via Google Earth along the route of the Pan American Highway throughout South America. Figure 5-11 illustrates the habitat loss that has occurred along a section of the Highway in Colombia between 1970 and 2011. There has been extensive conversion to agriculture in this area, as can be evidenced by the fields (Figure 5-11(b)), and from the many minor roads joining the highway that have been built to open up access to the surrounding countryside (Figure 5-11 (a)). In some areas this deforestation has been very rapid (Figure 5-7) and has occurred over as short a time period as five years.

Road building has made many areas more accessible than previously, not least to field botanists who have attempted to catalogue flora and fauna in these countries. For example, in Ecuador many of the orchid species discoveries have been made along Ecuador's road system and on the roads that surround the National Parks (Endara *et al*., 2007). These observations may explain why so many new *Restrepia* species have been discovered since 1960. The areas in which they grow were inaccessible prior to this and only became accessible due to the road building that accompanied that of the Pan American Highway.

5.4.3 National Parks

The maps (Figures 5-15 to 5-19) also provided evidence from which it was possible to establish which *Restrepia* locations were not facing immediate habitat loss. On each map the National Parks (protected areas, either state or privately owned) are shown as green areas. Many of the remaining locations for *Restrepia* species occur within National Parks. *Restrepia* species in these locations can be thought of as 'safe' or at a reduced risk, compared to those occurring elsewhere. This was important when assigning the final Red List Status for these species (Table 5-16, Final assessment for safe habitat). The number, type and area of National Parks vary from country to country and consequently the numbers of species protected by them also vary.

In Venezuela, there are 43 National Parks that cover 21.76% of the country (UNESCO, 2012); in Colombia there are 56 nationally protected areas representing more than 10% of the country's area (UNESCO, 2012); in Ecuador there are 30 National Parks that constitute 17% of the country (UNESCO, 2012); in Peru there are various natural protected areas preserved by the National Government and comprising 15.21% of the country (UNESCO, 2012); in Central America there are currently 26 National Parks in Costa Rica that make up 25% of the country (Baker,

2009) and 30% of the country in Panama has been given 'National Park status by the government (Baker, 2007). These figures affected the final Red List Status that was assigned to the *Restrepia* species in each country (Tables 5-11 to 5-16).

5.4.4 Red List categories

The initial geographical data presented problems, as they lacked latitude and longitude coordinates and the descriptions of the occurrence locations were not always clear. Although methods exist to correct for the uncertainty in such data (Solow and Roberts, 2003), they were not employed since the distribution maps and evidence from the initial data set had produced clear results. Some kinds of uncertainty may be overcome by collecting additional data (Burgman *et al.*, 1999; Akçakaya *et al.*, 2000), but in this case any extra data (should it have existed) would have contained the same uncertainty and could not have made the data more precise. The Red List Categories were established without any other corrections of the initial data, as recommended by the Guidelines (IUCN, 2012b; 2013b).

5.4.5 GeoCAT

The analysis of the data when entered into GeoCAT gave values for EOO and AOO. However, this in itself is not sufficient to assign Red List Categories and additional data were needed; i.e. the number of remaining locations for each species and any decline in EOO, AOO and number of locations (Table 5-3 and Table 5-5 (a) to (f)). When these calculations had been made (Table 5-8), it became possible to assign values for Criterion B, Criterion A and Criterion D (Table 5-10). Table 5-16 presents the final Red List Values, following corrections for Criterion B and additional sub-categories (a) and (b) (Table 5-3) and further corrections if the category of risk could be reduced due to the location being situated in a 'safe' area,

i.e. the risk could be regarded as having ceased. This was reflected in the Criterion A2c which applies to a past reduction in AOO, EOO or habitat quality which has not ceased (Table 5-3 and shown in Table 5-9, Population reduction).

Since a category of threat may be assigned from any of the criteria (Table 5-3), an analysis of the Red List category obtained (Table 5-16, final column) provides a picture of the degree of threat faced by each species. Every *Restrepia* species achieved a Red List Category of 'Vulnerable' or above for its complete range. Apart from *R. trichoglossa* and *R. contorta*, all species could be classified as endangered, with a Red List Category of 'Vulnerable', or above, in either one or more of their countries of origin (Table 5-16). *R. trichoglossa* is of Least Concern in the parts of its range that occur in Colombia and Ecuador, while *R. contorta* is of Least Concern in the part of its range occurring in Colombia.

In Ecuador, one species, *R. trichoglossa*, is categorized as Least Concern and one species,*R. howei,* as Data Deficient. All of the remaining 16 species are threatened: one species, *R. ephippium,* is Critically Endangered; 20% are Endangered and 80% are Vulnerable. The results for the distribution of *Restrepia* from this study correspond closely with the results of a Parsimony Analysis of Endemism (PAE) analysis carried out by field workers in Ecuador (Endara *et al.,* 2007). These indicate that the majority of endemic orchids occur in montane microhabitats between 1500 and 3000m, in the low montane and cloud montane forests: i.e. the same habitat and altitudes as identified for *Restrepia* from the initial data analysis. Only a small fraction of these species have been registered in the National System of Protected Areas (SNAP) in Ecuador. It is estimated that 85% of the endemic orchids of Ecuador are threatened: 2% are Critically Endangered, 11% are Endangered and 87% are Vulnerable (Endara *et al.,* 2007).

In Colombia, out of 30 species found to occur there, one species, *R. fritillina,* was considered to be Data Deficient and two species, *R. trichoglossa* and *R. contorta* were considered of Least Concern, or not under threat. All of the remaining species are threatened: 13% are Critically Endangered, 13% are Endangered and 63% are Vulnerable. When assigning the category Data Deficient, it is important to note that this does not imply that the species concerned is threatened, but rather that there is too little data from which to make an assessment. For this reason, the species *R. piperitosa* (Peru), *R. howei* (Ecuador) and *R. fritillina* (Colombia) are all categorized as Data Deficient. It is not possible to assign a category of threat from the currently available data although it is probable that they may prove to be in a threatened category, as they are known from so few locations.

In Venezuela there are three species categorized as Extinct: *R. guttulata*, *R. aspasiensis* and *R. roseola.* Of these, *R. guttulata* may be considered to be at the limit of its range in Venezuela and it is still found in Colombia as Endangered and in Ecuador as Vulnerable. *R. aspasiensis* also occurs in Colombia where it is Critically Endangered. *R. roseola* is only recorded from this one location in Venezuela and, although it may still persist elsewhere; there are currently no more recorded locations for this species. Hopefully, more sub- populations may be found in the protected areas for this species. All of the remaining 12 species are threatened: 13% are Critically Endangered, 13% are Endangered and 63% are Vulnerable.

Peru is nearly at the southern limit of the distribution range for this genus. Fewer *Restrepia* species occur here than in the previously mentioned countries and only two species are found further south in Bolivia. Of the nine species that have been found in Peru: two, *R. antennifera* and *R. brachypus* (at the extent of their respective ranges), are categorized as Extinct; *R. piperitosa* is categorized Data Deficient; *R. contorta* is categorized as Critically Endangered and *R. mohrii* as Endangered. The

remaining four species (50% of those found here) are all categorized as Vulnerable.

The smallest number of *Restrepia* species is in Central America. Only *R. muscifera*, *R. trichoglossa* and *R. aberrans* are found here. *R. aberrans* is arguably the rarest *Restrepia* species, as it is only known from one location in Panama. As there is so little data regarding this species, it could only be assigned the category D2VU (Vulnerable in Category D2) (Tables 5-5 (f) and 5-16). This is likely to be an underestimate of the threat to the species, as so few plants have found their way into collections and it is virtually unknown in cultivation.

Unlike other *Restrepia* species that have their centres of distribution in Colombia and Ecuador (Luer, 1996a), the centres of distribution for the other Central American species, *R. muscifera* and *R. trichoglossa*, are Panama and Costa Rica. For this reason, the countries Panama and Costa Rica have been considered separately from the remainder of the Central American countries. *R. muscifera* is not threatened in Costa Rica or Panama, but is categorized as Endangered in Mexico to Nicaragua. *R. trichoglossa* is categorized as Vulnerable in Costa Rica and Panama and as Extinct in Mexico to Nicaragua. Habitat destruction in some of these countries has been especially extensive and the few locations for *R. trichoglossa* have been lost (Figure 5-20).

In Bolivia, the only endemic species, *R. vasquezii*, is categorized as Vulnerable. *R. brachypus*, known from two locations, is classified as Critically Endangered here but this represents the limit of the range for the species, which is common in Colombia and Ecuador, although extinct in Peru.

5.4.6 How many records are needed to give an accurate estimate of threat?

This study presents a rather bleak review of the Red List Status for *Restrepia* species over their complete range, in which all species were classified as being threatened, one species as extinct and 51 species as Vulnerable to Critically Endangered. Unfortunately, this is probably an underestimate of the degree of threat that some species are facing.

The reason for this is apparent when assessing Criterion B. Although GeoCAT will calculate AOO for species with one occurrence, the EOO has to be considered as numerically equal to the AOO (IUCN, 2013b). This results in GeoCAT assigning categories for EOO and AOO values as Critically Endangered. However, any Category of threat under Criterion B cannot be assigned to the species as there is not enough data (i.e. more occurrences) from which to calculate values for the sub-categories (a) and (b) (Table 5-3 and Table 5-5 (a) to (f)). These are needed for a full Red List Assessment of Criterion B.

The adjustment for the final Criterion B assessment is shown in Tables 5-11 to 5-15 and Table 16. Even though GeoCAT had produced a preliminary Red List Status of Critically Endangered, this could not be assigned to these species. The only Criterion available for these species is Criterion D2. For *Restrepia* species with one location, this affects 20 out of 53 species across their entire range, 13 out of 30 species in Colombia, 5 out of 18 species in Ecuador, 4 out of 15 species in Venezuela, 4 out of 9 species in Peru, one species in Bolivia and one species in Central America. All of these species may in fact be more accurately classified as Critically Endangered, but this could not be done when the Red List guidelines were applied.

The criterion D2 was intended to identify taxa with very small or restricted populations. A taxon qualifies for Vulnerable D2 if its AOO and EOO are very limited (Table 5-5

(f)), and if there is a plausible, accompanying threat that would cause the taxon to become Critically Endangered or Extinct in a very short time period. Taxa with very limited AOO or EOO are particularly susceptible to such threat (IUCN, 2013b). However, it has been argued that the thresholds for AOO and the EOO, (Table 5-5 (f)) are frequently interpreted too literally and the sub-criterion is too inclusive resulting in excessive over-listing. It has also been argued that it is too exclusive thus leading to under-listing (IUCN, 2013b). For *Restrepia* species, however, an assessment of D2VU should be considered an underestimate of the Endangered status of the species. It is indicative of a species known from one recorded location that is threatened by habitat loss. The level of this threat depends upon the exact known location of the species. It is reduced for species in 'safe' areas and increased for those near urban areas or highways. This highlights the need for more precise geospatial data for these species and their locations.

Despite the fact that the majority of *Restrepia* species have poorly known distributions represented by few records, it has still been possible to make robust preliminary conservation assessments. This investigation has shown that an assessment is possible from very few records. Following the IUCN Red List guidelines, a species known only from a single locality can be assessed depending on its current status and possible threats (Rivers, *et al.,* 2011). Although more data would be desirable, it is important to perform assessments based on a small number of records when these represent all the available information for a species (Rivers, *et al.,* 2011).

Fortunately for some of these narrow endemic species, many of their countries of origin have large areas set aside as National Parks. When an occurrence for a species is within these areas, the risk may be substantially reduced. Figure 5-20 illustrates the percentage loss of locations and percentage safe locations for *Restrepia* species. The

worst example is found in Mexico to Nicaragua, which has lost 81% of *Restrepia* locations with only 10% remaining in protected habitats. Colombia, Ecuador and Peru have similar percentages of *Restrepia* locations remaining in protected habitats. In Venezuela, all the remaining locations for *Restrepia* species are in protected habitats, which suggest that these locations will remain 'safe' in the future. Unfortunately, this cannot be relied upon, as there are great pressures in some countries to clear the currently protected areas for farming and building. Critical problems facing such areas include inappropriate forms of administration, insecurity, encroachment, increasing human intervention and illegal activities (Eyre, 1990; Tranel and Hall, 2003).

5.4.7 The usefulness of online resources for conservation research

As mentioned previously, the past decade has seen an increasing number of online resources become available with which to investigate conservation threats to plant species. This study has shown that it is possible to produce evaluations, in line with Red List Categories, of the conservation status for *Restrepia* species using these resources (e.g. Tropicos, Kew Herbarium, GBIF, and GeoCAT).

5.4.7.1 GeoCAT

GeoCAT as a new, online resource has some unique problems concerning its use. The most important consideration of which is that although it performs analyses on spatially referenced primary data and produces values for EOO and AOO, these are preliminary values and only give an indication of the Red List value for Criterion B. In order to complete a full assessment under Criterion B a number of additional sub-criteria must be met. This was acknowledged by Bachman *et al.*, (2011) who anticipate making significant improvements in later versions of GeoCAT in order to incorporate additional range based analysis that will better inform these aspects of the

IUCN criteria (Bachman *et al.,* 2011). Complementary algorithms such as Alpha hulls (α-hulls - generalisations of convex hulls) may also be incorporated into GeoCAT to provide the user with a wider range of options for a more robust analysis (Bachman *et al.,* 2011). The use of α-hulls may be a more appropriate method for investigating reductions or continuing declines in EOO (IUCN 2013b). (*cf.* Introduction, 5.1.1.5 and Figure 5-2, the convex hull.)

The other major problem lies not with the tool itself, but with the quality of the data available for input. Much herbarium data lacks geospatial references; locations are description based and open to misinterpretation. This is particularly true of historic data. For this investigation the consequence was that data entry was time consuming with hundreds of data points having to be entered individually. For some species, with location details hard to interpret, this was especially onerous. The more powerful features allowing fast and reliable data input directly from online sources such as GBIF could not be utilized. For investigations with accurate geospatial references available which allow ease of data entry, GeoCAT would provide an excellent tool for data analysis with many additional benefits. One of these lies in the fact that the code is open source and the development of algorithms is encouraged so that the tool can develop towards a powerful automated assessment tool (Bachman *et al.,* 2011).

Since it was first made available online last year (2012) there have been various papers published citing GeoCAT (Pinzon *et al.,* 2012; Trias-Blasi and Suksathan, 2013) but the only orchid related reference was by Leopardi *et al.* (2012) who cited the tool in relation to the new genus *Amoana* (Orchidaceae, Laeliinae). It would seem that the current investigation represents the most comprehensive study to date to employ GeoCAT and review its usefulness when applied to primary biodiversity data.

The problem concerning lack of accurate geo-referenced collection data could be

overcome in the future by the growth of Global Positioning System (GPS) technology. Today's GPS receivers are accurate to within 15 metres, and may be found in many devices from car navigation systems, to mobile phones and cameras (El-Rabbany, 2006). Since GPS systems utilize the geographical coordinate system of latitude and longitude, this should make it much easier for field scientists to record location data accurately.

5.4.7.2 Online Herbarium data

The use of these online databases/resources has enabled a detailed picture of the current threats to *Restrepia* species in the wild to be produced. Surprisingly, it has proved possible to produce robust and reliable assessments from what were initially considered limited data which contained uncertainty for some species (this was explained previously). Many digitization projects are now underway around the world in herbaria, which will increase the availability of collection data (Rivers, *et al.,* 2011). While nothing can be done retrospectively to remove the uncertainty in such data, and especially that found in older records, these data are still important for conservation research. They are the only means of assessing how species distributions and hence biodiversity have changed over time. As newer data records are made and become available, it will become easier to perform increasingly accurate conservation and biodiversity assessments for more species.

The research presented in this chapter clearly demonstrates that *Restrepia* is an endangered genus, comprising many narrow endemics. In addition, the data presented do not take into account the effects of global warming, which are likely to exacerbate the threat to *Restrepia* and other plant and animal species in Central and South America. This, therefore, provides the rationale for both *in* and *ex situ* conservation of threatened *Restrepia* species.

"Semper Pleuro." Ann Jesup (2005)

6.1 Conspectus

Throughout these investigations the aim has been to add to the existing knowledge of the genus *Restrepia* so that the resultant data could be used to facilitate *ex situ* conservation initiatives. Implicit in this is the understanding that *ex situ* studies should ultimately help and support *in situ* conservation work. A consideration of the main findings from the study and the effect of other influences follows.

6.1.1 Propagation and cultivation

Fundamental to all *ex situ* plant conservation is the ability to propagate and grow the target species. The current study represents the first media trial for axenic seed germination and *in vitro* seedling development for any species in the genus *Restrepia* and has thus provided the methodology to produce *Restrepia* plant material for both scientific and horticultural purposes (e.g. production of new hybrids, now registered on the International Orchid Register). A time line from pollination/fertilisation to flowering was also established and a tested methodology developed for further investigation of the breeding system operating in the genus.

However, cultivation protocols for rare species, such as some *Restrepia* species, are often ill defined and produced without information on the provenance of the taxa involved (Maunder et al., 1997). Such inappropriate protocols will inevitably result in the loss of plants. Due to their rarity, such species may be impossible to replace and are lost to cultivation as a consequence. For this reason the specialist growing groups, The Pleurothallid Alliance (USA) and the Pleurothallid Alliance UK, play important roles in the *ex situ* conservation of Pleurothallid genera; their aim being to disseminate cultural knowledge so that more people can grow these orchids successfully.

6.1.2 Floral morphology and pollination

An understanding of floral morphology is needed in order to artificially pollinate species and the comparative vegetative and floral morphology of the genus has been well described by Luer (1996a). However, the labellar micromorphology and the micromorphology of the calli has not. The three regions of the labellum were therefore investigated using VPSEM, and their different surface micromorphology compared. The papillate structure of the calli and their cuticular features were established for the first time, together with their complex optical properties and possible 'structural reflection' in the UV range. For the first time the key role of the cirrhi in pollination was postulated. They are thought to act in several possible ways:

1. by protecting the column from predators;
2. by helping to funnel the fly through the flower;
3. by trapping the fly under the column so that pollination takes place;
4. by destabilising the fly in flight by interfering with the action of the halteres.

Luer (1996a) wrote that nothing was known about pollination in this genus although it was regarded as being myophilous. Since this time, further studies on pollination within the Pleurothallidinae have been reported (Blanco *et al.,* 2005; Borba *et al.,* 2001d; Borba and Semir, 2001; Borba *et al.,* 2002; Endara *et al.*, 2010) but very little about *Restrepia*. Although it has been impossible to observe *Restrepia* pollination in its natural habitat, the VPSEM study nevertheless enabled the construction of a pollination hypothesis.

6.1.3 Breeding systems

An orchid's breeding system is often considered to be related to its pollination syndrome; therefore, since little is known about pollination in *Restrepia* (Luer, 1996), it is not surprising that even less is known about their breeding system. None of the

studies published since 1996 regarding breeding systems in the Pleurothallidinae have dealt directly with *Restrepia* (Borba *et al.,* 2001a, b; 2011; Barbosa *et al.,* 2009). The current investigation aimed to address this. However, it was not possible to carry out the type of crosses regarded as standard practice in, for example, investigations on crop species. Many species of *Restrepia* may only produce one flower at a time, thus making inter-ramet pollinations impossible. To compensate for this, self-pollinations (within-flower) were performed across as many different species as possible and, subsequently, as many different cross-pollinations (inter-specific) as possible were performed for comparison. Over 50% of the genus was sampled using this technique, which has provided the most extensive survey of the genus to date and made the most efficient use of the plant material available. The main strength of the current study is that a wide selection of the genus was sampled, which is unusual for this type of investigation (*cf.* Stort and Galdino, 1984; Borba *et al.,* 1999b; Borba *et al.,* 2001a; Barbosa *et al.,* 2009; Gontijo *et al.,* 2010).

The methodology used was novel as there are no previous studies published involving dialellic crosses in the Orchidaceae (Barbosa *et al.,* 2009). Such crosses are fundamental to the understanding of the control of SI (Barbosa *et al.,* 2009). Complex diallelic crosses in progeny arrays are necessary for a more precise evaluation of the mechanisms involved in fruit failure and embryo abortion (Borba *et al.,* 1999b) in orchid species exhibiting such out breeding mechanisms. However, these are unlikely to be implemented due to the long period (about 5-8 years) required for maturation (Borba *et al.,* 1999b). Although the current study has taken eight years to complete, it has allowed data to be gathered which would otherwise have been impractical. It is unlikely that this type of study will be repeated due to the time scale involved.

This study has demonstrated that self-pollinations result in reduced seed filling compared to cross-pollinations. SI was confirmed in the genus by the study of pollen tube growth,

which indicated that, following self-pollination, pollen tubes did not grow fully into the ovary. The 'type' of SI observed is best explained as the operation of a gametophytic self-incompatibility system, which is in agreement with recent work on other Pleurothallid genera: *Stelis* (Christensen, 1992), *Lepanthes* (Tremblay and Ackerman, 2007), *Octomeria* (Barbosa et al., 2009), *Acianthera* and *Pleurothallis* (Borba *et al.*, 2001a).

In addition, specialist growers have observed (Barrow, 2006; Buckingham, 2008) that *Masdevallia* and *Dracula* do not set viable seed by self-pollination, suggesting that SI also operates in these genera. There is variability in the operation of SI across the range of species investigated, in line with previous studies that described 'plasticity' in the SI genes (Travers *et al.,* 2004). The pollen tube studies confirmed the time interval between pollination and fertilisation as nine days for *R. brachypus.* Observations made of similar capsule formation times for other species suggest that a similar time interval between pollination and fertilisation exists throughout the genus.

6.1.4 Conserving genetic diversity

Intra-specific hand pollination followed by subsequent axenic seed propagation cannot solve all the problems of circumventing SI in some species, as exemplified by *R. chocoënsis.* This species is a narrow endemic and obligate out breeder that does not form keikis. The provenance of all recorded plants of *R. chocoënsis* in the UK is known, as enquiry has revealed them all to be divisions and sub-divisions of the same plant. Genetic diversity achieved through sexual reproduction (seed production) was not possible for this species in the UK, as there were not enough unrelated plants available with which to perform intra-specific cross pollinations. In addition, vegetative reproduction for this species is very slow.

One solution was to hybridise *R. chocoënsis* with other members of the genus, thus

producing a hybrid F_1 generation, which would contain some of the gene pool for this endangered species. Currently, there are only two of these plants that have reached maturity and only one has flowered (Plate 6-1). This means that it has not yet been possible to perform backcrosses or other interspecific crosses to determine if these hybrids will prove easier to breed from. The aim of producing a 'hybrid' which closely resembles the original phenotype for this species but is more vigorous and productive is still ongoing.

This *R. chocoënsis* hybrid and other *Restrepia* hybrids produced in the current study are excellent plants for the collections of orchid enthusiasts. As hybrids, they have been found to exhibit improved fitness traits i.e. heterosis or hybrid vigour. This makes them more amenable to *ex situ* culture and ultimately more rewarding to grow.

Unfortunately, plants from cultivated collections of *Restrepia* species may never be suitable for future re-introduction and habitat restoration programmes, as their original habitat may be lost and they may have undergone artificial selection. However, these collections of cultivated *Restrepia* have served other important roles in the course of this study:

1. they have provided useful model populations for scientific investigation;
2. they have provided horticultural protocols for tissue culture of seeds;
3. they have provided data to support seed and germplasm collection initiatives;
4. they may help to relieve collection pressure on wild populations.

Consequently, the seedlings derived, using these protocols, from tissue culture of seeds harvested from wild species of *Restrepia* plants would be suitable for use in reintroduction programmes.

6.1.5 Trade in orchid species and impact on *ex situ* collections

For anyone wishing to grow species orchids, it has become increasingly difficult over the past few years to obtain plants within the UK. This has been due in part to increased production and import costs and also to the regulations now in place controlling importation.

The importation and exportation of orchid species across international borders are regulated by the Convention on Trade in Endangered Species (CITES) (Appendix I and II). CITES Appendix I (and EU Annex A[1]) regulations apply to those orchid species that are considered to be most endangered and at risk of extinction; e.g. all *Paphiopedilum* and *Phragmipedium*[2] species. All commercial trade in wild collected plants of these species and others on Appendix I is prohibited and is permitted only in exceptional circumstances (CITES, 2013).

All other orchid species are included in Appendix II. Although these orchids may not necessarily be threatened with extinction, their "trade must be controlled in order to avoid utilization incompatible with their survival" (CITES, 2013). The legal importation of these plants into the UK, from outside the EU, requires CITES permits from the Department for Environment, Food and Rural Affairs (Defra, 2013).

Seedlings or tissue cultures *in vitro* are exempt from this requirement, although documented proof may still be required that they have been artificially propagated from legally obtained plant material (CITES, 2013). Currently, seeds but not seed pods are exempt from CITES regulations for Appendix I orchids.

Restrepia species are therefore included on Appendix II and CITES permits are required to bring plants into the UK from their countries of origin. In contrast hybrid and species

[1] The EU has its own additional, more extensive list of orchid species (Annex A) which includes all native European orchids
[2] All *Paphiopedilum* and *Phragmipedium* species are included on Annex A

Restrepia seedlings *in vitro* are exempt from CITES regulations. The regulations governing the importation of *Restrepia* and other orchids illustrate the increasing importance of *in vitro* seed culture for all orchid species. This may become the only way that it is practical, given the cost of CITES import permits in this country, to import some of the rarer orchid species. The hybrid plants produced during this study provide an opportunity for enthusiasts to grow *Restrepia* plants more easily, with the added benefit that they will be better adapted to greenhouse conditions than imported plants. As a result of this study, flasks of hybrid *Restrepia* seedlings have been been exported to enthusiasts. For specialist growers in this country, many more species and hybrids have become available in the past few years through several nurseries. Of great interest is the breeding work that is currently being undertaken at the Eric Young Orchid Foundation (Purver, 2012). They have propagated plants that have been received awards from the RHS Orchid Committee and are currrently breeding plants to produce polyploid, 4N hybrids by using a colchicine protocorm treatment (Purver, 2012).

6.1.6 Criticisms of *ex situ* collections

While growing and studying rare species in *ex situ* collections appears to be purely beneficial, there are several important criticisms of such collections. The most common criticism is that the population sizes in ex situ collections are small, often derived from a few closely related individuals, and thus contain only a fraction of the genetic biodiversity of wild populations (Maunder et al., 1997). While this is often true, it ignores the fact that severely depleted wild populations may also contain relatively few individual plants (Tremblay et al., 1998). Therefore, cultivated collections of such species may form a useful genetic 'backup' from which plants can be studied and investigations performed without further depleting wild populations.

Further criticisms of *ex situ* collections, whether held by botanic gardens, specialist growers or enthusiasts, are based on the fact that they all share several adverse characteristics (Maunder *et al.,* 1997). They are susceptible to artificial selection, genetic drift, inbreeding and hybridising with congenerics. Horticulturally desirable taxa with commercial value survive the longest in collections, which makes such collections unrepresentative and biased by horticultural fashion (Maunder *et al.*, 1997). The smaller-flowered, less colourful species and genera are often overlooked. This can make a genus such as *Restrepia* a valuable tool for scientific research, however, as *Restrepia* species have not been subject to any of the effects noted above. Other genera within the Pleurothallidinae, such as *Masdevallia* and *Dracula*, which have undergone substantial hybridisation, would not have been as suitable for study.

6.1.7 Further study

There are several aspects of the current study that raise important questions and which would provide fruitful areas for further investigation.

- The first of these would be to confirm the nature of the labellar secretions in order to establish if these secretions are methyl eugenol, zingerone or raspberry ketone, as have been found in the genus *Bulbophyllum* (Tan, 2006; Tan and Nishida, 2006; Tan *et al.,* 2002). These chemicals are the precursors of the male pheromone and attract male fruit flies. As *Restrepia* are considered to be pollinated by Dipteran species (fruit flies), it would be of great interest to see if they have evolved the same biochemical mechanisms to attract pollinators as *Bulbophyllum* species.
- In addition, it is important to establish the presence or absence of calli papillae in other Pleurothallid genera and to elucidate if they have similar complex optical structures to those found in *Restrepia* and elsewhere.

- The two species, *R.chocoënsis* and *R. aberrans* were not included in the original systemic work on the subtribe (Pridgeon *et al.,* 2001) and are regarded as atypical in the genus. Further DNA studies would ascertain their genetic relationship to other members of the genus.

6.1.8 Are *Restrepia* Endangered?

Perhaps the most relevant question that this study set out to answer was - how endangered are *Restrepia* species in the wild? The use of online herbarium databases and the georeferencing tool, GeoCAT has made it possible to establish the Red List status for all the species. Despite the uncertainty in the geographical data the observed patterns of habitat loss were unexpectedly clear and correspond well to the building of the Pan American Highway and subsequent development since 1960. It was established that useful assessments could be made from only one or two data entries.

All *Restrepia* species were assessed as facing a significant degree of threat, although the category assigned to a species may be misleading. The category D2VU indicates a species with a small number of subpopulations and limited EOO. These species could be considered as Critically Endangered if they occur as narrow endemics in threatened locations, but the category of threat is reduced for those species in 'safe' or protected locations. Species listed as Endangered on national Red Lists are not eligible for entry into the global Red List produced by IUCN, unless they are endemics. Currently there are no *Restrepia* species listed on the IUCN Red List, although this research has clearly shown that most species should be listed both nationally and globally. This has resulted in an underestimate of the Endangered status of these species.

In comparison to the general acceptance that wild populations of orchids are under threat on many fronts, there is little general understanding of the corresponding threats to cultivated populations of orchid species. Collections worldwide, both private and public,

are disappearing as the fluctuating economic climate makes them difficult and uneconomic to run and maintain. The biodiversity of orchid species collections is dwindling as orchid species become rarer, more expensive and difficult to obtain. Conservation is needed for cultivated collections as well as wild populations and it is important to keep alive existing knowledge and expertise in growing these species.

In conclusion, although it is unlikely that the genus *Restrepia* as a whole is threatened with extinction in the near future, those species that are narrow endemics in threatened locations are certainly at risk. Bearing this in mind, *ex situ* conservation via axenic seed germination and subsequent seedling growth *in vitro*, as reported in this study, is of great importance in the conservation of the gene pool and for future studies of this exquisite genus. The understanding of both their floral morphology and the breeding systems that they have evolved is fundamental to the propagation of *Restrepia* species. Furthermore, the media and methodologies described in the current study may prove to be useful for the micropropagation and *ex situ* conservation of other orchid genera.

Addendum

Just after this manuscript was completed, the following details were published online:

> *"Drilling for oil is expected to start in the next few weeks in previously untouched areas of Yasuni Park, Ecuador, protected since 2010 by a UN backed funding initiative. Conservationists are concerned that now there is no viable way to stop the wave of oil drilling in this biodiverse region."*[1]

It does, indeed, induce a false sense of security to consider small orchid populations in currently 'protected' areas as not at risk. Are Restrepia *Endangered? Most certainly.*

H. Millner, 18th August, 2013

[1] BBC (2013) *Ecuador approves Yasuni Park oil drilling in Amazon rainforest* [online]. [Accessed 17th August, 2013]. Available at: www.bbc.co.uk/news/world-latin-america-23722204.

Plate 6-1: ***R. chocöensis* × *R. chameleon***

This hybrid may never be suitable for re-introduction to the wild, but it represents one means of conserving some of the genetic complement of *R chocoënsis by* careful hybridisation

Literature cited

Ackerman, J.D. (1985) Pollination of tropical and temperate orchids. Pp. 98-101 *in*: K.W. Tan (ed.), *Proceedings of the 11th World Orchid Conference.* American Orchid Society, Miami, Florida.

-------- (1998) Evolutionary potential in orchids: patterns and strategies for conservation. *Selbyana*, **19**: 8-14.

Adobe Photoshop CS6 [computer software]. Version 13.1.2 × 64. Adobe Systems Incorporated, San Jose, CA, US.

Agnew, J.D. (1986) Self-compatibility/incompatibility in some orchids of the subfamily Vandoideae. *Plant Breeding*, **97**(2): 183-186.

Akçakaya, H.R., Ferson, S., Burgman, M.A., Keith, D.A., Mace, G.M. and Todd, C.A. (2000) Making consistent IUCN classifications under uncertainty. *Conservation Biology*, **14**: 1001–1013.

Alcantara, S., Semir, J. and Solferini, V.N. (2006) Low genetic structure in an epiphytic Orchidaceae (Oncidium hookeri) in theAtlantic rainforest of South-eastern Brazil. *Annals of Botany*, **98**: 1207–1213.

Ames, O. and Schweinfurth, C. (1930) *Restrepia lankesteri. Schedulae Orchidianae,* **10**: 20-21.

Angiosperm Phylogeny Group (APG) (1998) An ordinal classification for the families of flowering plants. *Annals of the Missouri Botanical Garden*, **85**: 4.

Arcanelli (1883) Osservazioni sull'impollinazione in alcune Aracee. *Nuovo Giornale Botanico Italiano*, **15**: 72-97.

Arditti, J. (1967) Factors affecting the germination of orchid seeds. *Botanical Review*, **33** (1): 1-97.

-------- (1968) Germination and growth of orchid on banana fruit tissue and some of its extracts. *American Orchid Society Bulletin* **37**: 112-116.

-------- (1982) Orchid Seed Germination and seedling culture – a manual. *In:* Arditti, J. (ed.) *Orchid Biology: Reviews and Perspectives II.* Cornell University Press, London. pp 243-370.

-------- (1984) A history of orchid hybridization, seed germination and tissue culture. *Botanical Journal of Linnean Society*, **89**: 359–381.

-------- (1989) History of several important research contributions by South East Asia scientists. *Malayan Orchid Rev (Singapore)* **23**:64–80.

-------- (1990) Lewis Knudson (1884–1958): his science, his times and his legacy. *Lindleyana* **5**: 1–79.

-------- (1992) *Fundamentals of Orchid Biology.* Wiley and Sons. New York.

-------- (2008) *Micropropagation of orchids*, 2nd edn. Blackwell, Cambridge.

-------- and Ernst, R. (1984) Physiology of germinating orchid seeds. *In:* Arditti, J. (ed.) *Orchid Biology: Reviews and perspectives-III*, Cornell University Press, New York. pp. 177-222.

-------- and -------- (1993) *Micropropagation of orchids*. Wiley, New York.

-------- and Ghani, A.K.A. (2000) Numerical and physiological properties of orchid seeds and their biological implications. *New Phytologist,* **145**: 367-421.

-------- and Yam, T.W. (2010) From goats and thrushes to test tubes: development of sexual and clonal propagation methods for orchids. *Acta Horticulturae* **878**: 155-165.

Armenteras, D., Gast, F. and Villareal, H. (2003) Andean forest fragmentation and the representativeness of protected natural areas in the eastern Andes, Colombia. *Biological Conservation,* **113**: 245-256.

Ávila-Díaz, I., Oyama, K.,Gómez-Alonso, C. and Salgado-Garciglia, R. (2009) *In vitro* propagation of the endangered orchid *Laelia speciosa*. *Plant Cell and Tissue Organ Culture*, **99**: 335–343.

Bachman, S., Moat, J., Hill, A.W., de la Torre, J. and Scott, B. (2011) Supporting Red List threat assessments with GeoCAT: geospatial conservation assessment tool. *In*: Smith, V. and Penev, L. (eds) *e-Infrastructures for data publishing in biodiversity science. Zookeys,* **150**: 117–126.

Baker, C. (2007) *Panama 3rd Ed.* National Geographic, 272 pp.

Baker, C. (2009) *Costa Rica.* National Geographic Traveler, 276 pp.

Barbosa, A.R., Cabral do Melo, M. and Borba, E.L. (2009) Self-incompatibility and myophily in *Octomeria* (Orchidaceae, Pleurothallidinae) species. *Plant Systematics and Evolution,* **283** (1-2): 1-8.

Barrow, R. (2006) Proprietor Roydens' Orchids, email correspondence, April 2006.

Bataillon, T. and Kirkpatrick, M. (2000) Inbreeding depression due to mildly deleterious mutation in finite populations: size does matter. *Genetical Research,* **75**: 75-81.

Bechtel, H., Cribb, P. and Launert, E. (1992) *The Manual of Cultivated Orchid Species,* Blandford, London.

Bell, A. K., Roberts, D. L., Hawkins, J. A., Rudall, P. J., Box, M. S. and Bateman, R. M. (2009) Comparative micromorphology of nectariferous and nectarless labellar spurs in selected clades of subtribe Orchidinae (Orchidaceae). *Biological Journal of the Linnean Society* **160:** 369-387.

Benitez-Vieyra, S., Medina, A.M., Glinos, E. and Cocucci, A.A. (2006) Pollinator-mediated selection on floral traits and size of floral display in *Cyclopogon elatus*, a sweat bee-pollinated Orchid. *Functional Ecology* 1-10.

Benzing, D. (1990) *Vascular Epiphytes: General Biology and Related Biota,* Cambridge, University Press.

--------- (1998) Vulnerabilities of Tropical Forests to Climate Change: The significance of resident epiphytes. *Climate Change*, **39**: 519-540.

Bernard, N. (1899) Sur la germination de *Neottia nidus-avis*. *Comptes rendus hebdomadaires des séances de l'Académie des Sciences,* **128**: 1253–1255.

--------- (1900) Sur quelques germinations difficules. *Revue Génerale de Botanique*, **12**: 108–120.

Bhadra, S. K. and Bhowmik T. K. (2005) Axenic germination of seeds and rhizome-based micropropagation of an orchid *Arundina graminifolia* (D. Don.) Hochr. *Bangladesh Journal of Botany*, 34: 59-64.

Bijlsma, R., Bundgaard, J. and Van Putten, W.F. (1999) Environmental dependence of inbreeding depression and purging in *Drosophila melanogaster*. *Journal of Evolutionary Biology,* **12**: 1125-1137.

---------, --------- and Boerema, A.C. (2000) Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila. Journal of Evolutionary Biology*, **13**: 502-514.

Blake, I. (2012) *The Diptera Site* [online]. [Accessed July 2013]. Available at: http://diptera.myspecies.info/.

Blanco, M.A. and Barboza, G. (2005) Pseudocopulatory pollination in Lepanthes (Orchidaceae: Pleurothallidinae) by fungus gnats. *Annals of Botany,* **95**: 763-772.

Bnktop, B. (2010) *Wikimedia commons: Relief map of Central America* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013, under the terms and conditions of the Creative Commons Attribution-Share Alike 2.0 Generic License. Available at: http://commons.wikimedia.org/wiki/File:Relief_map_of_Central_America.jpg.

Bogner, A., Jouneau, P.H., Thollet, G., Basset, D. And Gauthier, **C.** (2007). A history of scanning electron miscroscopy developments: Towards “wet-STEM” imaging. *Micron.* **38:** 390-401.

Borba, E.L. and Semir, J. (1999) Temporal variation in pollinarium size in species of *Bulbophyllum*: a different mechanism preventing self-pollination in Orchidaceae. *Plant Systematics and Evolution*, **217**: 197-204.

--------- and --------- (2001) Pollinator specificity and convergence in fly-pollinated *Pleurothallis* (Orchidaceae) species: A multiple population approach. *Annals of Botany,* **88**: 75-88.

--------, Felix, J.M., Solferini, V.N. and Semir, J. (2001a) Fly-pollinated *Pleurothallis* (Orchidaceae) species have a high genetic variability: evidence from isozyme markers. *American Journal of Botany,* **83**(3): 419-428.

---------, Semir, J. and Shepherd, G.J. (2001b) Self-incompatibility, inbreeding depression and crossing potential in five Brazilian *Pleurothallis* (Orchidaceae) species. *Annals of Botany,* **88**: 89-99.

---------, Shepherd, G.J., Van de Berg, C. and Semir, J. (2002) Floral and vegetative morphometrics of five *Pleurothallis* (Orchidaceae) species: Correlation with taxonomy, phylogeny, genetic variability and pollination systems. *Annals of Botany,* **90**: 219-230.

---------, Barbosa. A.R., de Melo, M. C.,Gontijo, S.L. and de Oliveira, H.O. (2011) Mating systems in the Pleurothallidinae (Orchidaceae): Evolutionary and systematic implications. *Lankesteriana,* **11**(3): 207-221.

Botanic Gardens Conservation International, (BGCI) (2012). *International Agenda for Botanic Gardens in Conservation: 2nd edition.* BGCI, Richmond, UK.

--------- (2013) *Botanic Gardens Conservation International* [online]. [Accessed July 2013]. Available at: www.bgci.org.

Brains (2012) *Wikimedia commons: Topographical map of Colombia* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the Creative Commons Attribution-Share Alike 3.0 Unported License. Available at: http://commons.wikimedia.org/wiki/File:Campa%C3%B1a_libertadora_de_la_Nueva_Granada_(1819).png.

Breton, P.J., (1999). From microns to nanometers: early landmarks in the science of scanning electron microscope imaging. *Scanning Microscopy,* **13-1**: 1–6.

Brewbaker. J.L. (1957) Pollen cytology and self-incompatibility systems in plants. *Journal of hereditary,* **48**(6): 271-277.

Brooks, T.M., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Rylands, A.B., Konstant,W.R., Flick, P., Pilgrim, J., Oldfield, S., Magin, G. and Hilton-Taylor, C. (2002) Habitat loss and extinction in the Hotspots of Biodiversity. *Conservation Biology,* **16**: 909-923.

Bubb, P., May, I., Miles, L. and Sayer, J. (2004) Cloud forest agenda. UNEP-WMCM, Cambridge, UK.

Buckingham, M. (2008) Member of the Pleurothallid Alliance, UK, Personal communication, email October 2008.

Burgman, M.A., Keith, D.A. and Walshe, T.V. (1999) Uncertainty in comparative risk analysis of threatened Australian plant species. *Risk Analysis*, **19**: 585–598.

Busch, J.W. (2005) The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany*, **92**(9): 1503-1512.

Buyun, L., Lavrentyeva, A., Kovalska, L. and Ivannikov, R. (2004) *In Vitro* Germination of Seeds of Some Rare Tropical Orchids. *Acta Universitatis Latviensis, Biology*, **676**: 159-162.

Calderon, E. (1996) Listado preliminar de orquideas en peligro de extincion. *En: Instituto Alexander von Humboldt. Informe sobre el Estado de la Biodiversidad en Colombia*. 3 tomos. Inst, van Humboldt, Ministerio del Medio Ambiente, PNUD, Bogota.

Calderón-Sáenz, E. (ed.) (2007) *Libro rojo de plantas de Colombia. Volumen 6, Orquídeas: primera parte.* Instituto Alexander von Humboldt: Ministerio de Ambiente, Vivienda y Desarrollo Territorial, Bogotá, Colombia.

Central Intelligence Agency, (CIA) (1987) *World Factbook. Wikimedia commons: Maps of Pan American Highway* [online]. [Accessed July 2013]. Reused and modified by H. Millner, Public Domain material. Available at: http://commons.wikimedia.org/wiki/File:Central America_map_detail.PNG.

Charanasri, U. and Kamemoto, H. (1977) Self-incompatibility in the Oncidium alliance. *Hawaii Orchid Journal*, **VI**: 12–15.

Charlesworth, D. (2006) Evolution of plant breeding systems. *Current Biology*, **16:** R726–R735.

--------- (2010) Self-incompatibility *F1000 Biology Reports*, **2**: 68.

--------- and Willis, J.H. (2009) The genetics of Inbreeding Depression. Nature reviews/ dynamics and community interactions. *In*: Pridgeon, A.M., Suarez, J.P. (eds) *Proceedings of the second scientific conference on Andean orchids.* Universidad Técnica Particular de Loja. pp 197-207.

Chen, J.T. and Chang, W.C. (2000) Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). *Plant Science*, **160**(1): 87–9.

Chen, W. (2006) A structural and molecular investigation of 'autofertility' and 'autosterility' in *Vicia faba* (Faba bean). Unpublished PhD Thesis, University of Wolverhampton, UK.

---------, Stoddard, F.L. and Baldwin, T.C. (2011) Variable pressure scanning electron microscopy of *Vicia faba* stigmatic papillae. *Microscopy and Analysis*, **25**(7): 21-22.

Cheng, J., Shi, J. and Shangguan, F.Z. (2009) The pollination of a self-incompatible, food-mimic orchid, *Coelogyne fimbriata* (Orchidaceae), by female *Vespula* wasps. *Annals of Botany*, **104**(3): 565-571.

Christensen, D.E. (1992) Notes on the reproductive biology of *Stelis argentata* Lindl. (Orchidaceae, Pleurothallidinae) in eastern Ecuador. *Lindleyana*, **7**: 28-33.

-------- (1994) Fly pollination in the Orchidaceae. *In:* Arditti, J. ed. *Orchid Biology: Reviews and Perspectives VI.* John Wiley and Sons, Inc. U.S.A. pp. 415-454.

Clements, M.A., Muir, H. and Cribb, P.J. (1986) A Preliminary Report on the Symbiotic Germination of European Terrestrial Orchids. *Kew Bulletin*, **41**(2): 437-445.

Cock, J; Cabrillac, D; Giranton, J; Pastuglia, M; Ruffio-Chable, V; Miege, C; Dumas, C. and Gaude, T. (1999) Investigating the Molecular Mechanism of the Self-incompatibility Response in Brassica. *Annals of Botany*, **85** supplement A: 147-153.

Colinvaux, P.A. (1987). Amazon diversity in light of the paleoecological record. *Quarterly Science Reviews* **6**: 93-114.

Convention on Biological Diversity (CBD) (2006) *Updated Global Strategy for Plant Conservation, (GSPC)* [online]. [Accessed July 2013]. Available at: http://www.biodiv.org/programmes/cross-cutting/plant/default.shtml.

Convention on Trade in Endangered Species (CITES) (2013) *CITES* [online]. [Accessed July 2013]. Available at: http://www.cites.org/.

Correns, C. (1913) Selbststerilität und Individulstoffe. *Biologisches Zentralblatt*, **33**: 389-423.

Cribb, P. and Govaerts, R. (2005) Just how many Orchids are there? *In:* Raynal-Roques, A., Roguenant, A., and Prat, D. (eds.) *Proceedings of the 18th World Orchid Conference, March, 2005, Dijon, France.* Naturalia Publications, pp 161-172.

--------, Kell, S.P., Dixon, K.W. and Barrett, R.L. (2003) Orchid Conservation: a Global Perspective. *In:* Dixon, K.W., Kell, S.P., and Cribb, P.J. (eds.) *Orchid conservation.* Natural History Publications, Borneo, Kota Kinabalu, Sabah. pp 1-24.

C.V. Starr Virtual Herbarium (2013) The New York Botanical Garden, Virtual Herbarium [online]. [Accessed July, 2013]. Available at; http://sciweb.nybg.org/VirtualHerbarium.asp.

Curtis Botanical Magazine (1892) Vol. XLVIII 91.

Dafni, A. (1984) Mimicry and deception in pollination. *Annual Review of Ecology and Systematics,* **15**: 259–278.

Danilatos, G.D. (1991). Review and outline of environmental SEM at present. *Journal of Microsopy,*. **162:** 391–402.

-------- (1993). Introduction to the ESEM instrument. Microsc*opy, Research and Technique,* **25**: 354–361.

Darwin, C. (1876) *The effect of cross and self fertilization in the plant kingdom.* 2nd ed. 1900. J. Murray, London.

------- (1877) The various contrivances by which orchids are fertilised by insects. John Murray, London.

Davies, K.L. and Turner, M.P. (2004) Morphology of Floral Papillae in *Maxillaria* Ruiz. & Pav. (Orchidaceae). *Annals of Botany*, **93**(1): 75-86.

---------, Turner, M.P. and Gregg, A.(2003) Lipoidal labellar secretions in *Maxillaria* Ruisn & Pav. (Orchidaceae). *Annals of Botany,* **91**: 439-446.

de Nettancourt, D. (1997) Incompatibility in angiosperms. *Sexual Plant Reproduction,* **10**: 185-199.

Department for Environment, Food and Rural Affairs (Defra) (2013) *How to obtain import and export permits* [online]. [Accessed July 2013]. Available at: https://www.gov.uk/cites-controls-import-and-export-of-protected-species.

Department for Environment, Food and Rural Affairs (Defra) (2013) *The Darwin Initiative* [online]. [Accessed July 2013]. Available at: http://darwin.defra.gov.uk.

Dodson, C.H. (2003) Why are there so many orchid species? *Lankesteriana* **7:** 99-103.

Dressler, R.L. (1990) *The Orchids Natural History and Classification.* 2nd edn., Harvard University Press, Cambridge, MA USA.

---------(1993) *Phylogeny and classification of the Orchid family*. Cambridge University Press, Cambridge, UK.

East, E.M. and Mangelsdorf, A.J. (1925) A new interpretation of the hereditary behaviour of self-sterile plants. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences,* **II**: 166-171.

El-Rabbany, A. (2006) *Introduction to GPS: The Global Positioning System, Second Edition.* Artech House Publishers, Norwood, MA. 230 pp.

Endara, L., León-Yánez, S. and Williams, N. (2007) *Patterns of endemism of the orchids of Ecuador: Conservation perspectives and priorities. In:* Pridgeon,A.M. and Suarez, J.P. (eds.) *Proceedings of the Second Scientific Conference on Andean Orchids.* Universidad Técnica Particular de Loja, Loja, Ecuador. 127-147.

Endara, L., Grimaldi, D.A. and Bitty, A.R. (2010) Lord of the Flies: pollination of Dracula orchids. *Lankesteriana,* **10**(1): 1-11.

Ernst, R. (1967) Effect of select organic nutrient additives on growth *in vitro* of *Phalaenopsis* seedlings. *American Orchid Society Bulletin,* **36**: 386-394.

Eyre, A.L. (1990) The Tropical National Parks of Latin America and the Caribbean: Present Problems and the Future Potential. *In*: R.B. Kent, (ed.): *Conference of Latin Americanist Geographers,Yearbook 16.* Auburn University, Alabama, US. 15-33.

Fay, M.F. and Krauss, S.L. (2003) Orchid conservation genetics in the molecular age. *In:* Dixon, K.W., Kell, S.P., Barrett, R.L. and Cribb, P.J. (eds.) *Orchid Conservation.* Natural History Publications, Borneo.

Frank, B. (1885) Ueber die auf Wulzelsymbiose beruhende Ernährung geiwsser Bäume durch unterirdische Pilze. *Ber Dtsch Bot Ges* **3**: 128–129.

---------. (1892) *Lehrbuch der Botanik*. Wilhelm Engelmann, Leipzig.

Frankham, R. (1995) Inbreeding and extinction. *Biological Conservation,* **126**: 131-140.

Franklin-Tong, V.E. (2008). *Self-Incompatibility in Flowering Plants: Evolution, Diversity, and Mechanisms.* Springer, Nature, 313 pp.

Franklin-Tong, V.E. and Franklin, F.C.H., (2003) The different mechanisms of gametophytic Self-Incompatibility. *Philosophical Transactions of the Royal Society of London: Series B,* **358**: 1025–1032.

Gentry, A.H. (1982). Neotropical floristic diversity: phytogeographical connections between Central and South America, Pleistocene climatic fluctuations, or an accident of the Andean orogeny? *Annals of the Missouri Botanical Garden* **69:** 557–593.

Gentry, A.H. and Dodson, C.H. (1987). Diversity and biogeography of Neotropical vascular epiphytes. *Ann. Missouri Bot. Gard.* **74**: 205-233.

Gilpin, M.E. and Soulé, M.E. (1986) Minimum viable populations: processes of species extinction. *In:* Soulé, M.E. (ed.) *Conservation biology: the science of scarcity and diversity*. Sinauer Associates, Inc., Sunderland, Massachusetts. pp 19-34.

Glémin, S., Bataillon, T., Ronfort, J., Mignot, A. and Olivier, I. (2001) Inbreeding depression in small populations of self-incompatible plants. *Genetics,* **159**: 1217-1229.

Global Biodiversity Information Facility (GBIF) (2013) *GBIF Data Portal* [online]. [Acessed July 2013]. Available at: http://data.gbif.org/welcome.htm.

Global Land Cover Facility (2013) *TREE's project map - Hot spots of deforestation* [online]. [Accessed July 2013]. Available at: www.landcover.org.

Glover, B. J. (2009) My favourite flowering image. *Journal of Experimental Botany: Flowering Newsletter Review*, 1-3.

--------- and Martin, C. (1998) The role of petal cell shape and pigmentation in pollination success in *Antirrhinum majus*. *Heredity*, **80**: 778-784.

---------, Moyroud, E. and Vignolini, S. (2012) Modelling the formation of diffraction gratings on top of iridescent petal epidermis. *5th Mathematics in the Plant Sciences, Study Group*.

Goldberg, E. E., Kohn, J. R., Lande, R., Robertson, K. A., Smith, S. A. and Igić, B. (2010) Species selection maintains self-incompatibility. *Science*, **330**: 493-5.

Gontijo, S.L., Barbosa, A.R., Cabral de Melo, M. and Borba, E.L. (2010) Occurrence of different sites of self-incompatibility reaction in four *Anathallis* (Orchidaceae, Pleurothallidinae) species. *Plant Species Biology*, **25** (2): 129-135.

Google Earth (2013a) *Colombia 6º 08'22. 58" S, 76º 11'13. 74" W, elevation 177m.* [online]. Google Earth 7.02. [Accessed January 2013]. Available at: http://www.google.co.uk/intl/en_uk/earth/index.htm.

--------- (2013b) *Costa Rica 9º 58'59. 87" N, 84º 11'41. 18" W, elevation 921m.* [online]. Google Earth 7.02. [Accessed January 2013]. Available at: http://www.google.co.uk/intl/en_uk/earth/index.htm.

--------- (2013c) *Venezuela 10º 21'22. 15" N, 66º 57'49. 89" W, elevation 1460m.* [online]. Google Earth 7.02. [Accessed January 2013]. Available at: http://www.google.co.uk/intl/en_uk/earth/index.htm.

--------- (2013d) *Colombia 0º 26'54. 90" N, 78º 13'51. 59" W, elevation 2815m.* [online]. Google Earth 7.02. [Accessed January 2013]. Available at: http://www.google.co.uk/intl/en_uk/earth/index.htm.

--------- (2013e) *Colombia 0º 41'49. 89" S, 79º 27'47. 51" W, elevation 138m.* [online]. Google Earth 7.02. [Accessed January 2013]. Available at: http://www.google.co.uk/intl/en_uk/earth/index.htm.

Graph Pad Prism software, San Diego, California, USA [online]. [Accessed July 2013]. Available at: www.graphpad.com.

Greatwood, A. (2010) *Orchid Growing at Charlesworths' – 1920 -1960.* Lecture to Birmingham and Midland Orchid Society, September, 2010.

Griesbach, R.J. (2002) Development of *Phalaenopsis* Orchids for the Mass-Market. *In:* J. Janick and A. Whipkey (eds.) *Trends in new crops and new uses*. Ashs Press, Alexandria, VA.

Gullan, P.J. and Cranston, P.S. (2005) *The insects: An outline of Entomology. 3rd ed.* Wiley-Blackwell Publishing Ltd. New York, pp 282.

Haffer, J. and Prance, G.T. (2001). Climatic forcing of evolution in Amazonia during the Cenozoic: on the refuge theory of biotic differentiation. *Amazoniana-*

Limnologia et Oecologia Regionalis Systemae Fluminis. Amazonas **16**: 579–605.

Haguenau, F., Hawkes, P.W., Hutchison, J.L., Satiat-Jeunemaître, B., Simon, G.T., and Williams, D.B., (2003). Key events in the history of electron microscopy. *Microscopy Microanalysis*, **9**: 96–138.

Hamrick, J.L. and Godt, M.J. (1990) Allozyme diversity in plant species. *In:* Brown, A.H.D., Clegg, M.T., Kahler, A.L., and Weir, B.S., (eds.) *Plant population genetics, breeding and genetic resources.* Sunderland, Sinauer, pp 43-63.

Hassig, S.M. and Quek, L. (2007) *Cultures of the World, Panama.* Marshall Cavendish, Tarrytown, NY. pp144.

Hawkes, A.D. (1989) *Encyclopaedia of Cultivated Orchids*, Faber and Faber, London, pp 602.

Hawkes, P. (2004) Recent advances in electron optics and electron microscopy. *Annales de la Fondation Louis de Broglie* 29-1.

Hayman, D.L. (1956) The genetical control of incompatibility in *Phalaris coeruleus*. *Australian Journal of Biological Scienc*e, **9**: 321-333.

Hedrick, P.W. and Kalinowski, S.T. (2000) Inbreeding depression in Conservation Biology. *Annual Review Ecology and Systematics*, **31**: 139-162.

Heywood, V.H. (ed). (1995) *The Global Biodiversity Assessment.* The United Nations Environment Programme. Cambridge University Press, Cambridge, UK.

Hilton-Taylor, C. (2000) *2000 IUCN Red List of Threatened Species.* IUCN, Gland, Switzerland and Cambridge, UK.

Hiscock, S. J. and Tabah, D. A. (2003) The different mechanisms of sporophytic self-incompatibility. *Philosophical transactions of the Royal Society of London. Series B*, **358**: 1037-45.

Howe, C. (2013) *Restrepia* [online]. [Accessed July 2013]. Available at: http://www.restrepia.co.uk .

--------- (2005) Personal communication. Email to H. Millner, December 2005.

--------- (2006) Personal communication. Email to H. Millner, February 2006.

--------- (2010) Personal communication. Email to H. Millner, March 2010.

Humboldt, Bonpland and Kunth. (1816) *Nova Genera et species plantarum quas in peregrinatione ad plagam aequinoctialem orbis novi collegerent Bonpland et Humboldt* (7 vols., Paris, 1815-1825).

Ibisch, P.L., Nowicki, C., Muller, R. and Araujo, N. (2003) Methods for the assessment of habitat and species conservation in data-poor countries - case study of

Pleurothallidinae (Orchidaceae) of the Andean rain forests of Bolivia. *Lyonia,* **2**: 109-132.

Ingrouille, M. and Eddie, B. (2006) Plants: Diversity and Evolution. Cambridge University Press UK.

International Union for Conservation of Nature (IUCN) (2012a) *IUCN Red List Categories and Criteria: Version 3.1.* Second edition. Gland, Switzerland and Cambridge, UK: IUCN. iv + 32pp.

--------- (2012b) *Guidelines for Application of IUCN Red List Criteria at Regional and National Levels: Version 4.0.* Gland, Switzerland and Cambridge, UK: IUCN. iii + 41pp.

--------- (2013a) *The IUCN Red List of Threatened Species. Version 2013.1* [online]. [Accessed, July 2013]. Available at: http://www.iucnredlist.org.

--------- (2013b) *Guidelines for Using the IUCN Red List Categories and Criteria. Version 10.* Prepared by the Standards and Petitions Subcommittee [online]. [Accessed July 2013]. Available at: http://www.iucnredlist.org/documents/RedListGuidelines.pdf.

--------- (2013c) *Red List Criteria Summary Sheet* [online]. [Accessed July 2013]. Available at: http://www.iucnredlist.org/documents/2001CatsCrit_Summary_EN.pdf.

Jersáková, J. and Johnson, S.D. (2006) Lack of floral nectar reduces self-pollination in a fly-pollinated orchid. *Oecologia,* **147**: 60-68.

Jesup, A. (2005) Trustee American Orchid Society, Pleurothallid grower, motto reused with permission, email 2005.

Johannsen, D. (1974) Ecology of vascular epiphytes in West African rain forest. *Acta Phytogeographica Suecica,* **59.**

Johansen, B. (1990) Incompatibility in Dendrobium (Orchidaceae). *Botanical Journal of the Linnean Society,* **103:** 165–196.

Karnstedt, A. (2009) *Wikimedia commons: Map of the National Parks in Panama* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the Creative Commons Attribution-Share Alike 3.0 Unported License. Available at: http://en.wikipedia.org/wiki/File:National_parks_of_Panama_map.png.

Kay, K.M., Reeves, P.A., Olmstead, R.G. and Schemske, D.W. (2005). Rapid speciation and the evolution of hummingbird pollination in Neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *American Journal of Botany,* **92**(11): 1899–1910.

Kerry, S. and Gillett, W.H.J. (1998) *1997 IUCN Red List of Threatened Plants.* World Conservation Monitoring Centre IUCN, Gland, Switzerland and Cambridge, UK.

Knudson, L. (1922) Nonsymbiotic Germination of Orchid Seeds. *Botanical Gazette,* **73**(1): 1-25.

------- (1924) Further Observation on Nonsymbiotic Germination of Orchid Seeds. *Botanical Gazette,* **77**(2): 212-219.

------- (1946) A new nutrient solution for the germination of orchid seed. American Orchid Society Bulletin **15**: 214–217.

Koopowitz, H. (2001) *Orchids and their Conservation.* Timber Press. Portland.

---------, Thornhill, A., and Anderson, M. (1993) Species distribution profiles of the neotropical orchids *Masdevallia* and *Dracula* (Pleurothallidinae, Orchidaceae); implications for conservation. *Biodiversity and Conservation,* **2**: 681-690.

---------, --------- and ---------, (1994) A General Stochastic Model for the Prediction of Biodiversity Losses Based on Habitat Conversion. *Conservation Biology,* **8**: 2 425.

---------, Lavarack, P.S. and Dixon, K.W. (2003) The Nature of Threats to orchid conservation. *In:* Dixon, K.W., Kell, S.P., Barrett, R.L. and Cribb, P.J. (eds.) *Orchid Conservation.* Natural History Publications, Borneo.

Kuper, W., Kreft, H., Nieder, J., Koster, N. and Barthlott, W. (2004) Large-scale diversity patterns of vascular epiphytes in Neotropical montane rain forests *Journal of Biogeography*, **31**: 1477-1487.

Kyte, L. and Kleyn, J. (1999) *Plants from Test Tubes. Third ed.* Timber Press, Portland, Oregon, pp 239.

Lamoureux, J., Akçakaya, H.R., Bennun, L., Collar, N.J., Boitani, L., Brackett, D., Bräutigam, A., Brooks, T.M., da Fonseca, G.A.B., Mittermeier, R.A., Rylands, A.B., Gärdensfors, U., Hilton-Taylor, C., Mace, G., Stein, B.A. and Stuart, S. (2003) Value of the IUCN Red List. *Trends in Ecology and Evolution,* **18(**5): 214-215.

Larson, B.M.H., Kevan, P.G. and Inouye, D.W. (2001) "Flies and flowers: taxonomic diversity of anthophiles and pollinators". *Canadian Entomologist,* **133**(4): 439–465.

Lawrence, M. J. (2000) Population Genetics of the Homomorphic Self-incompatibility Polymorphisms in Flowering Plants. *Annals of Botany*, **85** (supplement A): 221-226.

Lehman (1901) *Restrepia trichoglossa,* Sanders' Orchid Guide, 215. Sanders, St. Albans.

León-Yánez, S., Valencia, R., Endara, L., Pitman, N. and Ulloa-Ulloa, C. (eds.) (2011) *The Red Book of the Endemic Plants of Ecuador*. Publications of QCA Herbarium, Pontifical Catholic University of Ecuador, Quito, Ecuador.

Leopardi, C., Carnevali, G. and Romero-Gonzalez, G.A. (2012) : *Amoana* (Orchidaceae, Laeliinae), a new genus and species from Mexico. *Phytotaxa*, **65**: 23-35.

Lewis, D. (1979) *Studies in Biology No. 10: Sexual incompatibility in plants*. Edward Arnold Ltd. London, UK. 59 pp.

Lindley, J. (1859) Folia Orchidacea 8-9. London.

Lo, S.F., Nalawade, S.M., Kuo, C., Chen, C. and Tsay, H. (2004) Asymbiotic germination of immature seeds, plantlet development and *ex vitro* establishment of plants of *Dendrobium tosaense* 'Makino' - A medicinally important orchid. *In vitro Cellular & Developmental Biology - Plant*, **40**: 5.

Luer, C. A. (1986a) Icones Pleurothallidinarum I. Monographs in Systematic Botany of the Missouri Botanical Garden 15.

-------- (1986b) Icones Pleurothallidinarum III. Monographs in Systematic Botany of the Missouri Botanical Garden 20.

-------- (1996a) *Icones Pleurothallidinarum XIII, Systematics of the genus Restrepia (Orchidaceae).* Monographs in Systematic Botany. Missouri Botanical Gardens, St. Louis, MO.

-------- (1996b) Nuevas especies de *Restrepia* / New species of *Restrepia*. Orquideología **20**(2): 117–182.

-------- (1998) Icones *Pleurothallidinarum XVI,* Monographs in Systematic Botany **65**: 119 Missouri Botanical Gardens, St. Louis, MO.

-------- (2002) *Icones Pleurothallidinarum XXIV,* Monographs in Systematic Botany **88**: 109 Missouri Botanical Gardens, St. Louis, MO.

-------- (2003) *Icones Pleurothallidinarum XXV: Systematics of Masdevallia Part 5.* Monographs in Systematic Botany, Missouri Botanical Gardens, St. Louis, MO.

-------- (2004) *Icones Pleurothallidinarum XXVI: Pleurothallis subgenus Acianthera and three allied subgenera. A second century of new species of Stelis of Ecuador. Epibator, Ophidion, Zootrophion. Addenda to Brachionidium, Dracula, Lepanthes, Platystele, Pleurothallis, Porroglossum, and Masdevallia. New genera and combinations.* Monographs in Systematic Botany, Missouri Botanical Gardens, St. Louis, MO.

-------- (2005) *Icones Pleurothallidinarum XXVII,* Monographs in Systematic Botany **103**: 279 Missouri Botanical Gardens, St. Louis, MO.

------- (2006) *Icones Pleurothallidinarum XXVIII. Reconsideration of Masdevallia and the Systematics of Specklinia and Vegetatively Similar Taxa.* Monographs in Systematic Botany, Missouri Botanical Gardens, St. Louis, MO.

------- (2007) *Icones Pleurothallidinarum XXIX. A Third Century of Stelis of Ecuador, Systematics of Apoda-Prorepentia, Systematics of Miscellaneous Small Genera, Addenda New Genera, Species and Combinations (Orchidaceae).* Monographs in Systematic Botany, Missouri Botanical Gardens, St. Louis, MO.

Lundquist, A. (1956) Self-incompatibility in rye. I. Genetic control in the diploid. *Hereditas*, **42**: 293-348.

Lynch, M. (1991) The genetic interpretation of inbreeding depression and outbreeding depression. *Evolution*, **45**(3): 622-629.

Manning, S. (2010) Discovering New World Orchids. Cambrian Press, Aberystwyth, Wales, pp.669.

Matton, D., Nass, N., Clarke, A. and Newbigin, E. (1994) Self-incompatibility : How plants avoid illegitimate offspring. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, **91:** 1992-1997.

Mau, S. L., Anderson, M. A., Heisler, M., Haring, V., McClure, B. A. and Clarke, A. E. (1991) Molecular and evolutionary aspects of self-incompatibility in flowering plants. *Symposia of the Society for Experimental Biology*, **45**: 245-69.

Maunder, M., Culham, A. and Hankamer, C. (1997) Picking up the pieces: Botanical conservation on degraded oceanic islands. *In:* Fiedler PL, Kareiva PM, (eds.) *Conservation Biology: for the coming decade.* Springer 2nd ed. pp. 333.

McKrath, K., (2004) Market Outlook Report: Hawaii's Potted Orchids. *Hawaii Department of Agriculture.*

McMichen, M. (2005) Formerly of the Micropropagation Unit, Kew. Visit in 2005.

Mena-Ali, J.I. and Stephenson, A.G. (2007) Segregation Analyses of partial self-incompatibility in self and cross progeny of *Solanum carolinense* reveal a leaky *S*-allele. *Genetics*, **177**: 501-510.

Millner, H.J., Obeng, A., McCrea, A.R. and Baldwin, T.C. (2008) Axenic seed germination and in vitro seedling development of *Restrepia brachypus* (Orchidaceae). *Journal of the Torrey Botanical Society*, **135**(4): 497–505.

Ministero del Ambiente (2012) *Mapa del sistema nacional de areas protegidas del Ecuador* [online]. [Accessed July 2013]. Available at: www.ambiente.gob.ec.

Ministero del Ambiente, Perú (2008) *Áreas naturales protegidas; Perú 2008* [online]. [Accessed July 2013]. Available at: http://sinia.minam.gob.pe/public/docs/738.jpg.

Mittermeier, R.A., Mittermeier, C.G. and Myers, N. (1999) *Hotspots: earth's biologically richest and most endangered terrestrial ecoregions*. CEMEX, Mexico City, Mexico.

Moore, D. (1849) On growing orchids from seeds. *Gardeners' Chronicle* **35**: 549.

Morel, G. (1960) Producing virus-free cymbidiums. *American Orchid Society Bulletin,* **29**: 495-497.

-------- (1965) Clonal propagation of orchids by meristem culture. *Cymbidium Society News,* **20**: 87-93.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum,* **15**: 473- 497.

Myers, N. (1988) Threatened Biotas: "Hotspots" in Tropical Forests. *The Environmentalist,* **8**: 187-208.

-------- (1990) The Biodiversity Challenge: Expanded Hot-Spots Analysis. *The Environmentalist,* **10**: 1243-256.

---------, Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B. and Kent, J. (2000) Biodiversity Hotspots: Threatened Species and Conservation Priorities. *Nature*, **403**: 853-858.

Nasrallah, J. B. (2000) Cell-cell signalling in the self-incompatibility response, *Current opinion in plant biology*, **3**: 368-73.

Neiland, M.R.M. and Wilcock. C.C. (1998) Fruit set, nectar reward, and rarity in the Orchidaceae. *American Journal of Botany,* **85**: 1657–1671.

Neyland, R. and Urbatsch, L.E. (1993) Anatomy and morphology of the articulation between ovary and pedicel in *Pleurothallidinae. Lindleyana,* **8**(4): 189-192.

------- (1995) A terrestrial origin for the Orchidaceae suggested by phylogeny inferred from ndhF chloroplast gene sequences. *Lindleyana* **10**(4): 244-251.

Oakeley, H.F. (2003) Infrageneric classification of *Ida* and the description of a New Hybrid genus - *Lycida* (Oakeley), *Orchid Digest,* **67**: 1.

-------- (2005) *Anguloa tognettiae* (Orchidaceae) *Curtis's Botanical Magazine,* **22**(1): 63.

O'Shaughnessy, L. (2010) *Orchids of the Pleurothallid Alliance* [online]. [Accessed July 2013]. Available at: http://www.pleurothallids.com/restrepia.htm.

Oatley, C.W. (2004). The Early History of the Scanning electron microscope. *Advances in imaging and electron physics.* **133**: 7-34.

Pand, M.J. and van Stade, J. (1998) The use of charcoal in *in vitro* culture – a review. *Plant Growth Regulation,* **26**: 155-163.

Pease, R.F.W. and Nixon, W.C**.** (1965). High resolution scanning electron microscopy. *Journal of Scientific Instrumentation,* **42:** 31-35.

Phafl, J. (2013) *Orchid Species Encyclopedia* [online]. [Accessed July 2013]. Available at: http://www.orchidspecies.com.

Philips, O. (1997) Biodiversity and Conservation of Neotropical Montane Forests: Proceedings of the Neotropical Montane Forest Biodiversity and Conservation Symposium, New York Botanical Garden 21-26 June 1993. *Biodiversity and Conservation,* **6**: 8.

PhytoTechnology Laboratories (2013) *Product Information Sheet, P668* [online]. [Accessed July 2013]. Available at: http://www.phytotechlab.com/TechInfo/P668-Info.pdf.

Pinheiro, F. and Cozzolino, S. (2013). *Epidendrum* (Orchidaceae) as a model system for ecological and evolutionary studies in the Neotropics. *Taxon,* **62**(1): 77-88.

Pinzon, J.P., Ramirez, I.M. and Carnevali, G. (2012) The re-establishment of *Tillandsia cucaensis* (Bromeliaceae), a good species formerly confused with a new species from the Gulf of Honduras. *Phytotaxa,* **61**: 1-16.

Plant Heritage (NCCPG) (2013) *National collections* [online]. [Accessed July 2013]. Available at: www.nccpg.com/National-Collections.aspx.

Pridgeon, A.M. (1981) Absorbing trichomes in the Pleurothallidinae (Orchidaceae). *American Journal of Botany,* **68**(1): 64-71.

-------- (1982a) Diagnostic anatomical characters in the Pleurothallidinae (Orchidaceae) *American Journal of Botany,* **69**: 921-938.

-------- (1982b) Numerical analyses in the classification of the Pleurothallidinae (Orchidaceae). *Botanical Journal of the Linnaean Society,* **85**(2): 103-131.

-------- (1992) *The illustrated encyclopaedia of orchids.* Timber Press, Portland, Oregon.

-------- (2003) Modern species concepts and practical considerations for conservation of Orchidaceae, *In:* Dixon, K.W., Kell, S.P., Barrett, R.L., and Cribb, P.J. (eds.) *Orchid Conservation.* Natural History Publications, Borneo pp 43-54.

-------- (2004) Email to Dr F. Stoddard and H Millner, November 2004.

--------- and Chase, M.W. (2001) A phylogenetic reclassification of Pleurothallidinae (Orchidaceae). *Lindleyana,* **16**: 235-271.

--------- and Stern, W.L. (1983) Ultrastructure of osmophores in *Restrepia* (Orchidaceae). *American Journal of Botany*, **70**(8): 1233-1243.

--------- and ---------, (1985) Osmophores of scaposepalum (Orchidaceae). *Botanical Gazette,* **146**(1): 115-123.

--------- and Williams, N.H. (1979) Anatomical aspects of *Dresslerella. Selybana,* **5**: 120-134.

---------, Solano, R. and Chase, M.W. (2001) Phylogenetic relationships on Pleurothallidinae (Orchidaceae): Combined evidence from nuclear and plastic DNA sequences. *American Journal of Botany*, **88**: 2286-2308.

---------, Cribb, P.J., Chase, M.W. and Rasmussenn, F.N.(2010) *Genera Orchidacearum. Volume 4: Epidendroideae Part 1*. Oxford University Press, pp 672.

Primack, R. and Corlett, R. (2005) *Tropical Rain Forests: An Ecological and Biogeographical Comparison.* Blackwell Publishing, UK.

Pritchard, H. (2007) *Orchid seed stores for sustainable use, The Darwin Initiative* [online]. [Accessed July 2013]. Available at: http://darwin.defra.gov.uk/project/16012.

Purver, C. (2012) Curator Eric Young Orchid Foundation, Jersey. Personal communication at British Orchid Congress, Writhlington School , October, 2012.

Rands, S.A., Glover, B.J. and Whitney, H.M. (2011) Floral epidermal structure and flower orientation: getting to grips with awkward flowers. *Athropod interactions*, **5**: 279-285.

Reed, D.H., Briscoe, D.A. and Frankham, R. (2002) Inbreeding and extinction: the effect of environmental stress and lineage. *Conservation Genetics*, **3**: 301-307.

---------, Lowe, E.H., Briscoe, D.A. and Frankham, R. (2003) Inbreeding and extinction: effects of rate of inbreeding. *Conservation Genetics,* **4**: 405-410.

Rice, R. (2006) *Oasis the Journal, the* Restrepia *Gallery* [online]. [Accessed July 2013]. Available at: http://www.oasisjournal.com/restrepia/index.html.

Richards, A.J. (1997) *Plant Breeding Systems*. 2nd Ed. Chapman and Hall, London UK.

Riley, M. (2012). Owner 'Riley orchid growers' Ltd., Newent, UK. Visited 2012.

Rivers, M.C., Taylor, L., Brummitt, N.A., Meagher, T.R., Roberts, D.L. and Lughadha, E.N. (2011) How many herbarium specimens are needed to detect threatened species? *Biological Conservation,* **144**: 2541-2547.

Roberts, D.L. (2003) Pollination biology: the role of sexual reproduction in orchid conservation. *In:* Dixon, K.W., Kell, S.P., and Cribb, P.J. (eds.) *Orchid*

conservation. Natural History Publications, Borneo, Kota Kinabalu, Sabah. pp 113-136.

Rothschild, W. and Jordan, K. (1903) A revision of the lepidopterous family Sphingidae. *Novitates Zoology* **9**, suppl.

Rotor, J.G. (1949) A method for vegetative propagation of Phalaenopsis species and hybrids. *Amerian Orchid Society Bulletin* **18**: 738–739.

Royal Botanical Garden (RBG) Kew (2013a) *GIS Unit – Red Listing (GeoCAT)* [online]. [Accessed July 2013]. Available at: http://www.kew.org/science-research-data/kew-in-depth/gis/species-conservation/red-listing/index.htm.

--------- (2013b) *Herbarium Collections* [online]. [Accessed July 2013]. Available at: http://www.kew.org/collections/herbcol.html.

--------- (2013c) *Millennium Seed Bank Project Kew* [online]. [Accessed July 2013]. Available at: http://www.kew.org/msbp/index.htm.

Royal Horticultural Society (RHS) (2013) *International Orchid Register* [online]. [Accessed July 2013]. Available at: http://www.rhs.org,uk/plants/registerpages/orchidsearch.asp.

Ruiz, H. and Pavon, J. (1798) *Humboltia contorta,* Systema vegetabilum florae peruvianae et chilensis 235.

Runkle, E., Wang, Y., Blanchard, M. and Lopez, R. (2007) Growing the Best Phalaenopsis Part 1: An Introduction to Potted Phalaenopsis Orchids. *AOS Orchids,* Jan. 24-29.

Ruska, E. (1993) The development of the electron microscope and of elctronmicroscopy. *In: Nobel Lectures Physics 1981-1990,* Scientific Publishing Company, Singapore.

Ryan, A. and Oakeley, H. (2003) *Ida,* a new genus, based on *Lycaste* section Fimbriate Fowlie. *Orchid Digest,* **67**: 1.

Sander (1906) *Sander's Complete List of Orchid Hybrids*. Sander and Sons, St Albans, UK. 123pp.

Sazima, M., Vogel, S., Cocucci, A.A. and Hausner, G. (1993) The perfume flowers of *Cyphomandra* (Solanaceae); pollination by Euglossine bees, bellows mechanism, osmophores and volatiles. *Plant systematics and evolution,* **187**: 51-88.

Schiestl, F.P. (2005) On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften,* **92**: 255-264.

Schlecter. (1918) Barbosella Nov.Gen. *Repertorium Specierum Novarum Regni Vegetabilis,* **15**: 259-260.

Schuiteman, A. and de Vogel, E. (2003) Taxonomy for Conservation. *In:* Dixon, K.W., Kell, S.P., and Cribb, P.J. (eds.) *Orchid conservation.* Natural History Publications, Borneo, Kota Kinabalu, Sabah. 55-68.

Seaton, P.T. (2007) Establishing A Global Network Of Orchid Seed Banks. *Lankesteriana*, **7**(1-2): 371-375.

Seaton, P.T. (2010) Seed Stores for Sustainable Use, RBG, Kew. Email correspondence, February 2010.

Seaton, P. and Ramsay, M. (2005) *Growing orchids from seeds.* Royal Botanic Gardens, Kew.

Seaton, P.T., Marks, T., Perner, H., Jijon, C. and Pritchard, H. (2007) Orchid Seed Banking Takes Off. *In:* Pridgeon, A. M., Suarez, J.P. (eds) *Proceedings of the Second Scientific Conference on Andean Orchids*, Universidad Técnica Particular de Loja, Loja, Ecuador. pp 173-183.

Shabecoff, P. (1978) New York Times, 4th June, 1978.

Shadowxfox (2008a) *Wikimedia commons: Parques Naturales Venezuela* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the GNU Free Documentation License Version 1.2 Available at: http://commons.wikimedia.org/wiki/File:ParquesNaturalesVenezuela.png.

-------- (2008b) *Wikimedia commons:* Parques Nacionales Naturales de Colombia [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the Creative Commons Attribution-Share Alike 3.0 Unported License. Available at: http://commons.wikimedia.org/wiki/File:ParquesNaturalesColombia.png.

-------- (2012) *Wikimedia commons: Topographical map of Ecuador* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the Creative Commons CC0 1.0 Universal Public Domain Dedication. Available at: http://commons.wikimedia.org/wiki/File:Ecuador_relief_location_map.svg.

-------- (2013) *Wikimedia commons: Topographical map of Venezuela* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the GNU Free Documentation License Version 1.2 Available at: http://commons.wikimedia.org/wiki/File:Venezuela_relief_location_map_(%2Bclaimed).jpg.

Shimada, T., Otani, M. and Morii, M. (2001) *In vitro* propagation and recovery of a habitat of *Habenaria radiata* (*Orchidaceae*). *Acta Horticulturae*, **560**: 481-484.

Silva, N.F. and Goring, D.R. (2001) Mechanisms of self-incompatibility in flowering plants. *Cellular Molecular Life Science*, **58**: 1988-2007.

Singer, R.B. and Cocucci, A.A. (1999) Pollination mechanism in four sympatric southern Brazilian Epidendroideae orchids. *Lindleyana*, **14**: 47-56.

Smith, R. and Gould, J.H. (1989) Introductory essay for a reprinting of Murashige T, Skoog F, 1962. A revised medium for rapid growth and bioassays withtobacco tissue cultures. *Physiol Plant* 15: 473–497. *In:* Janick, J. (ed) *Classic papers in horticultural science,* Prentice-Hall, Englewood Cliffs. pp 59–88.

Snowman, B. N., Geitmann, A., Clarke, S., Staiger, C. J., Franklin, F. C. H., Emons, A. M. and Franklin-Tong, V. E. (2000) Signalling and the Cytoskeleton of Pollen Tubes of *Papaver rhoeas*. *Annals of Botany*, **85**: 49-57.

Solow, A.R., Roberts, D.L. (2003) A nonparametric test for extinction based on a sighting record. *Ecology*, **84**: 1329-1332.

Stenzel, H. (2000) Pollen morphology of the subtribe Pleurothallidinae Lindl. (Orchidaceae). Grana **39** Issue 2 and 3.

--------- (2004) Systematics and evolution of the genus *Pleurothallis* R.Br. (*Orchidaceae*) in the Greater Antilles. PhD thesis, Humboldt University, Berlin.

Stephenson, A.G., Good, S.V. and Vogler, D.W. (2000) Interrelationships among inbreeding depression, plasticity in the self-incompatibility system and the breeding system of *Campanula rapunculoides* L. (Campanulaceae). *Annals of Botany*, **85**: 211-220.

Stern, W.L., Pridgeon, A.M. and Luer, C.A. (1985) Stem structure and its bearing on the systematics of Pleurothallidinae (Orchidaceae). *Botanical Journal of the Linnaean Society,* **91**(4): 457-471.

Stewart, S.L. and Kane, M.E. (2006) Asymbiotic seed germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae). *Plant Cell and Tissue Organ Culture*, **86**: 147-158.

Stoddard, F.L. (1986) Floral viability and pollen tube growth in *Vicia faba* L. *Journal of Plant Physiology*, **123:** 249-262.

Stone, J.L., Sasuclark, M.A. and Blomberg, C.P. (2006) Variation in the self-incompatibility response withing and among populations of the tropical shrub *Witheringia solanacea* (Solanaceae). *American Journal of Botany,* **93**: 592-598.

Stort, M.N.S. and de Lima Galdino, G. (1984) Self- and cross-pollination in some species of the genus *Laelia,* Lindl., (Orchidaceae). *Brazilian Journal of Genetics,* **VII** (4): 671–676.

Stout, A.B. (1917) Fertility in *Cichorium intybus*: The sporadic occurrence of self-fertile plants among the progeny of self-sterile plants. *American Journal of Botany,* **4**(4): 375-395.

Stpiczynska, M., Davies, K.L. and Gregg, A. (2003) Nectary Structure and Nectar Secretion in *Maxillaria coccinea* (Jacq.) L.O.Williams *ex* Hodge (Orchidaceae). *Annals of Botany* **93**: 87-95.

Sutherland, W.J. (2000) *The Conservation Handbook: Research, Management and Policy.* Blackwell Publishing, UK.

Tan, K. H. (2006) Fruit Fly Pests as Pollinators of Wild Orchids. *In:* Sugayama R.L., Zucchi R.A., Ovruski S.M., and Sivinski, J. (Eds.) *Fruit Flies of Economic Importance: From Basic to Applied Knowledge.* Proceedings of the 7th International Symposium on Fruit Flies of Economic Importance, 10-15 Sept 2006, Brazilian Association for the Advancement of Science (SBPC), Salvador, Brazil. pp 195-206.

--------- and Nishida, R. (1995) Incorporation of raspberry ketone in the rectal glands of males of the Queensland fruit fly, Bactrocera tryoni Froggatt (Diptera: Tephritidae). *Applied Entomology and Zoology,* **30**(3): 494-497.

--------- and Nishida, R. (2007) Zingerone in the floral synomone of *Bulbophyllum baileyi* (Orchidaceae) attracts *Bactrocera* fruit flies during pollination. *Biochemical Systematics and Ecology*, **35**(6)**:** 334-341.

---------, --------- and Toong, Y. C. (2002) Floral synomone of a wild orchid, *Bulbophyllum cheiri*, lures Bactrocera fruit flies for pollination. *Journal of chemical ecology*, **28**(6): 1161-72.

Teixeira, S., Borba, E.L. and Semir, J. (2004) Lip anatomy and its implications for the pollination mechanism of *Bulbophyllum* species (Orchidaceae). *Annals of Botany,* **93**: 499-505.

Thomale, H. (1956) Neue Wege der Orchideenvermehrung. *Orchidee* **7**: 104–105.

Thompson, P.A. (1980) *Orchids from Seed*. Royal Botanic Gardens Kew.

Toucan Guides (2006) *Costa Rica's National Parks* [online]. [Accessed July 2013]. Available at: http://costa-rica-guide.com/travel/index.php?Itemid=563&id=340&option=com_content&task=view.

Tranel, M.J. and Hall, A. (2003) Parks as Battlegrounds: Managing Conflicting Values. *In:* Harmon D.P. and Putney, A.D. (eds.) *The Full Value of Parks: From Economics to the Intangible.* Rowman & Littlefield, Lanham, Maryland, US. pp 253-267.

Travers, S.E., Mena-Ali, J. and Stephenson, A.G. (2004) Plasticity in the self-incompatibility system of *Solanum carolinense*. *Plant Species Biology,* **19**(3): 127-135.

Tremblay, R.L. (1997) Distribution and dispersion patterns of individuals in nine species of *Lepanthes* (Orchidaceae). *Biotropica,* **29**: 38-45.

--------- and Hutchings, M.J. (2003) Population dynamics in orchid conservation: a review of analytical methods based on the rare species *Lepanthes eltoroensis*. *In:* Dixon, K.W., Kell, S.P., and Cribb, P.J. (eds.) *Orchid conservation.* Natural History Publications, Borneo, Kota Kinabalu, Sabah. pp 183-204.

--------- and Ackerman, J.D. (2007) Floral color patterns in a tropical orchid: Are they associated with reproductive success? *Plant Species Biology,* **22**: (2) 95-105.

--------- and Otero, J.T. (2009) Orchid conservation biology: predicting species dynamics and community interactions. *In:* Pridgeon, A.M., Suárez, J.P. (eds) *Proceedings of the Second Scientific Conference on Andean Orchids*. Universidad Técnica Particular de Loja, Loja, Ecuador.

---------, Zimmermann, J.K., Lebrón, L., Bayman, P., Sastre, I., Axelrod, F. and Alers-García, J. (1998) Host specificity and low reproductive success in the rare endemic Puerto Rican orchid *Lepanthes caritensis. Biological Conservation,* **85**: 297-304.

Tremblay, R.L., Ackerman, J.D., Zimmerman, J.K. and Calvo, R.N. (2004). Variation in sexual reproduction in orchids and its evolutionary consequences: A spasmodic journey to diversification. *Biological Journal of the Linnaean Society,* **84**: 1–54.

---------, Pomales-Hernández, G. and Méndez-Cintrón, M. de L. (2006) Flower Phenology and Sexual Maturation: Partial Protandrous Behaviour in Three Species of Orchids. *Caribbean Journal of Science,* **42**(1): 75-80.

Treub, M. (1890) Étues sur les Lycopodiacées. *Annales du Jardin botanique de Buitenzorg,* **8**: 1–37.

Trias-Blasi, A. and Suksathan, P. (2013) Delimitation in the *Veratrum mengtzeanum - V. chiengdaoense* complex (Melanthiaceae) in Thailand based on morphology, with commentary on conservation status. *Phytotaxa,* **96**(1): 54–62.

Tropicos (2013) *Missouri Botanical Garden, Tropicos. org.* [online]. [Accessed July 2013]. Available at: http://www.tropicos.org.

United Nations Educational, Scientific and Cultural Organization, (UNESCO) (2012) *The World's Heritage, New Second Edition*. UNESCO, Paris and Harper Collins, Glasgow, UK. 872 pp.

United Nations Environment Programme, (UNEP) (2001) Convention on Biological Diversity, (CBD)[online]. [Accessed July 2013]. Available at: www.biodiv.org.

United Nations Environment Programme - World Conservation Monitoring Centre (UNEP-WCMC) (2013) *Biodiversity and Ecosystems* [online].[Accessed July 2013]. Available at: www.unep-wcmc.org.

-------- (2013) Distribution map of montane forest vegetation [online]. [Accessed July 2013]. Available at: http://www.unep-wcmc.org/tropical-montane-cloud-forest_229.html.

Urutseg (2011) *Wikimedia commons: Topographical map of Peru* [online]. [Accessed July 2013]. Reused and modified by H. Millner, 2013 under the terms and conditions of the Creative Commons CC0 1.0 Universal Public Domain Dedication. Available at: http://commons.wikimedia.org/wiki/File:Peru_physical_map.svg.

Vacin, E and Went, F.W. (1949) Some pH changes in nutrient solutions. *Botanical Gazette*, **110**: 605 – 613.

Valencia, R., Pitman, R.N., León-Yánez, S. and Jφrgensen, P.M. (2000). *Libro Rojo de las Plantas Endémicas del Ecuador 2000.* QCA Herbarium, Pontificia Universidad Católica del Ecuador, Quito.

van der Cingel, N.A. (2001) *An Atlas of Orchid Pollination: America, Africa, Asia and Australia.* A.A. Balkema, Rotterdam, Netherlands.

van der Pijl, L. and Dodson, C.H. (1966) *Orchid flowers: their pollination and evolution,* Coral Gables, University of Miami Press.

van Treuren, R., Bulsma, R., Ouborg, N.J. and van Delden, W. (1993) The significance of genetic erosion in the process of extinction. Inbreeding depression and heterosis effects caused by selfing and outcrossing in *Scabiosa columbaria. Evolution*, **47**(6): 1669-1680.

Vogel, S. (1990) *The role of scent glands in Pollination*. 1st edn. Smithsonian Institution, Washington, D.C. USA.

-------- and Renner, S. (1992) The role of Scent Glands in Pollination: On the structure and function of osmophores. *Taxon,* **41**(2): 400.

Waide, R.B., Willig, M.R., Steiner, C.F., Mittelbach, G., Gough, L., Dodson, S.I., Juday, G.P. and Parmenter, R. (1999) The relationship between Productivity and Species Richness. *Annual Review of Ecology and Systemics,* **30**: 257-300.

Warcup, J.H. (1973) Symbiotic germination of some Australian terrestrial orchids. *New Phytologist,* **72**(2): 387-392.

Warren, R. (2010) Proprietor of 'Equatorial Plants'. Personal communication, London Orchid Show.

Webb, M. (1985) Propagation of *Restrepia* by leaf cuttings. *American Orchid Society Bulletin*, **50**(4): 419.

Wheeler, M.J. and Franklin-Tong, V.E. (2007) Specifying self-recognition: peptides lead the way. *The New Phytologist*, **175**(4): 597-9.

---------, de Graaf, B.H.J., Hadjiosif, N., Perry, R., Poulter, N.S., Osman, K., Vatovec, S., Harper, A., Franklin, F.C.H. and Franklin-Tong, V.E. (2009) Identification of the pollen self-incompatibility determinant in *Papaver rhoeas. Nature*, **459**: 992-995.

Whitmore, T.C. (2001) *An Introduction to Tropical Rain Forests, 2nd edn.* Oxford University Press.

Whitney, H.M., Chittka, L., Bruce, T.J.A., and Glover, B.J. (2009a) Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. *Current Biology*, **19:** 48-53.

---------, Federle, W. and Glover, B.J. (2009b) Grip and slip: mechanical interactions between insects and the epidermis of flowers and flower stalks. *Communication and Integrative Biology*, **2**(6): 505-508.

---------, Kolle, M., Alvarez-fernandez, R., Steiner, U. and Glover, B.J. (2009c) Contributions of iridescence to floral patterning. *Communication and Integrative Biology*, **2**(3): 230-232.

---------, Bennett, K.M.V., Dorling, M., Sandbach, L., Prince, D., Chittka, L. and Glover, B.J. (2011) Why do so many petals have conical epidermal cells? *Annals of Botany*, **108**: 609-16.

Wieczorek, J., Guo, Q. and Hijmans, R. (2004) The point-radius method for georeferencing locality descriptions and calculating associated uncertainty. *International Journal f Geographical Information Science*, **18**(8): 745-767.

Wiemer, A.P., Moré, M., Benitez-Vieyra, S., Cocucci, A.A., Raguso, R.A. and Sérsic, A.N. (2009) A simple floral fragrance and unusual osmophore structure in *Cyclopogon elatus* (Orchidaceae). *Plant Biology*, **11**(4): 506-514.

Williams, H. (1983) Floral fragrances as cues in animal behavior. *In*: C. E. Jones and R. J. Little (eds.), *Handbook of experimental biology*, pp. 50-72. Van Nostrand Reinhold, New York.

Williams, L.O. (1940) *Pleurothallis amesiana.* Botanical Museum Leaflets, **8**: 143.

World Checklist of Selected Plant Families, (WCSP) (2013) *World Checklist of Selected Plant Families, facilitated by the Royal Botanic Gardens, Kew* [online]. [Accessed July 2013]. Available at: http://apps.kew.org/wcsp/ Retrieved 2013-04-22.

Wright, L.I., Tregenza,T. and Hosken, D.J. (2008) Inbreeding, inbreeding depression and extinction. *Conservation Genetics*, **9**(4): 833-843.

Wright, S. (1977). *Evolution and the genetics of populations. Vol 3. Experimental results and evolutionary deductions.* U. of Chicago Press, Chicago.

Yam, T.W. and Arditti, J. (2009) History of orchid propagation: a mirror of the history of biotechnology. *Plant Biotechnology Reports*, **3**(1): 1-56.

Zettler, L.W., Stewart, S.L., Bowles, M.L. and Jacobs, K.A. (2001) Mycorrhizal Fungi and Cold-assisted Symbiotic Germination of the Federally Threatened Eastern Prairie Fringed Orchid, *Platanthera leucophaea* (Nuttall) Lindley. *American Midland Naturalist*, **145**(1): 168-175.

Zhang, Z.H., Ping, L., Wang, L.X., Hu, Z.L., Zhu, L.H. and Zhu, Y.G. (2004) Genetic dissection of the relationships of biomass production and partitioning with yield and yield related traits in rice. *Plant Science*, **167**(1): 1-8.

Appendices:

Appendix 1:

Millner, H.J., Obeng, A., McCrea, A.R. and Baldwin, T.C. (2008)

Axenic seed germination and in vitro seedling development of *Restrepia brachypus* (Orchidaceae).

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Journal of the Torrey Botanical Society 135(4), 2008, pp. 497–505

Axenic seed germination and in vitro seedling development of *Restrepia brachypus* (Orchidaceae)

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Millner H. J., A. Obeng, A. R. McCrea, and T. C. Baldwin. (School of Applied Sciences, University of Wolverhampton, Wulfruna Street, Wolverhampton, WV1 1SB, United Kingdom). Axenic seed germination and in vitro seedling development of *Restrepia brachypus* (Orchidaceae). J. Torrey Bot. Soc. 135: 497–505. 2008.—Montane rain forests in Central and South America are threatened by human activities and climate change. Consequently, epiphytic plant genera such as *Restrepia* are also endangered, making their ex-situ conservation vital. For success, this conservation strategy requires affordable, efficient, and reliable protocols for axenic seed germination as well as protocorm and seedling development prior to establishment ex vitro. In our study, effects of four asymbiotic media (Murashige and Skooge, Phytotech P668, Vacin and Went, and Western) on seed germination and early protocorm development of *Restrepia brachypus* were compared. In addition, their effects with and without banana pulp were examined on in vitro seedling development. Western medium produced the highest mean germination rate (53%), the second highest mean protocorm diameter (325 μm) and, with banana, the largest mean seedling length (3.6 mm). These data provide a simple protocol using commercially available media that is suitable for ex-situ conservation of *Restrepia*. These media may also be of use for the micropropagation and conservation of other related orchid genera.

Key words: asymbiotic, conservation, epiphyte, orchid, *Restrepia*.

Montane rain forest areas contain high concentrations of endemic species facing significant threats, which are classified as ecological 'hotspots' (Myers 1988, 1990, Mittermeier et al. 1999). Such 'hotspots' are defined as containing at least 1500 species of vascular plants (> 0.5 percent of the world's total) as endemics and must have lost at least 70% of their original habitat (Myers 1988).

Orchid 'hotspots' in South America coincide with the centres of plant diversity: for example the Northern Andes contain high numbers of endemic orchid species (Cribb and Govaerts 2005). In the Ecuadorian Andes to which *Restrepia* are endemic, epiphytes constitute 30% of the vascular plant species in biodiversity hotspots (Kuper et al. 2004), which puts orchid genera such as *Restrepia* at risk.

Restrepia is a small epiphytic orchid genus (50 species) (Luer 1996, Govaerts 2005) in the sub-tribe Pleurothallidinae (Dressler 1990, 1993), of horticultural interest (Bechtel et al. 1992, Pridgeon 1992, O'Shaughnessy 2005, NCCPG 2008), which possesses small but attractive flowers (Phafl 2008, Rice 2006, Howe 2008). Many species of this genus are currently threatened with population decline or extinction by destruction and fragmentation of their montane rain forest habitats (Venezuela, Ecuador, Columbia, and Peru).

Unfortunately, micropropagation techniques, developed since 1960 (Morel 1960, 1965), as commonly used for commercial orchid propagation, result in plants with identical genotypes, making application of these techniques inappropriate to maintaining genetic diversity in threatened genera. Therefore, plants produced by these means cannot be used for reintroduction to the wild, or for ex situ conservation. Seed propagated plants, however, can be used for this purpose. To date, axenic seed germination and in vitro protocorm and seedling development of *Restrepia* have not been reported in the literature. Many *Restrepia* are easy to propagate by

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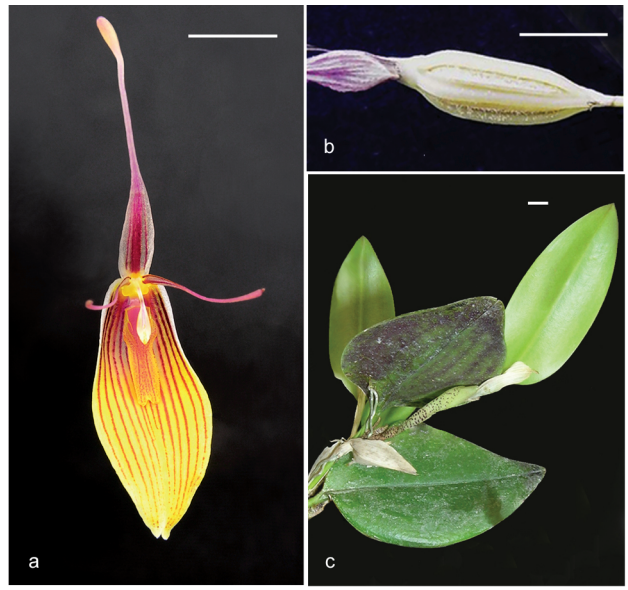

FIG. 1. a) *Restrepia brachypus* flower. b) Seed capsule at dehiscence. c) Leaf with keiki forming in the leaf axil. Internal scale bars = 1 cm.

plantlets that form on the base of the leaves, called 'keikis' (Fig. 1c; Webb 1981), the ease with which most species form these structures may explain why to date little effort has been made to propagate them from seed.

In light of this, the primary objectives of the current study were to establish a protocol for the axenic seed germination of *Restrepia brachypus* using commercially available media, and then to study the subsequent seedling development on these media in vitro. The longer term aims were concerned with ex situ conservation of the genus via the establishment of species in 'living' collections around the world (without increasing collection pressures on already endangered populations) and the eventual reintroduction of species to their native habitat.

As such, here we describe an economical, simple, and reliable protocol for the asymbiotic axenic seed germination and in vitro protocorm and seedling development of *Restrepia brachypus*, (Fig. 1a) a relatively widely distributed species (Luer 1996) that is well known in cultivation using commercially available media.

Methods and Materials. AXENIC SEED GERMINATION AND INITIAL PROTOCORM DEVELOPMENT. When working with rare species of any plant genus it is often impossible, due to the shortage of material, to perform the requisite number of replications required for statistical analysis. Since *Restrepia* forms hundreds of seeds per capsule (Arditti 1992, Arditti and Glaini 2000), statistical evaluation of results from a comparatively small number of seed capsules is feasible.

All seeds and plant material for germination and subsequent protocorm and seedling development were incubated in an illuminated growth chamber at 21°C with 16 h day length. The media used in the current study and their abbreviations are listed in Table 1.

Two of the media were commercially prepared and modified by Duchefa labs: Vacin and Went (Vacin and Went 1949), and Murashige and Skooge (Murashige and Skooge 1962). The Murashige and Skooge (MS) medium was used at one half strength (Seaton and Ramsay 2005, Kyte and Kleyn 1999) and both MS and Vacin and Went (VW) were further modified by the addition of 3% sucrose. The P668 medium (P668) was supplied by PhytoTechnology Laboratories and Western medium (W) was supplied by Western Laboratories, Australia. Media were adjusted to pH 6.0 after the addition of sucrose and agar with 0.1N KOH and were autoclaved for 20 min at 120 °C. Four 30 ml replicates were then prepared of each medium in 90 mm diameter sterile plastic Petri dishes.

Table 1. Media used in the study.

Medium (abbreviations used in text shown in parentheses)		Modifications in study	Original citations
PhytoTechnology P668 (P668)	used commercially by orchid flasking companies in the UK, very similar to 1/2 strength MS and identical to Sigma P-6668	0.5% TC agar, Duchefa	http://www.phytotechlab.com/TechInfo/P668-Info.pdf (accessed 1 May 2008)
Murashige and Skooge (MS)	preliminary experiments showed *Restrepia* seeds would germinate on 1/2 strength macro elements MS (Thompson 1974, Seaton et al. 2005)	½ strength macro nutrients (Duchefa catalogue, Seaton et al. 2005), 0.5% TC agar, 3% sucrose	Murashige and Skooge (1962)
Vacin and Went (VW)	used by RGB Kew in propagation of orchids (M. McMichen, pers. comm.)	0.5% TC agar, Duchefa, 3% sucrose	Vacin, E., and Went, F. W. (1949)
Western (W)	a comparatively new medium, developed to overcome changes in pH of medium during growth and development of seedlings.	none	proprietory brand, supplied by Western Orchid Laboratories, Australia

The seeds used in these experiments were from capsules produced by hand cross-pollination of two different, unnamed, clones of *Restrepia brachypus*. Since the pollinator for *Restrepia* is not known, (although it is assumed they are fly pollinated; Pridgeon 1985), preliminary hand pollinations were carried out. Visual estimates of seed filling using a dissecting microscope showed that these hand pollinations produced a high percentage of 'filled' seeds (i.e., seeds which contained embryos; Fig. 2b), often $> 85\%$. When the seed capsules were approaching dehiscence, they were inspected daily and were collected just as the capsules began to split (Fig. 1b). Seeds were then sown within 24 hours in order to ensure that seed storage methods had no influence upon the germination results. Seeds were surface sterilised in 0.5% v/v sodium hypochlorite solution for 10 minutes and allowed to settle. This solution was then decanted in the laminar air flow cabinet (LAF) and the seeds washed twice in sterile distilled water (SDW). The seeds were then resuspended in 5 ml SDW and spread evenly across the prepared media plates. Germination was recorded with the use of a dissecting microscope.

Since *Restrepia* seeds do not display synchronous germination, germination rates were recorded every week for five weeks. A count of > 150 filled (containing embryos, stage 1, Table 2, Fig. 2) seeds per plate was sampled each time. The number of swollen embryos (stage 2, Table 2, Fig. 2) was recorded, and full germination (stage 3, Table 2, Fig. 2) was assumed when the developing protocorms had split their testa. Final full germination rates were calculated for all replicates of each medium each week.

The diameters of the protocorms after five weeks were recorded using the Spot RT Colour camera (with integrated software Version 4.02) manufactured by Diagnostic Instruments Inc., Michigan, USA, mounted on a Nikon 'Eclipse' ME600 (Nikon Corp., Tokyo, Japan). The image analysis software used was Image Pro Plus (Version 5.0.1) manufactured by Media Cybernetics Inc. Maryland, USA. All statistical analysis was performed on SPSS, Version 12 (SPSS Inc., Chicago, USA). The Kolmogorov-Smirnov test and Levene's test were used to test for normality and homogeneity of variances respectively. A Kruskal-Wallis test was used to compare the germination percentage means, as the sample sizes were too small to ensure normality. Initial protocorm diameters were log transformed for normality and analysed by one-way ANOVA; Tamhane's T2 was used post hoc where differences were highly significant but variances were not homogeneous.

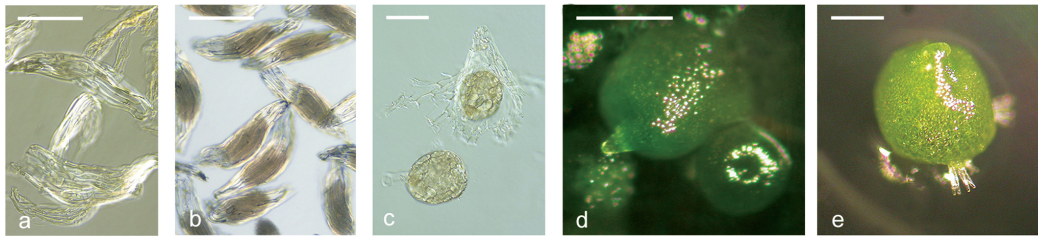

FIG. 2. Stages of germination, adapted from Stewart et al. 2006. a) Stage 0, empty embryos. b) Stage 1 with viable embryos. c) Stage 2, embryos have begun to swell, and stage 3 one embryo has burst its testa. d) Stage 4, leaf primordial just visible. e) Stage 5, rhizoids have emerged. Internal scale bars a, b, and c = 50 μm; d and e = 5 mm.

SEEDLING DEVELOPMENT. Once the optimal medium for axenic seed germination had been established (see Results section) the following protocol was used to determine the best medium on which to replate protocorms for subsequent seedling development. Seeds from the same interclonal cross of *Restrepia brachypus* were therefore pre-germinated on Western medium for this part of the study.

The media and culture vessels used for replating were the same as used for the axenic seed germination except that each medium was also tested with the addition of banana pulp at the rate of 60 g L^{-1} of medium. This addition was recommended by K. Western for the Western medium (pers. comm.) and its addition to all the other media was also tested. The pulp was derived from fresh bananas which were liquefied in a food blender and then added to the media prior to autoclaving. Banana supplementation (although considered to be an undefined additive) of media is commonly used in orchid replate/subculture media and has been consistently shown to promote rooting and development (Arditti 1967, 1982).

After six weeks when protocorms were large enough to be replated (> 1 mm diameter, Fig. 3 week 1), replicates were subcultured onto this range of media. Microscopic examination of the individual protocorms confirmed that they were all at a similar stage of development, Stage 5, (Table 2): i.e., they had initiated rhizoids (Arditti 1992, Withner 1974) and leaf primordia and were undergoing photosynthesis. Twenty five protocorms per standard 90 mm Petri dish containing 30 ml of medium were arranged in a numbered grid formation. This enabled random selection of individual protocorms later in the study. Duplicate grids of 25 protocorms were prepared for each medium, plus and minus banana.

Table 2. Seed germination and protocorm development in *Restrepia brachypus*, adapted from Stewart and Kane (2006).

Stage	Description
0	Empty seed, no embryo
1	Viable embryo, no germination
2	Swollen embryo, testa not split
3	Further swelling, testa split, embryo (protocorm) is photosynthetic (= germination)
4	Leaf primordia visible
5	Rhizoids visible

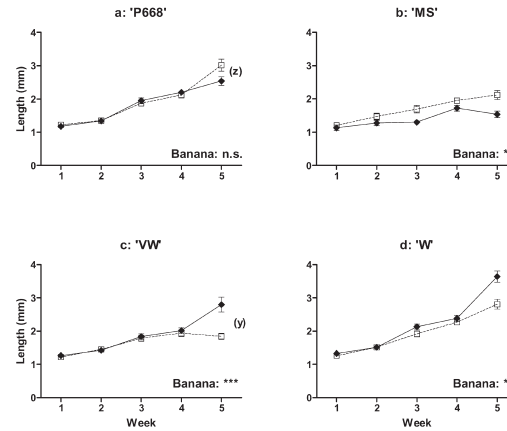

FIG. 3. Growth rates of *Restrepia brachypus* protocorms in four different media: 'P668', 'MS', 'VW', and 'W' for the five weeks following germination. Banana additions are depicted as ◆ with banana, and □ without banana; error bars represent ± 1 SE. Two-way ANOVA on log-transformed data indicated a highly significant difference between media after five weeks ($P < 0.001$); media means with the same label (x, y, or z) are not significantly different from each other (Tamhane's T2, $P < 0.05$). Results of media-specific *t*-tests on the banana additions are displayed as: 'n.s.', non-significant; **, $P < 0.01$; ***, $P < 0.001$.

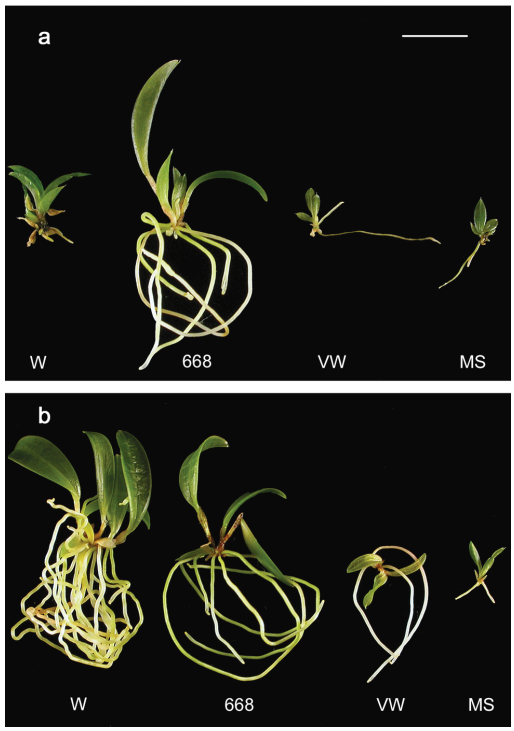

FIG. 4. Comparison of seedling growth on all media after six months. a) media without banana supplement, and b) media with banana supplement. Internal scale bar = 1 cm.

The length of each protocorm was measured weekly using the Spot RT Colour camera. The image analysis software was Image Pro Plus (Version 5.0.1). These measurements were taken for four weeks (Fig. 3, weeks 1–4). By this time the vertical growth of the seedlings made valid comparisons of the measurements impossible.

After five weeks ten seedlings were randomly selected from each plate, removed, laid horizontally and measured accurately, (Fig. 3, week 5). Mean protocorm lengths on the different media were calculated and the effects of media and banana were compared using two-way ANOVA. Tamhane's T2 was used post hoc where differences were highly significant but variances were heterogeneous and *t*-tests were used to distinguish the different effects of banana additions for each medium, where these were obscured in two-way ANOVA by an interaction between factors. The remaining seedlings were replated onto 10 ml of their respective media in 30 ml sterile, plastic, universal tubes and allowed to develop further in the growth chamber for another six months (Fig. 4). Establishment of seedlings ex vitro was not studied as it is well documented elsewhere (Seaton and Ramsey 2005, Thompson 1980).

Results. AXENIC SEED GERMINATION AND INITIAL PROTOCORM DEVELOPMENT. A summary of axenic seed germination rates on all four media is presented in Table 3. *Restrepia* seeds do not show synchronous germination (H. Millner, pers. comm.) and P668 and W were found to support maximum germination rates (26.7% and 53.1%) after four weeks and VW and MS after five weeks (16.6% and 8.0%), respectively.

Comparison by Kruskal-Wallis (Table 3a) of germination percentages showed a highly significant difference between means: χ^2 (3) = 12.794, $P = 0.005$. The media 'W' (53.05%) and 'P668' (26.66%) support the largest means, with much smaller means from 'VW' (16.61%) and 'MS' (7.6%).

One-way ANOVA (Table 3b) of initial protocorm diameter indicated a highly significant difference between means: $F_{3,314} = 58.345$; $P < 0.001$. The medium 'P668' produced significantly larger protocorms at the end of the germination period than any other (410.01 μm; Tamhane's T2, $P < 0.05$). The medium 'W' produced the next largest (325.10 μm), which were significantly larger than those produced by either 'MS' (235.20 μm) or 'VW' (225.32 μm). The means of 'MS' and 'VW' were not significantly different from each other.

Since 'W' medium gave the highest mean germination rates and, on microscopic examination, protocorms at the most consistent stage of development (stage 5, Table 2), it was chosen as the medium of choice to pregerminate seeds for the seedling development study.

SEEDLING DEVELOPMENT. Growth rates for the different media, with and without the banana addition, are presented in Figs. 3a–d. Two-way ANOVA showed a highly significant main effect due to the growth media: $F_{3,151} = 35.085$, $P < 0.001$. The overall effect of adding banana to the media was not significant: $F_{1,151} = 1.559$, $P = 0.214$; however a highly significant interaction ($P < 0.001$) between 'banana' and 'media' indicated the likely confounding effect of media on banana. The mean protocorm lengths at the end of the

Table 3. Results of media comparisons for germination and diameter of *Restrepia* protocorms after four weeks' germination on four different media: 'P668', 'MS', 'VW', and 'W'.

Media	Mean	SE	df	χ^2/F	P
Germination rate (%)[a]					
P668	26.66	3.35	3	12.794	0.005**
MS	7.96	2.17			
VW	16.61	4.25			
W	53.05	6.38			
Protocorm diameter (μm)[b]					
P668	410.01 c	34.15	3, 314	58.345	< 0.001***
MS	235.20 a	10.52			
VW	225.32 a	9.12			
W	325.10 b	26.47			

[a] Comparison of percentage germination (Kruskal-Wallis, $P < 0.01$) shows a very significant difference between media (** $P < 0.01$).

[b] Comparison of protocorm diameter by one-way ANOVA on log-transformed data also indicates very significant differences between media (*** $P < 0.001$). Media means with the same label (a, b, or c) are not significantly different from each other (Tamhane's T2; $P < 0.05$).

treatment were smallest for 'MS' (1.82 mm) and greatest for 'W' (3.23 mm); 'VW' was intermediate and significantly different from the rest (2.32 mm) but there was no significant difference between 'P668' (2.78 mm) and 'W'. (Tamhane's T2, $P < 0.05$). The separate effects of banana additions on the different media investigated by *t*-test showed a very significant difference for 'MS': $t\ (37) = 3.687$, $P = 0.001$; 'VW': $t\ (29.07) = -4.04$, $P < 0.001$ and 'W': $t\ (38) = -3.72$, $P = 0.001$. However, there was no significant difference for 'P668': $t\ (38) = 1.99$, $P = 0.054$.

Fig. 4 shows a comparison of seedlings grown on all four media after six months in culture; on media with and without banana supplementation. The growth of the seedlings especially in terms of root development, have benefited markedly from the addition of banana to the replating media. When establishing seedlings ex vitro, well developed roots are an advantage in rapid acclimatization and in uptake of nutrients from the chosen substrate: (i.e., orchid bark, sphagnum moss, or any proprietary orchid seedling mix). The larger, more vigorous seedlings have a much increased chance of survival once they are transplanted. *Restrepia* seedlings have been successfully 'deflasked' into pure sphagnum moss (data not shown) and seedlings resulting from the current study are being grown by the *Restrepia* National Collection Holder in the United Kingdom, and by members of the Pleurothallid Alliance U.K.

Discussion. The conservation threats to orchid species are many and varied, with the main impacts being habitat destruction, modification, and fragmentation, unsustainable wild collection, and the effects of global climate change (Bubb et al. 2004). Species within the genus *Restrepia* are indigenous to the montane rain forests of Central and South America and are distributed alongside other epiphytes throughout the canopy on the basis of available moisture—either as rainfall or atmospheric humidity, which affects their diversity, abundance, and distribution. Such species are known to be early indicators of climate change, being among the first to be affected by phenomena associated with global warming, such as changes in temperature and precipitation (Benzing 1990, 1998). These forests have also come under threat from changes in land use, such as the felling of trees for timber, farming, or mining, all of which lead to deforestation. For example, in Eastern Columbia, the most altered and fragmented ecosystems have been found to correspond to montane and sub-montane rain forests (Armenteras et al. 2003).

The result in a country such as Costa Rica, where rain forest was widespread until fifty years ago, is that the forest cover has now been reduced to isolated regions unevenly distributed throughout the country. Similar habitat fragmentation is happening throughout South America. How this is affecting extinction rates in *Restrepia* is currently unknown. Data have, however, been published on other genera in the sub-tribe Pleurothallidinae such as *Masdevallia* and *Dracula*. In common with *Restrepia*, the majority of *Masdevallia* and *Dracula* species have been found in single localities

(Koopowitz et al. 1993). Using published deforestation rates and species distribution profiles, Koopowitz et al. (1994) calculated that 402 of the total 3,405 species within the Pleurothallidinae may already have been driven to extinction by random deforestation events. He later demonstrated these estimates to be too high (Koopowitz et al. 2003), since they relied upon the premises of total deforestation, even distribution of orchid plants, and random felling. Nevertheless, this work represents the first data-based estimate of the threat of extinction to genera within the Pleurothallidinae, including *Restrepia*.

Since the elegant, classic experiments performed by Knudson (1922, 1924, 1925), asymbiotic axenic seed culture has long been known to be a powerful method with which to perform genetic conservation of orchids and epiphytic species in particular (Wang et al. 2007). For example in a recent study, Buyun et al. (2004) concluded that *Cattleya* species could be effectively propagated by in vitro seed culture with the aim of ex-situ biodiversity conservation. As a result, a wide range of tissue culture media with the aim of optimizing asymbiotic seed germination and seedling growth rates has been developed.

Since very little has been published on the growth and development of *Restrepia* in its natural habitat, and nothing with regards these processes in vitro, the current study is of importance, as it represents the first media trial for axenic seed germination and in vitro seedling development for any species in this genus. The results obtained demonstrate that 'W' medium produced the highest axenic seed germination rates (53%; Table 3), the second largest (325 μm) and third most consistent (SE = 26.5) protocorm growth (Table 3), the best early seedling growth (3.6 mm; Fig. 3d), and subsequently the best seedling development after six months in culture (Fig. 4).

These data demonstrate that *Restrepia brachypus* seeds can be effectively and efficiently propagated in vitro using 'W' medium for germination and with banana pulp supplement for ongoing growth and development; and that 'P668' medium would be an adequate alternative used without banana supplement throughout (Table 3; Figs. 3c and 4). Alternative concentrations of these media or other media may also prove to produce acceptable results, but the current data provide a valuable starting point for axenic seed protocols within this genus and its subtribe.

To put the current study in to the broader context of ex situ conservation of orchid species, there are several aspects to be considered. The primary objective of such programmes is to cultivate plants outside their natural habitat in order to preserve them for posterity. As mentioned previously in relation to the data presented, seedlings of *Restrepia brachypus* produced using the methodology described have successfully been grown in flask and sent to the National Plant Collection of *Restrepia*, NCCPG, U.K. and distributed to members of the Pleurothallid Alliance U.K. In addition, *Restrepia* seedlings micropropagated using the technique described will form part of the living orchid collection at the Royal Botanic Gardens, Kew (H. Millner, pers. comm.).

One other important aspect of ex situ conservation is that of raising awareness of the orchid genus in question and the threats posed to these species in their natural habitat. As such, the current study has enabled the authors to present educational displays describing the genus and its endangered status in the wild to the Royal Horticultural Society, U.K. and at the Royal Chelsea Flower show, U.K., thus raising both the public and scientific profile of this beautiful and little known genus. Thirdly, ex situ conservation also aims to relieve pressure on wild populations by producing material for scientific research and for horticultural purposes. This study has provided the methodology to supply *Restrepia* material for both these purposes.

Lastly, this approach to conservation has led to ongoing initiatives to develop world wide seed banks of orchid and other plant species: for example the Millennium Seed Bank Project, Kew, which is the 'largest ex-situ conservation project ever conceived'. This project aims to have banked seed from 10% of the world's wild plant species by the end of the decade (RBG Kew 2008).

In relation to all these aspects, the Convention on Biological Diversity (CBD), (UNEP 2001) recognised that ex situ conservation complements in situ conservation by providing material for a recovery programme and in developing research and education programmes. CBD supports the sustainable use of the habitat as a last line of defence against extinction in the wild in contrast to the

previous conservation ideal of a return to a pristine 'original' state. The primary objective of the CBD is that 60% of threatened plant species should be held in accessible ex situ collections, preferably in the country of origin, with 10% of these included in recovery and restoration programmes by 2010 (Target 8, Global Strategy for Plant Conservation 2006).

The Darwin Initiative (DEFRA 2008) assists countries that are rich in biodiversity but poor in financial resources to implement the Convention on Biological Diversity (CBD) through the funding of collaborative projects which draw on U.K. biodiversity expertise. One current project is the creation of orchid seed banks in their countries of origin for 'sustainable use' (Pritchard 2007). Furthermore, in order to reintroduce plants derived from such seed banks to reclaimed habitats, research such as that described here is required and is complementary to the seed bank approach.

In conclusion, although it is unlikely that the genus *Restrepia* as a whole is threatened with extinction in the near future, those species which are narrow endemics in threatened locations are almost certainly at risk. Bearing this in mind, ex-situ conservation via axenic seed germination and subsequent seedling growth in vitro, as reported in our study, is of great importance in the conservation of the gene pool and for future studies of this exquisite genus. Furthermore, the media and methodology described in the current study may prove to be useful for the micropropagation and ex-situ conservation of other orchid genera.

Literature cited

ARDITTI, J. 1967. Factors affecting the germination of orchid seeds. Bot. Rev. 33: 1–97.

ARDITTI, J., M. A. CLEMENTS, G. FAST, G. HADLEY, G. NISHIMURA, AND R. ERNST. 1982. Orchid Seed Germination and seedling culture–a manual, p. 243–370. *In* J. Arditti [ed.], Orchid biology: reviews and perspectives, Vol. II. Cornell University Press, Ithaca, NY.

ARDITTI, J. 1992. Fundamentals of Orchid Biology. John Wiley and Sons, New York, NY. 691 p.

ARDITTI, J. AND G. AKA. 2000. Numerical and physiological properties of orchid seeds and their biological implications. New Phytol. 145: 367–421.

ARMENTERAS, D., F. GAST, AND H. VILLAREAL. 2003. Andean forest fragmentation and the representativeness of protected natural areas in the eastern Andes, Colombia. Biol. Conserv. 113: 245–256.

BECHTEL, H., P. CRIBB, AND E. LAUNERT. 1992. The Manual of Cultivated Orchid Species. Blandford Press, London, UK. 444 p.

BENZING, D. 1990. Vascular Epiphytes: General Biology and Related Biota. Cambridge University Press, Cambridge, UK. 354 p.

BENZING, D. 1998. Vulnerabilities of tropical forests to climate change: the significance of resident epiphytes. Climatic Change. 39: 519–540.

BUBB, P., I. MAY, L. MILES, AND J. SAYER. 2004. Cloud Forest Agenda. UNEP-WCMC, Cambridge, UK. 36 p.

BUYUN, L., A. LAVRENTYEVA, L. KOVALSKA, AND R. IVANNIKOV. 2004. In vitro germination of seeds of some rare tropical orchids. Acta U. Latviensis Biol. 676: 159–162.

CRIBB, P. AND R. GOVAERTS. 2005. Just how many orchids are there?, p. 161–172. *In*: A. Raynal-Roques, A. Roguenant, and D. Prat [eds.], Proceedings of the 18th World Orchid Conference, March, 2005. Naturalia Publications, Dijon, France.

DEFRA. 2008. The Darwin Initiative. Retrieved July 1, 2008 from Department for Environment Food and Rural Affairs (DEFRA). <http://darwin.defra.gov.uk/>

DRESSLER, R. L. 1990. The Orchid's Natural History and Classification 2nd ed. Harvard University Press, Cambridge, MA. 332 p.

DRESSLER, R. L. 1993. Phylogeny and classification of the Orchid family. Cambridge University Press, Cambridge, UK. 314 p.

GOVAERTS, R. L. S. 2005. World checklist of monocots–Orchidaceae. Retrieved May 1, 2008 from the online publication by The Board of Trustees of the Royal Botanic Gardens, Kew. <http://www.kew.org/monocotChecklist/orchids>

HOWE, C. 2008. National Collection of *Restrepia*. Retrieved May 1, 2008 from the National Council for the Conservation of Plants and Gardens. <http://www.restrepia.co.uk/>

KNUDSON, L. 1922. Nonsymbiotic germination of orchid seeds. Bot. Gaz. 73: 1–25.

KNUDSON, L. 1924. Further observation on nonsymbiotic germination of orchid seeds. Bot. Gaz. 77: 212–219.

KNUDSON, L. 1925. Physiological study of the symbiotic germination of orchid seeds. Bot. Gaz. 79: 345–359.

KOOPOWITZ, H., A. THORNHILL, AND M. ANDERSON. 1993. Species distribution profiles of the neotropical orchids *Masdevallia* and *Dracula* (Pleurothallidinae, Orchidaceae); implications for conservation. Biodivers. Conserv. 2: 681–690.

KOOPOWITZ, H., A. THORNHILL, AND M. ANDERSON. 1994. A general stochastic model for the prediction of biodiversity losses based on habitat conversion. Conserv. Biol. 8: 1–25.

KOOPOWITZ, H., P. S. LAVARACK, AND K. W. DIXON. 2003. The nature of threats to orchid conservation, p. 25–42. *In*: K. W. Dixon, S. P. Kell, R. L. Barrett, and P. J. Cribb [eds.], Orchid Conservation. Natural History Publications, Kota Kinabalu, Sabah. 418 p.

KUPER, W., H. KREFT, J. NIEDER, N. KOSTER, AND W. BARTHLOTT. 2004. Large-scale diversity pat-

Appendix 2:

Millner, H.J., McCrea, A.R. and Baldwin, T.C. (2012)

The use of *ex situ* orchid collections in conservation research with reference to *Restrepia* (Ochidaceae).

In: Proceedings of the 20th World Orchid Conference, 2011, Singapore (in press).

terns of vascular epiphytes in Neotropical montane rain forests. J. Biogeogr. 31: 1477–1487.

KYTE, L. AND J. KLEYN. 1999. Plants from Test Tubes. 3rd ed. Timber Press, Portland, OR. 240 p.

LUER, C. A. AND R. E. RESTREPO. 1996. Icones Pleurothallidinarum XIII, Systematics of the genus *Restrepia* (Orchidaceae). Monogr. Syst. Bot. Missouri Bot. Gard. 59: 1–168.

MITTERMEIER, R. A., C. G. MITTERMEIER, AND N. MYERS. 1999. Hotspots: Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions. CEMEX, Mexico City, Mexico. 432 p.

MOREL, G. 1960. Producing virus-free Cymbidiums. A.O.S. Bulletin. 29: 495–497.

MOREL, G. 1965. Clonal propagation of orchids by meristem culture. Cymbidium Soc. News. 20: 87–93.

MURASHIGE, T. AND F. SKOOGE. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum. 15: 473–497.

MYERS, N. 1988. Threatened biotas: ''hotspots'' in tropical forests. Environmentalist 8: 187–208.

MYERS, N. 1990. The biodiversity challenge: expanded hot-spots analysis. Environmentalist 10: 243–256.

NCCPG. 2008. National Council for the Conservation of Plants and Gardens. Retrieved May 1, 2008. <http://www.nccpg.com>

NILSSON, L. A. 1992. Orchid pollination biology. Trends. Ecol. Evol. 7: 255–259.

O'SHAUGHNESSY, L. 2005. Orchids of the Pleurothallid Alliance. Retrieved May 1, 2008 from the Pleurothallid Alliance website. <http://www.pleurothallids.com/restrepia.htm>

PHAFL, J. 2008. Orchid Species Encyclopedia. Retrieved May 1, 2008. <http://www.orchidspecies.com>

PRIDGEON, A. M. 1992. The Illustrated Encyclopedia of Orchids. Timber Press, Portland, OR. 304 p.

PRITCHARD, H. 2007. Orchid seed stores for sustainable use. Retrieved July 1, 2008 from the Darwin Initiative, Project 16012. <http://darwin.defra.gov.uk/project/16012/>

RICE, R. 2006. Oasis the Journal, The *Restrepia* Gallery. Retrieved May 1, 2008 from Oasis the Journal. <http://www.oasisjournal.com/restrepia/index.html>

RBG Kew. 2008. The Millennium Seed Bank Project Kew. Retrieved July 12, 2008 from website of the Royal Botanic Gardens, Kew. <http://www.kew.org/msbp/index.htm>

SEATON, P. AND M. RAMSAY. 2005. Growing Orchids from Seeds. Royal Botanic Gardens, Kew, U.K. 83 p.

STEWART, S. L. AND M. E. KANE. 2006. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae). Plant Cell Tiss. Org. 86: 147–158.

THOMPSON, P. A. 1980. Orchids from Seed. Royal Botanic Gardens, Kew, U.K. 30 p.

VACIN, E. F. AND F. W. WENT. 1949. Some pH changes in nutrient solutions. Bot. Gaz. 110: 605–613.

WANG, M. AND Y. HA. 2007. An electrochemical approach to monitoring pH change in agar media during plant tissue culture. Biosens. Bioelectron. 22: 2718–2723.

WEBB, M. 1981. Propagation of *Restrepias* by leaf cuttings. A.O.S. Bulletin. 50: 419.

WITHNER, C. L. 1974. The Orchids: Scientific Studies. John Wiley and Sons, New York, NY. 604 p.

The Use of *Ex-Situ* Orchid Collections in Conservation Research with Reference to *Restrepia* (Orchidaceae).

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ABSTRACT

Private collections of Restrepia were used to study axenic seed germination, establish horticultural protocols for ex- and in-situ conservation and to investigate breeding barriers within the genus. A programme of plant breeding experiments was performed in concert with axenic seed culture in order to propagate this genus in-vitro. A variety of interclonal crosses; and interspecific crosses producing primary hybrids have been carried out.

Self-pollinations were found to produce few viable seeds. Interclonal and interspecific cross-pollinations produced >90% seeds with embryos which germinated well.

Restrepia grow in endangered montane forest habitats. With habitat fragmentation and dwindling populations successful cross-pollination is reduced. This in turn reduces viable seed production and reduces chances of maintaining a self-sustaining population. The value of ex-situ conservation and in-vitro propagation in the conservation of this genus are discussed.

THE GENUS *RESTREPIA*

The sub-tribe Pleurothallidinae is one of the most diverse groups in the family Orchidaceae. It comprises >4000 species in 31 genera (Pridgeon and Chase, 2001; Govaerts, 2003). Since the 1980's Pleurothallids have been the subject of various morphological and anatomical studies (Luer, 1986 – 2007; Pridgeon and Williams, 1979; Pridgeon, 1981a, b, c; Pridgeon and Stern, 1982, 1983, 1985; Stern *et al.,*1985; Neyland and Urbatsch, 1993; Stenzel, 2004). The overall result has been further taxonomic splitting increasing the number of genera to 129 (Luer, 2003, 2004, 2006, 2007; Tropicos, 2010). This exclusively Neotropical subtribe constitutes more than 15% of the world's orchid flora, Stenzel (2004), and has its ecological centre of diversity lying in the montane and cloud forest of the Central American and South American Andes. Pleurothallids grow primarily in epiphytic habitats, although some taxa are found in rupiculous and pseudoterrestrial niches as well.

One small genus within this group, that to date has escaped taxonomic revision is *Restrepia* which comprises ~50 species. The first species to be discovered was *R. contorta*, described as *Humboltia contorta* (Ruiz and Pavon, 1798). Subsequently, Humboldt and Bonpland discovered another species near Popáyan, Colombia, in 1801 and named the genus, *Restrepia* in honour of José Manuel Restrepo, an early

Colombian botanist (Bechtel *et al*., 1992; Pridgeon, 1992; Manning, 2010). The plant they discovered was *Restrepia antennifera* and their description published in 1816 by Humboldt, Bonpland and Kunth, remains the type species for the genus (Humboldt *et al.*, 1816). Since these first discoveries, many more species of *Restrepia* have been identified. The majority of which have been discovered since 1980.

Figure 1: (a) *R. contorta*; (b) *R.antennifera*

The genus is split into two monotypical sections and two main sections, Restrepia and Pleurothallopsis. The two sections are distinguished by the peduncle. The section Restrepia has an elongated peduncle which holds the flower erect above the leaf (e.g *R. contorta, R. antennifera* and *R. brachypus)* and the section Pleurothallopsis has a short peduncle and the flower is often held against the back of the leaf (e.g. *R. muscifera*). The flowers have a unique morphology in which the dorsal sepal and two lateral petals are modified into long apices which terminate in osmosphores, (Pridgeon and Stern, 1985; Luer, 1996). The two lateral sepals are joined forming the synsepal, which is the largest and most colourful part of the flower.

RESTREPIA RESEARCH

At the University of Wolverhampton in the U.K. research has involved the species *R. brachypus.* This is a species that is common in cultivation in the U.K., with several different clones. Cultivated collections of this species were used as model populations to investigate firstly axenic seed germination, Millner *et al.* (2008); and secondly to establish horticultural protocols for the resulting seedlings which would aid both *ex*- and *in-situ* conservation. Currently research is continuing into studying breeding barriers within the genus.

Table 1: Media used in the study

Media and abbreviations		Modifications in study	Original citations
PhytoTechnology P 668 (668)	Used commercially by orchid flasking companies in the U.K., very similar to 1/2 strength MS and identical to Sigma P-6668	0.5% TC agar	http://www.phytotechlab.com/TechInfo/P668-Info.pdf (accessed 1 May 2008)
Murashige and Skoog (MS)	Preliminary expts showed Restrepia seeds would germinate on 1/2 strength macro elements MS (Thompson, 1974, Seaton *et al,* 2005)	½ strength macro nutrients (Duchefa catalogue, Seaton *et al,* 2005) 0.5% TC agar, 3% sucrose	Murashige and Skoog (1962)
Vacin and Went (VW)	Used by RGB Kew in propagation of orchids (M. McMichen – pers. comm.)	0.5% TC agar, 3% sucrose	Vacin, E., and Went, F. W. (1949)
Western (W)	A comparatively new medium, developed to overcome changes in pH of medium during growth and development of seedlings.	None	Proprietary brand, supplied by Western Orchid Laboratories, Australia

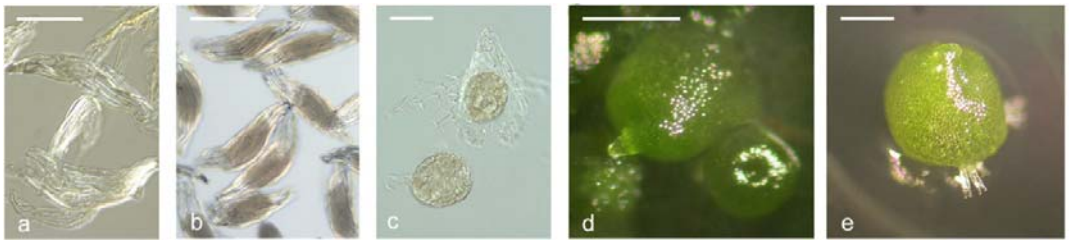

Figure 2: Stages of germination

(a) *Stage 1*, empty embryos; (b) *Stage 2* with viable embryos; (c) *Stage 3* embryos have begun to swell, and *Stage 4* one embryo has burst its testa; (d) *Stage 5* leaf primordial just visible; (e) *Stage 6* rhizoids have emerged. Internal scale bars (a), (b) and (c) represent 50µm and (d) and (e) represent 5mm

Figure 3: Six months culture *in-vitro*

These data demonstrate that *Restrepia brachypus* seeds can be effectively and efficiently propagated *in-vitro* using Western medium for germination and with banana pulp supplement for ongoing growth and development; and that 'P668' medium would be an adequate alternative used without banana supplement throughout. Alternative concentrations of these media or other media may also prove to produce acceptable results, but the current data provide a valuable starting point for axenic seed protocols within this genus and its subtribe.

Figure 4: Seedling to flowering plant

To date studies of breeding barriers have shown that cross-pollinations either between species or between clones produce viable seeds with embryos and germination rates of typically >60%. This was found to apply equally to intra- and intersectional cross-pollinations and there was little variation found with reciprocal crosses. Healthy, vigorous seedlings were produced from these pollinations. In contrast self-pollinations, in which the pollinia are placed on the stigmatic surface of the same flower, produce seeds with few viable embryos of typically <1%. There is some interspecifc and interclonal variation in this figure with individual species and individual clones producing higher percentages of viable embryos. It is worth noting that no seedlings were successfully raised to maturity from self-pollinations during the course of this research; in contrast many seedlings resulting from cross-pollinations were. There is currently a variety of new *Restrepia* hybrids registered on the RHS International Orchid Register, (RHS, 2012; Howe, 2012) that have been produced through this research

project. Research is continuing in order to establish the scientific basis for these findings.

CONSERVATION CONSIDERATIONS

The implications of these findings for both *ex-* and *in-situ* conservation of the genus and potentially other genera are considerable given the nature of threats to orchid habitats worldwide.

Orchid 'hotspots' in South America (Myers, 1988; 1990) coincide with centres of plant diversity: for example the Northern Andes contain high numbers of endemic orchid species, Cribb and Govaerts (2005). In the Ecuadorian Andes to which *Restrepia* are endemic, epiphytes constitute 30% of vascular plant species in biodiversity hotspots, Kuper *et al*. (2004), which puts orchid genera such as *Restrepia* at risk.

Many species of this genus are currently threatened with population decline or extinction by destruction and fragmentation of their montane rain forest habitats (in Venezuela, Ecuador, Columbia, and Peru). Unfortunately micropropagation techniques, developed since 1960 (Morel 1960, 1965) as commonly used for commercial orchid propagation, result in plants with identical genotypes, making application of these techniques inappropriate to maintaining genetic diversity in threatened genera. Therefore plants produced by these means cannot be used in *ex-situ* conservation for reintroduction to the wild; whereas seed propagated plants may be used for this purpose. To date this study of axenic seed germination, *in-vitro* protocorm and seedling development of *Restrepia* represents the first reported in the literature. It should be noted that many *Restrepia* are easy to propagate from plantlets that form on the base of the leaves, called 'keikis', Webb (1981) and the ease with which most species form these structures may explain why little effort has been made to propagate them from seed.

Whether the offspring from cultivated collections of species can be used in re-introduction initiatives depends on their provenance and the degree to which they have undergone artificial selection in cultivation. Many cultivated species could not be reintroduced to the wild for these reasons. Alongside this there is an increased awareness of the importance of the role of *ex-situ* orchid collections – they help to relieve collection pressure on wild populations, they provide useful model population for scientific study, they help to establish horticultural protocols for tissue culture of seeds from wild plants (the resulting seedlings from which *may* be used in reintroduction programmes) and they provide data to support seed and germplasm collection

initiatives such as the Darwin Initiative and the Millennium Seed Bank (Pritchard, 2008; RGB Kew, 2008).

The significance of the pollination biology and breeding systems operating within orchid species has become more evident. Although not all orchid species fail to set viable seed by self-pollination in the literature there are growing reports of reduced fruit set linked to self-incompatibility. For instance within the Pleurothallidinae this has been reported in five *Pleurothallis* species, Borba *et al.* (2002), three *Octomeria* species, Barbosa *et al.* (2009), four *Anathallis* species, Gontijo *et al.* (2010), *Stelis argentata*, Christenen (1992) and *Lepanthes* species (Blanco and Barbosa, 2005; Tremblay *et al.*, 2007). These reports have not been limited to Neotropical orchids and include *Coelogyne,* Cheng *et al.* (2009), *Oncidium*, Charanasri *et al.* (1977), *Dendrobium*, Johansen (1990), 30% of species in the former subfamily 'Vandoideae', Agnew (1986) and the Laeliinae, Stort and de Lima Galdino (1984). The effect on conservation is not fully known, Roberts (2003) and research at the University of Wolverhampton is continuing to identify the extent and nature of these effects within the genus *Restrepia.*

It is possible to postulate is that for any orchid populations already under threat from increased habitat loss and fragmentation, the incidence of self-pollination within these populations will increase. If such species also have a reduced ability to set viable seed through self-pollination then this will impose an extra pressure on the self-sustainability of the population. As self-incompatibility effects are identified in more and more species and genera it raises the possibility that orchid populations are in even graver danger than from habitat loss alone.

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REFERENCES

Agnew, J.D. (1986). Self-compatibility/incompatibility in some orchids of the subfamily Vandoideae. *Plant Breeding* 97:(2)183-186.

Barbosa, A.R., Cabral do Melo, M. and Borba, E.L. (2009). Self-incompatibility and myophily in Octomeria (Orchidaceae, Pleurothallidinae) species. *Plant Syst Evol* 283:(1-2)1-8.

Bechtel, H., Cribb, P. and Launert, E. (1992). *The Manual of Cultivated Orchid Species,* Blandford, London

Blanco, M.A. and Barboza, G. (2005). Pseudocopulatory pollination in Lepanthes (Orchidaceae: Pleurothallidinae) by fungus gnats. *Annals of Botany* 95: 763-772.

Borba, E.L., Shepherd, G.J., van den Berg, C. and Semir, J. (2002). Floral and vegetative morphometrics of five *Pleurothallis* (Orchidaceae) species: Correlation with taxonomy, phylogeny, genetic variability and pollination systems. *Annals of Botany*. 90: 219-230.

Charanasri, U. and Kamemoto, H. (1977). Self incompatibility in the Oncidium alliance. *Hawaii Orchid J*. VI:12–15.

Cheng, J., Shi, J. and Shangguan, F.Z. (2009). The pollination of a self-incompatible, food-mimic orchid, *Coelogyne fimbriata* (Orchidaceae), by female *Vespula* wasps. *Annals of Botany* 104:(3)565-571.

Christensen, D.E. (1992). Notes on the reproductive biology of *Stelis argentata* Lindl. (Orchidaceae, Pleurothallidinae) in eastern Ecuador. *Lindleyana* 7:28-33.

Cribb, P. and Govaerts, R. (2005). Just how many Orchids are there? In: Raynal-Roques A, Roguenant A, Prat D, eds *Proceedings of the 18th World Orchid Conference, March, 2005, Dijon -France* Naturalia Publications, Pp161-172

DEFRA. (2008). The Darwin Initiative. http://darwin.defra.gov.uk (accessed 01 Jan 2012)

Gontijo, S.L., Barbosa, A.R., Cabral de Melo, M. and Borba, E.L. (2010). Occurrence of different sites of self-incompatibility reaction in four*Anathallis* (Orchidaceae, Pleurothallidinae) species. *Plant Species Biol* 25:(2) 129-135.

Govaerts, R.L.S. (2005). World checklist of monocots Orchidaceae, published online by The Board of Trustees, RBG, Kew. http://www.kew.org/monocotChecklist/orchids (accessed 01 Jan 2012).

Howe, C. (2008). Plant Heritage Collection of Restrepia http://www.restrepia.co.uk/ (accessed 01 Jan 2012).

Humboldt, Bonpland and Kunth. (1816). *Nova Genera et species plantarum quas in peregrinatione ad plagam aequinoctialem orbis novi collegerent Bonpland et Humboldt.* Vol. 1. *Nov Gen Sp.* Paris.

Johansen, B. (1990). Incompatibility in Dendrobium (Orchidaceae). *Bot J Linn Soc* 103:

Kuper, W., Kreft, H., Nieder. J., Koster, N. and Barthlott, W. (2004). Large-scale diversity patterns of vascular epiphytes in Neotropical montane rain forests. *Journal of Biogeography* 31: 1477-1487.

Luer, C.A. (1986a). Icones Pleurothallidinarum I. *Monogr Syst Bot Missouri Bot Gard 15 .* St. Louis, MO.

…… (1986b). Icones Pleurothallidinarum III. *Monogr Syst Bot Missouri Bot Gard 20.* St. Louis, MO

…….(1990). Icones Pleurothallidinarum VII. *Monogr Syst Bot Missouri Bot Gard 38* St. Louis, MO

…….(1991). Icones Pleurothallidinarum VIII. *Monogr Syst Bot Missouri Bot Gard 39* St. Louis, MO

……...(1994). Icones Pleurothallidinarum XI. *Monogr Syst Bot Missouri Bot Gard 52* St. Louis, MO

……(1996). Icones Pleurothallidinarum XIII, Systematics of Restrepia (Orchidaceae). *Monogr Syst Bot Missouri Bot Gard* St. Louis, MO.

……(1998a). Icones Pleurothallidinarum XVII. *Monogr Syst Bot Missouri Bot Gard 72* St. Louis, MO.

…….(1998b). New Species of Orchids from Cuba. *Lindleyana* 13(3): *138*-147.

…….(1999a). New species of Pleurothallids from Cuba and Hispaniola. *Lindleyana* 14(2): 106-121.

…….(1999b). Icones Pleurothallidinarum XVIII. 76 *Monogr Syst Bot Missouri Bot Gard* St. Louis, MO

…….(2000). Icones Pleurothallidinarum XX. *Monogr Syst Bot Missouri Bot Gard* 79 St. Louis, MO.

…….(2001). Miscellaneous new species in the Pleurothallidinae. *Revista de la Sociedad Boliviana de Botánica* 3(1-2): 37-63.

……(2002). A systematic method of classification of the Pleurothallidinae versus a strictly phylogenetic method. *Selbyana* 23(1): *57*-110.

…….(2003). Icones Pleurothallidinarum XXV: Systematics of Masdevallia Part 5. *Monogr Syst Bot Missouri Bot Gard* St. Louis, MO.

…….(2004). Icones Pleurothallidinarum XXVI: Pleurothallis subgenus Acianthera and three allied subgenera. A second century of new species of Stelis of Ecuador. Epibator, Ophidion, Zootrophion. Addenda to Brachionidium, Dracula, Lepanthes, Platystele, Pleurothallis, Porroglossum, and Masdevallia. New genera and combinations*. Monogr. Syst. Bot. Missouri Bot. Gard.* St. Louis, MO. 165-196

……(2006). Icones Pleurothallidinarum XXVIII. Reconsideration of Masdevallia, and the systematic of Specklinia and vegetatively similar genera (Orchidaceae). *Monogr Syst Bot Missouri Bot Gard* St. Louis, MO

……(2007). Icones Pleurothallidinarum XXIX. A Third Century of *Stelis* of Ecuador. Systematics of *Apoda-Prorepentia*. Systematics of miscellaneous small genera. Addenda: new genera, species, and combinations. *Monogr Syst Bot Missouri Bot Gard* St. Louis, MO

Manning, S. (2010). *Discovering New World Orchids.* Manning U.K.

Millner. H.J., Obeng, A., McCrea, A.R. and Baldwin, T.C. (2008). Axenic seed germination and in vitro seedling development of *Restrepia brachypus* (Orchidaceae). *J Torrey Bot Soc* 135:(4)497–505.

Morel, G. (1960). Producing virus-free cymbidiums. *Am. Orch. Soc. Bull.* 29:495-497.

.....(1965). Clonal propagation of orchids by meristem culture. *Cymbidium Soc. News.* 20:87-93.

Murashige, T. and Skooge, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum. 15: 473–497.

Myers, N. (1988). Threatened Biotas: “Hotspots” in Tropical Forests. *The Environmentalist,* 8: 187-208

……(1990). The Biodiversity Challenge: Expanded Hot-Spots Analysis. *The Environmentalist,* 10: 1243-256

Neyland, R. and Urbatsch, L. E. (1993). Anatomy and morphology of the articulation between ovary and pedicel in Pleurothallidinae. *Lindleyana* 8(4):189-192.

Pridgeon, A.M. (1981a). *Contribution to the systematics of the Pleurothallidinae: anatomical evidence and numerical analyses*. Unpublished Ph.D. thesis. The Florida State University, Tallahassee.

…….(1981b). Absorbing trichomes in the Pleurothallidinae. *American Journal of Botany* 68: 64-71.

……(1981c). Shoot anatomy of two additional species of Dresslerella. *Selbyana* 5: 274-278.

……(1992). *The illustrated encyclopedia of orchids.* Timber Press, Portland, Or.

Pridgeon, A.M. and Chase, M.W. (2001). A phylogenetic reclassification of Pleurothallidinae (Orchidaceae). *Lindleyana* 16: 235-271

Pridgeon, A.M. and Stern, W.L. (1982). Vegetative anatomy of Myoxanthus. *Selbyana* 7: 55-63.

…….(1983). Ultrastructure of osmophores in *Restrepia* (Orchidaceae). *American Journal of Botany,* 70:(8) 1233-1243

…….(1985). Osmophores of Scaphosepalum. *Bot. Gaz.* 146(1): 115-123.

Pridgeon, A.M. and Williams, N.H. (1979). Anatomical aspects of Dresslerella. *Selbyana* 5: 120-134.

Pritchard, H. (2008). *Orchid seed stores for sustainable use*. The Darwin Initiative. http://darwin.defra.org.uk (accessed 01 Jan 2012).

RBG Kew. (2008). The Millennium Seed Bank Project Kew. Website of the Royal Botanic Gardens, Kew. www.kew.org/msbp/index.htm *(accessed 01 Jan 2012).*

RHS. (2004). www.rhs.org.uk/plants/registerpages/orchidsearch.asp *(accessed 01 Jan 2012).*

Roberts, D.L. (2003). *Pollination biology: the role of sexual reproduction in orchid conservation*. Pages 113-136 *in* KW Dixon, SP Kell, PJ Cribb, eds. Orchid conservation. Natural History Publications, Borneo, Kota Kinabalu, Sabah.

Ruiz, H. and Pavon, J. (1798). *Humboltia contorta,* Systema vegetabilum florae peruvianae at chilensis 235

Seaton, P. and Ramsay, M. (2005). Growing orchids from seeds. Royal botanic gardens, Kew.

Stenzel, H. (2004). *Systematics aand evolution of the genus Pleurothallis R. Br. (Orchidaceae) in the Greater Amtilles.* Berlin, Humboldt-Universïat, Diss.

Stern, W.L., Pridgeon, A.M. and Luer, C.A. (1985). Stem structure and its bearing on the systematics of Pleurothallidinae. *Botanical Journal of the Linnaean Society* 91:457-471.

Stort, M.N.S. and de Lima Galdino, G. (1984). Self- and cross-pollination in some species of the genus Laelia Lindl. (Orchidaceae). *Braz J Genet* VII 4:671–676.

Tremblay, R.L., Ackerman, J.D. (2007). Floral color patterns in a tropical orchid: Are they associated with reproductive success? *Plant species biol.* 22:(2) 95-105

Tropicos. (2010). Tropicos.org. Missouri Botanical Garden. http://www.tropicos.org *(accessed 01 Jan 2012).*

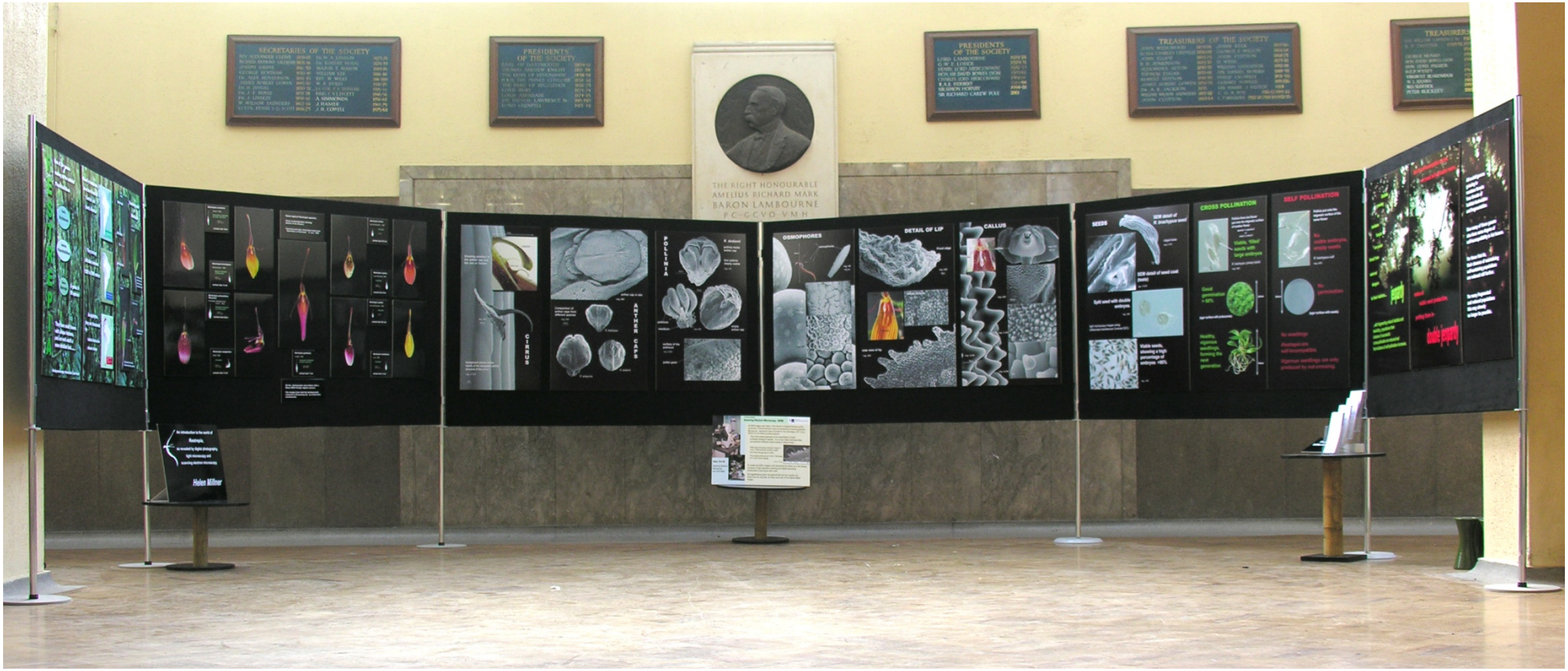
Vacin, E.F. and WENT, F.W. (1949). Some pH changes in nutrient solutions. Bot. Gaz. 110:605–613.

Webb, M. (1981). Propagation of Restrepias by Leaf Cuttings*. Am. Orchid Soc. Bulletin* 50(4):419

Appendix 3:

Gold medal exhibition on *Restrepia* conservation staged by H Millner.

RHS London Orchid Show, 2006.



RHS London Orchid Show.

Restrepia Conservation –Gold Medal Award

Boards 1 and 2:

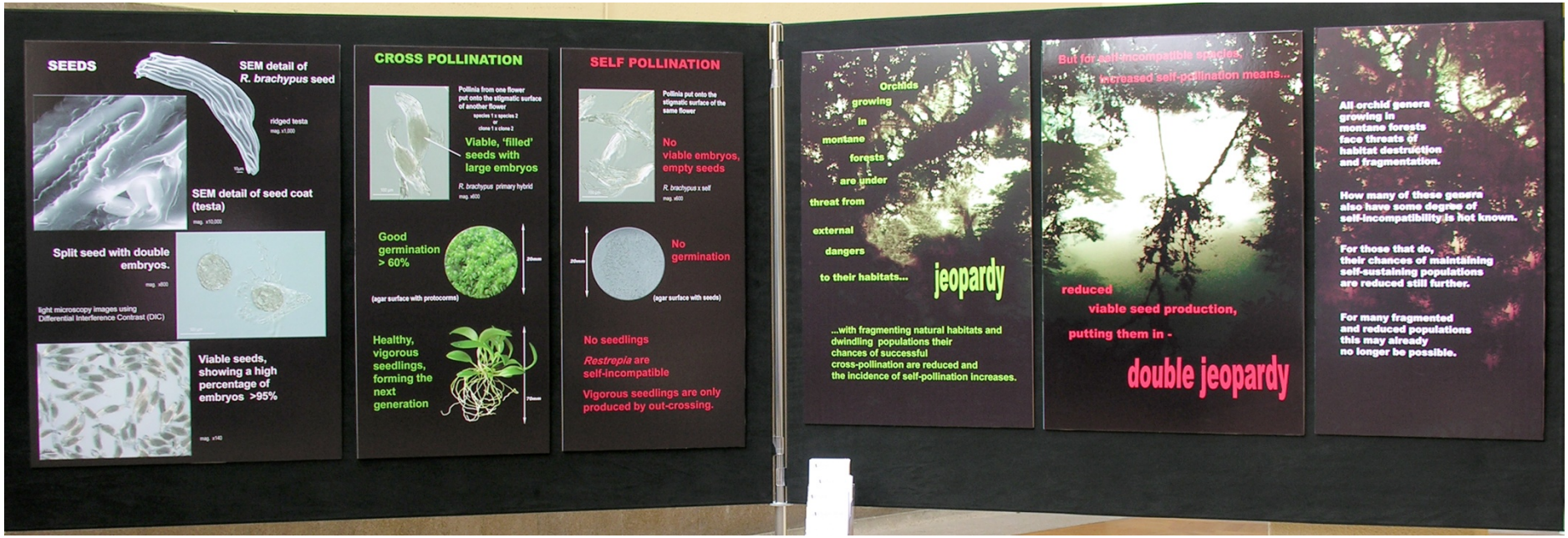
Board 1: Habitat and morphology

Board 2: *Restrepia* species

Boards 3 and 4: SEM

Board 3: Cirri, anther caps and pollinia

Board 4: Osmophores, lip and callus



Boards 5 and 6:

Board 5: Seeds and pollination Board 6: Jeopardy and double jeopardy

Appendix 4:

Silver- gilt medal exhibition on *Restrepia* conservation staged by H Millner.

The Chelsea Flower Show, Life Long Learning, 2007.

RHS Chelsea Flower Show, Life Long Learning. *Restrepia* Conservation. Silver-Gilt Award

Appendix 5:

Gold medal exhibition on *Restrepia* pollination staged by H Millner.

RHS London Orchid Show, 2012

RHS London Orchid Show 2012. Fly pollination in *Restrepia*