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Oreocharis × *heterandra* (Gesneriaceae): a natural hybrid from the Shengtangshan Mountains, Guangxi, China

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Abstract

Macro- and micro-morphological characters, molecular nuclear ribosomal internal transcribed spacer and chloroplast *trn*L-F intron-spacer data confirmed the hybrid status of *Oreocharis* × *heterandra*. Cytological studies showed that the parental species and the hybrid possess *2n*=34 chromosomes, suggesting that chromosome translocations, not dysploid or ploidy level changes, are the cause of the high hybrid sterility. Recurrent reciprocal hybridisation between its parental species *O. argyreia* and *O. magnidens* in an area of secondary contact is apparently responsible for the persistent presence of the hybrids, though at low levels. As a consequence the name *Oreocharis heterandra* has to be changed to *Oreocharis* × *heterandra*.

Key words: Cytology, hybrid sterility, ITS, molecular data, morphology, *Oreocharis argyreia*, *Oreocharis magnidens*, recurrent hybridisation, *trn*L-F

Introduction

The significance of interspecific hybridization for plant evolution has long been recognised (Stebbins 1950, 1959, Lewis 1966, Grant 1981). Hybridization can lead to the establishment of new species through polyploid or homoploid speciation and introgression (Arnold 1997, 2006, Abbott 1992, Rieseberg & Carney 1998, Buerkle *et al*. 2000, Rieseberg *et al.* 2003). Interspecific crosses do not occur uniformly across angiosperms but are more frequent in certain families (Ellstrand *et al.* 1996). They do not necessarily need to result in the establishment of a new species or hybrid swarm if polyploidization is not involved (e.g. Rieseberg *et al.* 2003, Mallet 2007, Rieseberg & Willis 2007, Soltis & Soltis 2009), and the first generation plants are completely or almost fully sterile (e.g. Saito *et al.* 2006, 2007).

Gesneriaceae is one of the families where hybridization is common (Ellstrand *et al.* 1996): genera in which natural hybrids have been found are the New World *Columnea* Linnaeus (1753: 638; Morley 1976) and *Sinningia* Nees von Esenbeck (1825: 297; Clayberg 1996) and the Old World *Monophyllaea* Brown (1838: 121; Okada 1990) and *Cyrtandra* Forster & Forster (1776: t. 3; Ellstrand *et al*. 1996, Smith *et al*. 1996, Schlag-Edler & Kiehn 2001, Kiehn 2005). Particularly in the African genus *Streptocarpus* Lindley (1828: pl. 1173), hybrid origin for some species has been suspected (Hilliard & Burtt 1971) and then demonstrated to play a significant role in the evolution of the genus (Möller *et al.* 2004, Hughes *et al*. 2005, de Villiers 2008). While hybridisation is widespread in some Gesneriaceae genera, examples from China are rare; none are listed in the Flora of China (Wang *et al.* 1998).

During fieldwork in China in 2006, specimens of three species of *Oreocharis* Bentham (1876: 995, 1021) were collected in Jinxiu, Shengtangshan, in the Dayao Shan Mountains in Central Guangxi (Fig. 1). In

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consultation with Prof D. Fang at the herbarium in Nanning, and through comparison with herbarium specimens, they were later identified as *O. argyreia* Chun ex Pan (1987: 283), *O. magnidens* Chun ex Pan (1987: 276) and *O. heterandra* D.Fang & D.H.Qin in Fang *et al.* (1994: 563) (Fig. 2).

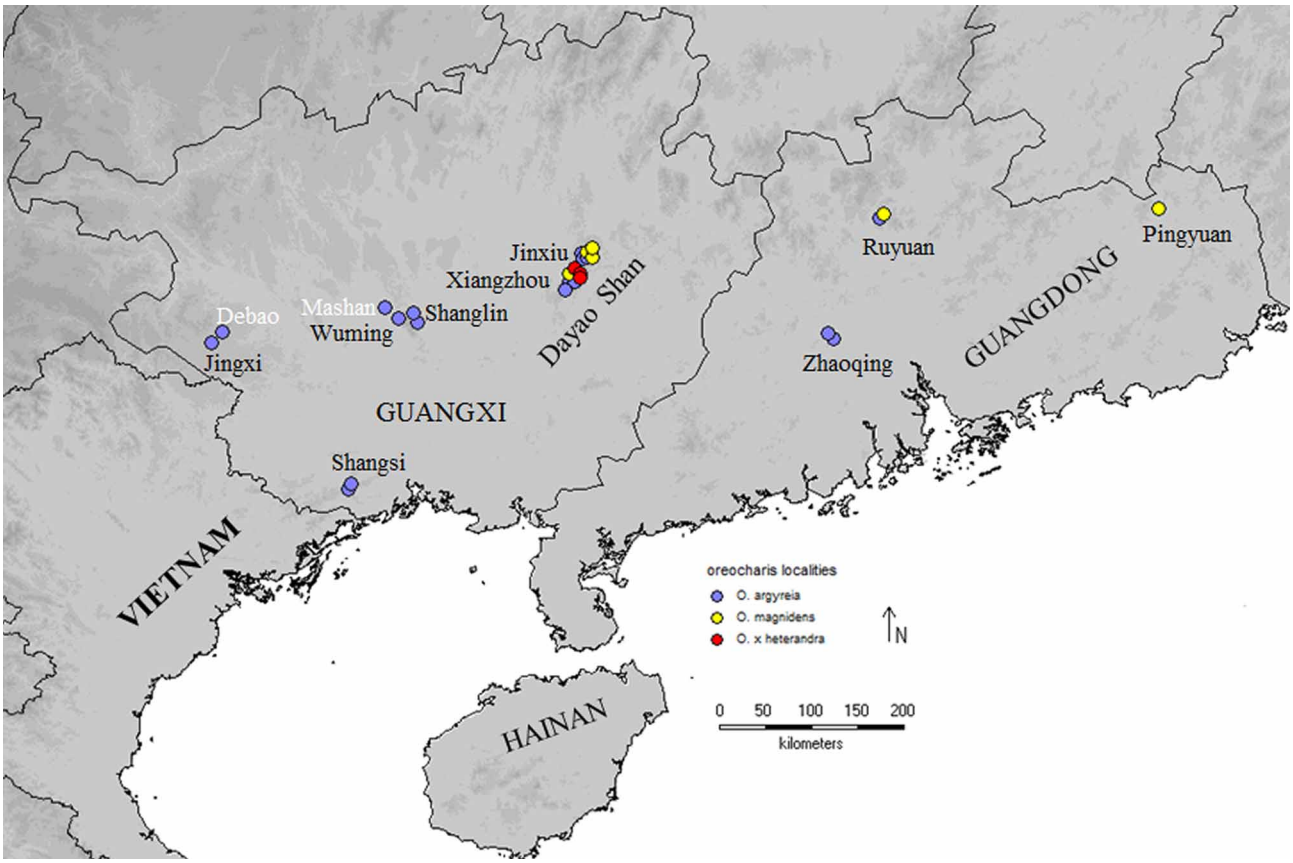

FIGURE 1: Distribution of *Oreocharis argyreia*, *O. magnidens* and *O.* × *heterandra* in Guangxi and Guangdong provinces, China, indicating areas of overlap in Dayao Shan (Guangxi) and Ruyuan (Guangdong). At localities in white print (Debao and Mashan) *O. argyreia* is likely extinct (see also Wei *et al*. 2010).

At the collection site, the plants were growing on rocks in moss in the forest along paths to the top of the mountains. Plants of *O. argyreia* and *O. magnidens* were present in high numbers (thousands), while only three individuals of *O. heterandra* were found. Morphologically, this species appeared to be intermediate between the two abundantly occurring species. The most conspicuous characters were the flower shape and colour and the leaf texture and margin (Figs. 2, 3); *Oreocharis argyreia* has a smooth leaf texture with entire margins (Fig. 3G, K) and a purple corolla tube with short purple lobes (Fig. 3A, D); *Oreocharis magnidens* has a narrow white corolla tube with long white lobes purple at the tips (Fig 3B, E) and a bullate leaf with a strongly dentate margin (Fig. 3H, L); *Oreocharis heterandra* has an open-tubed purple corolla with long purple lobes (Fig. 3C, F) and a slightly bullate leaf surface with a finely serrulate margin (Fig. 3J, M). The low number of individuals found in Jinxiu and their intermediate macro-morphology suggested a hybrid origin for *O. heterandra*.

Oreocharis argyreia is a very widespread species occurring with many populations across Guangxi and in West Guangdong at altitudes of 600–1600m. The holotype is from Jingxi (Lu Shan). *Oreocharis magnidens* has a scattered disjunct distribution, growing in Guangxi only in the Dayao Shan area and in two locations in North Guangdong at 1400–1718m (Fig. 1). The holotype is from Xiangxian, Jinxiu County. *Oreocharis heterandra* is only described from the Jinxiu County in Guangxi, growing at an elevation of 1300m.

The type specimen of *O. heterandra* was collected by a student of Prof D. Fang, Qin De-Hai (coll. no. 64868), on 18 October 1981. It was described in September 1992 and published as *Oreocharis heterandra* D.Fang & D.H.Qin in 1994. It has not been collected again until our fieldwork in 2006.

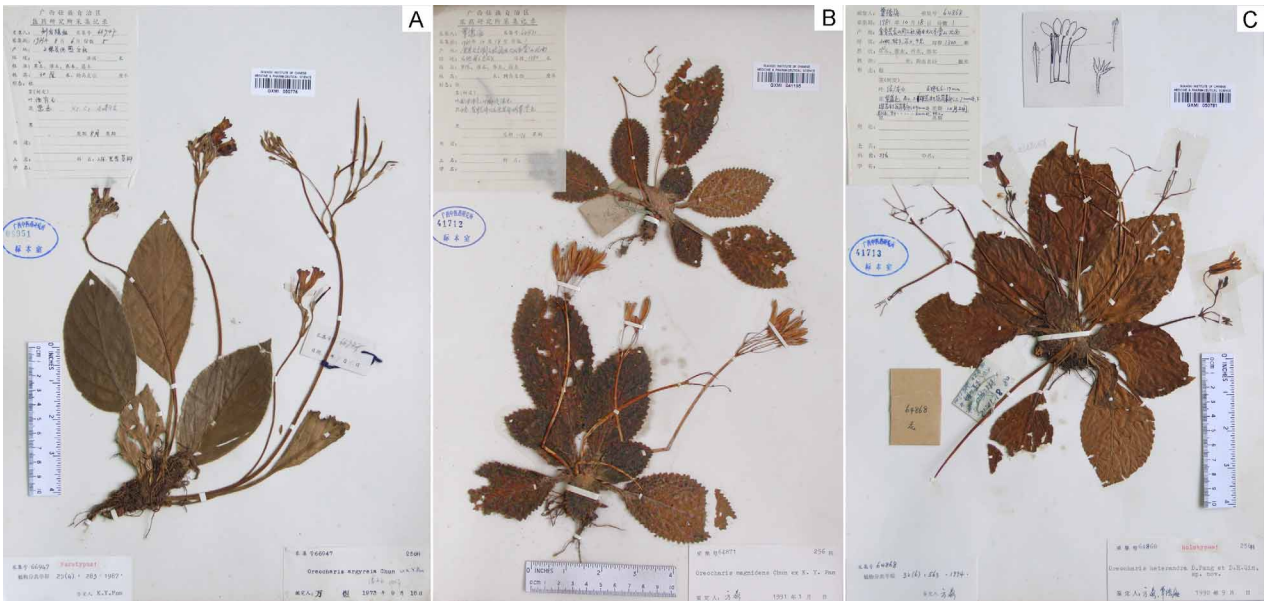

FIGURE 2: Herbarium sheets of the type specimens: A) *Oreocharis argyreia*; B) *O. magnidens*; (C) *O.* × *heterandra*.

In order to verify the hybrid origin of *O. heterandra* and parentage of *O. magnidens* and *O. argyreia*, we used molecular and morphological data analyses. Following Sang *et al.* (1995) and Saito *et al.* 2006, 2007, we sequenced the nuclear ribosomal internal transcribed spacer (ITS) and searched for nucleotide additivity. The chloroplast *trn*L-F intron-spacer, uniparentally inherited in Gesneriaceae (Möller *et al.* 2004), was sequenced additionally to obtain an insight into the direction of hybridization. The micro-morphological characters were examined in detail, somatic chromosome numbers were determined and pollen viability was investigated, which in early hybrids would be expected to be very low (e.g. Saito *et al.* 2007, Moyle & Nakazato 2010).

Material and methods

Plant material

Samples of the putative parents, *O. magnidens* and *O. argyreia*, were collected together with three individuals identified as *O. heterandra* in Jinxiu, Shengtangshan, Dayao Shan, in Guangxi, China. For each plant a herbarium voucher, leaves dried in silica gel and flowers fixed and preserved in 70% ethanol were collected (Table 1). Vouchers are deposited in E. Material for chromosome investigations came from plants growing in RBGE's living research collection raised from seeds collected in the field; for *O. argyreia* from MMO 06-892 (CH-66) and *O. magnidens* from MMO 06-896 (CH-23). Among the 20 seedlings of *O. magnidens*, one *O. heterandra* plant (MMOG 47) was detected and used for chromosome studies.

TABLE 1: Type of study material of *Oreocharis argyreia*, *O. magnidens* and *O.* × *heterandra* collected in Jinxiu, Chang Dong, Shengtangshan, Dayao Shan, Guangxi, China (+ present; - absent due to too few flowers present).

taxon	identifier	coll. number	DNA number	voucher	silica gel	flowers fixed
O. argyreia	CH-66	MMO 06-892 [1]	EDNA08-01092	E	+	+
O. magnidens	CH-23	MMO 06-896 [2]	EDNA08-00322	E	+	+
O. × *heterandra*	CH-68	MMO 06-897	EDNA08-01094	E	+	-
O. × *heterandra*	CH-69	MMO 06-898	EDNA08-01095	E	+	-
O. × *heterandra*	CH-70	MMO 06-900	EDNA08-01096	E	+	+

[1] *O. argyreia* plants raised from seeds used for chromosome counts
[2] *O. magnidens* and *O.* × *heterandra* plants raised from seeds used for chromosome counts; the individual *O.* × *heterandra* plant was vouchered as MMOG 47.

FIGURE 3: Photographic images of *Oreocharis argyreia* (A, D, G, K), *O. magnidens* (B, E, H, L), *O.* × *heterandra* (C, F, J, M). A–F. flowers; G–J. habit; K–M. leaves.

Molecular analyses

Silica-dried leaves were used for total genomic DNA extraction from all samples using a modified CTAB protocol (Doyle & Doyle 1987). For one sample of *O. heterandra,* CH-69, a second DNA extraction was carried out using the DNeasy plant mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The nuclear ITS and the chloroplast *trn*L-F regions were amplified using the primers "5P" and "8P" (Möller & Cronk 1997) and "c" and "f" (Taberlet *et al.* 1991), respectively. The PCR reaction mix contained 2µl 10×NH4 buffer, 2µl dNTPs, 0.6µl $MgCl_2$, 2µl forward primer, 2µl reverse primer, 0.4µl BioTaq polymerase, 0.2µl BSA and 9.8µl dH_2O, with 1µl of isolated DNA. For some reactions the addition of 2M Betaine, up to 10µl, improved the PCR results.

For amplifying the nuclear ITS region, the following PCR profile was used: initial denaturation for 3min at 94°C, then 30 cycles of 1min at 94°C, 1min at 55°C and 1.5min at 72°C and a final extension step of 5min at 72°C. The profile for *trn*L-F was: initial denaturation for 4min at 94°C, then 35 cycles of 45sec at 94°C, 45sec at 55°C and 3min at 72°C with a final extension step of 10min at 72°C.

Both DNA and PCR product quality was checked on agarose gels. PCR products were purified either with GFX PCR purification kits (GE Healthcare, Buckinghamshire, UK) or with ExoSAP IT (Affymetrix, Santa Clara, California, USA) following the manufacturer's protocols. For the sequencing reactions we used 0.5µl BigDye mix (Applied Biosystems, Carlsbad, California, USA), 2µl 5× sequencing buffer, 0.32µl primer, 0.4µl DMSO (in ITS reactions only), 1 or 2µl purified PCR product and dH_2O to make up 10µl. The sequencing profile used consisted of 25 cycles of 30sec at 95°C, 20sec at 50°C and 4min at 60°C. The products of this reaction were sequenced on an ABI 3730 DNA Analyser (Applied Biosystems, Carlsbad, California, USA), at the GenePool facility (University of Edinburgh, UK). Sequence contigs were edited and electropherograms checked in Sequencher v. 4.7 (Gene Code Corporation, Ann Harbor, Michigan, USA).

Divergence time estimations

To estimate the divergence time between the putative parents of *O. heterandra*, we used the uncorrected distance between *O. argyreia* (CH-66) and *O. magnidens* (CH-23), and calculated the average of 11 evolutionary rates for ITS for herbaceous plants from Kay *et al.* (2006) and three for *trn*L-F from Richardson *et al.* (2001).

Micro-morphological analyses

Micro-morphological data were obtained from herbarium specimens and associated material preserved in 70% ethanol as listed in Table 1. Light and scanning electron microscopes (SEM) were used. For the latter, material was fixed in 3 : 1 absolute ethanol : glacial acetic acid, de-hydrated through an ethanol series and acetone, critical point dried in CO_2 with an Emitech K850 (Quorum Technologies Ltd, Ashford, UK), coated with platinum for 2min at 25mA using an Emitech K575X sputter coater (Quorum Technologies Ltd, Ashford, UK), and then examined with a LEO Supra 55VP Scanning Electron Microscope (Zeiss, Cambridge, UK). We observed leaf and corolla indumentum, gynoecium, androecium and pollen characters.

Pollen stainability

We sampled anthers from herbarium specimens and flowers fixed in 3 : 1 absolute ethanol : glacial acetic acid and stored in 70% ethanol (Table 1). Pollen was stained on microscope slides with 0.25% acetocarmine. To prepare permanent slides we used a vapour exchange method; slides were left vertical in an ethanol saturated environment overnight, then placed horizontally in a mild ethanol environment overnight and then sealed with Euparal (Agar Scientific, Stansted, UK). The pollen samples were observed under a Zeiss Axiophot microscope and photographed with a Zeiss Axiocam MRc5 (Zeiss, Cambridge, UK). Pollen grains were scored as potentially viable when fully formed and stained red and as sterile when unstained and collapsed.

Chromosome investigations

The cytological methods followed Jong & Möller (2000). Root tips were pre-treated in 0.002M 8-hydroxyquinoline for 4–5 hours at room temperature, and fixed in freshly prepared Farmer's Fluid (3 parts ethanol : 1 part glacial acetic acid). After hydrolyzation for 30 min in 5M HC1 at room temperature, they were stained with Feulgen Reagent (prepared according to Fox, 1969) for 2 hours in the dark. After softening in a 1 : 1 enzyme mixture (4% pectinase, Sigma 2401 : 4% cellulose, Calbiochem 21947) at 36 °C for 30 minutes, roots were squashed in 0.4% acetocarmine counterstain. Images were captured using Zeiss AxioVision 4.7 with an AxioCam MRc5 mounted on an AxioSkop brightfield microscope (Zeiss, Cambridge, UK).

Results

Molecular data

ITS: *Oreocharis argyreia* (CH-66) and *O. magnidens* (CH-23) differed in their ITS sequences in 34 positions, 21 in ITS1 and 13 in ITS2 (Table 2). All but one were base changes; at position 412, *O. magnidens* had a single base pair deletion. The electropherograms of the ITS sequences we obtained for the three *O. heterandra* samples showed sequence additivity at each position with the two peaks representing the two nucleotides found in the parental genomes. The only exceptions were two positions (533 and 633) where the putative parents differed (e.g. Fig. 4) and the putative hybrids showed only one parental nucleotide (Table 2).

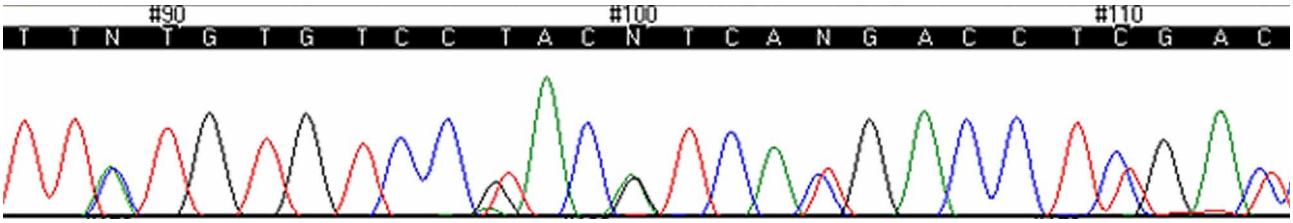

FIGURE 4: Example of a section of a Sanger sequencing trace file of internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) sequences of *Oreocharis* × *heterandra* (CH-70), showing additivity of signals at positions (89, 97, 100, 104, 110 and 113, equivalent to positions 67, 75, 78, 82, 88 and 91 in the matrix, Table 2) where the putative parents *O. argyreia* and *O. magnidens* differ (Table 2).

TABLE 2: Variable sites in ITS sequences of the three taxa of *Oreocharis* analysed. Identification numbers refer to Table 1.

ITS1

taxon (identifier) / position	11	29	44	46	52	67	75	78	82
O. argyreia (CH-66)	T	A	T	C	T	C	T	A	T
O. magnidens (CH-23)	C	G	C	T	C	A	G	G	C
O. × *heterandra* (CH-68)	C/T	G/A	C/T	C/T	C/T	A/C	G/T	G/A	C/T
O. × *heterandra* (CH-69)	C/T	G/A	C/T	C/T	C/T	A/C	G/T	G/A	C/T
O. × *heterandra* (CH-70)	C/T	G/A	C/T	C/T	C/T	A/C	T/G	G/A	C/T

taxon (ident.)/ position	88	91	98	102	109	117	127	134	150	162	193	233
(CH-66)	C	C	T	G	G	G	A	T	C	C	C	A
(CH-23)	T	T	G	A	T	A	C	C	A	T	A	G
(CH-68)	T/C	T/C	G/T	A/G	T/G	A/G	C/A	C/T	A/C	T/C	A/C	G/A
(CH-69)	T/C	T/C	G/T	A/G	T/G	A/G	C/A	C/T	A/C	T/C	A/C	G/A
(CH-70)	T/C	T/C	G/T	A/G	T/G	A/G	C/A	C/T	A/C	T/C	A/C	G/A

ITS2

taxon (ident.)/ position	412	447	460	461	467	492	513	514	515	533	561	589	590	633
(CH-66)	T	T	A	T	G	T	A	T	G	T	A	A	G	T
(CH-23)	-	A	T	G	A	G	T	C	T	G	T	T	T(G)	A
(CH-68)	T/-	A/T	T/A	G/T	A/G	G/T	T/A	C/T	T/G	G	T/A	T/A	T/G	T
(CH-69)	T/-	A/T	T/A	G/T	A/G	G/T	T/A	C/T	T/G	T	T/A	T/A	T/G	A
(CH-70)	T/-	A/T	T/A	G/T	A/G	G/T	T/A	C/T	T/G	G	T/A	T/A	T/G	T/A

*trn*L-F: The chloroplast *trn*L-F regions of *O. argyreia* (CH-66) and *O. magnidens* (CH-23) differed by twelve mutations, eight being base changes, and four indels, two of one base pair and two of five base pairs in length (both duplications) (Table 3). Two (CH-69, CH-70) of the three *O. heterandra* samples had sequences identical to *O. argyreia*, while CH-68 was identical to *O. magnidens*.

TABLE 3: Variable sites in the *trn*L-F sequences of the three taxa of *Oreocharis* analysed. Identification numbers refer to Table 1. Sequences of and identical to *O. magnidens* highlighted in grey.

taxon (identifier)/ position	204	262	276	408-412	479	538	554	555	662	744	745	817-821
O. argyreia (CH-66)	T	A	G	-----	A	-	C	C	A	A	A	AATTG
O. magnidens (CH-23)	C	G	C	TTATT	G	A	A	T	G	C	-	-----
O. × *heterandra* (CH-68)	C	G	C	TTATT	G	A	A	T	G	C	-	-----
O. × *heterandra* (CH-69)	T	A	G	-----	A	-	C	C	A	A	A	AATTG
O. × *heterandra* (CH-70)	T	A	G	-----	A	-	C	C	A	A	A	AATTG

Estimation of divergence time

The divergence time estimates suggest that the parents *O. argyreia* and *O. magnidens* diverged 5.88MY (±3.45SE) based on ITS rates, and 5.98MY (±0.95SE) based on *trn*L-F rates.

Micro-morphology

Leaf indumentum: All plants had leaves with multicellular hairs on both sides, with little interspecific variation (Table 4). Glands were also observed. In *O. argyreia* (CH-66), the upper surface had long hairs (c. 860μm), all 3-4-celled and covered with small knobs, and sporadic glands. On the lower surface the indumentum was much denser along the leaf venation; glands were more abundant and scattered between stomata which were up to 29μm long.

The upper leaf surface of *O. magnidens* (CH-23) was characterised by 1-2mm long, smooth trichomes distributed in patches between the veins and by glands scattered across the lamina. The lower surface appeared lanate, with hairs of a different type, smooth, flat and thin. Their distribution mostly matched that of the leaf veins. Because of their length and abundance, they covered most of the lamina. In the less covered areas, glands and stomata (c. 14μm long) were observed.

The three *O. heterandra* plants had an indumentum similar to that of *O. argyreia* in hair type, distribution and density. However, in samples CH-68 and CH-69, the hair surface appeared generally smoother. Glands were numerous on both sides of the leaf. The average length of the stomata was c. 33μm in sample CH-68, c. 22μm in CH-69 (leaves of this plant had more glands) and 20μm in *O. heterandra* CH-70.

Corolla indumentum: The most relevant cryptic character of the corolla observed was the indumentum on the outside of the tube (Table 4). In *O. argyreia* (CH-66) it consisted of multicellular glands. Similar in size (30-40μm), these glands had heads made up of two to four elongated cells (Fig. 5A, B–D). In *O. magnidens* (CH-23) the corolla tube had long (330μm) glandular trichomes (Fig. 5E). Each had a stalk with up to 3 cells with a 6-8-celled head (Fig 5F). *Oreocharis heterandra* (e.g. CH-70, Fig. 5G) had glandular trichomes like those of *O. magnidens*, although with sometimes unusually sculptured head-cells (Fig. 5H).

Gynoecium: The main difference found between the putative parent species in the gynoecia was the stigma. *Oreocharis argyreia* (CH-66) had very long stigmatic papillae, unlike *O. magnidens* (CH-23), whose papillae were short and seemingly fewer. The stigma of *O. heterandra* (e.g. CH-70) resembled that of *O. argyreia*. The disc surrounding the ovary base had a variable edge: it was irregularly lobed in *O. argyreia*, entire in *O. magnidens* and deeply lobed in *O. heterandra*. In all taxa this area possessed scattered stomata.

Androecium: The anthers of *O. argyreia* (CH-66) were 1.4mm long and in *O. magnidens* (CH-23) 0.8mm. In the *O. heterandra* samples the length of the anther was 1.3mm.

TABLE 4: Summary of macro- and micro-morphological characters of three *Oreocharis* taxa collected in Jinxiu, Chang Dong, Shengtangshan, Dayao Shan, Guangxi, China.

	O. argyreia (CH-66)	*O. magnidens* (CH-23)	*O.* × *heterandra* (CH-68 to CH-70)	putative inheritance
macro-morphological characters				
corolla				
lobes:	short	long	long	*magnidens*
colour:	purple	white with purple tipped lobes	purple	*argyreia*
leaf				
margins:	entire	dentate	finely serrulate	intermediate
texture:	smooth	bullate	medium bullate	intermediate
colour:	greyish green	bright green with pale veins	shiny green with slightly paler veins	intermediate
micro-morphological characters				
indumentum				
leaf				
upper side:	long egl. & glands	long egl. & glands	long egl. & glands	all identical
lower side:	long egl. & glands fewer and shorter than *O. magnidens*	woolly & glands	long egl. & glands fewer and shorter than *O. magnidens*	*argyreia*
flower				
corolla outside:	2–4 celled glands	glandular trichomes	glandular trichomes	*magnidens*
gynoecium:	long stigmatic papillae	short stigmatic papillae	long stigmatic papillae	*argyreia*
disc	irregularly lobed	entire	deeply lobed	intermediate
androecium	anthers 1.4mm	anthers 0.8mm	anthers 1.3mm	*argyreia*
pollen				
shape:	spheroidal to oblate	spheroidal	variable	variable
exine:	reticulate	reticulate	reticulate	all identical
colpi:	tricolpate	tricolpate	1–4-colpate	unique
orbicules:	spheroidal	toroidal	absent	unique

Pollen morphology: Two sets of pollen samples were investigated. The first was extracted from anthers fixed in the field, the second from the herbarium voucher specimens. Pollen from herbarium specimens were slightly smaller (~7%), perhaps due to them being stored dried before staining in acetocarmine. This might also explain the somewhat smaller sizes reported by Pan (1987) for the two species (*O. argyreia*: 17.1µm; *O. magnidens*: 11.7µm).

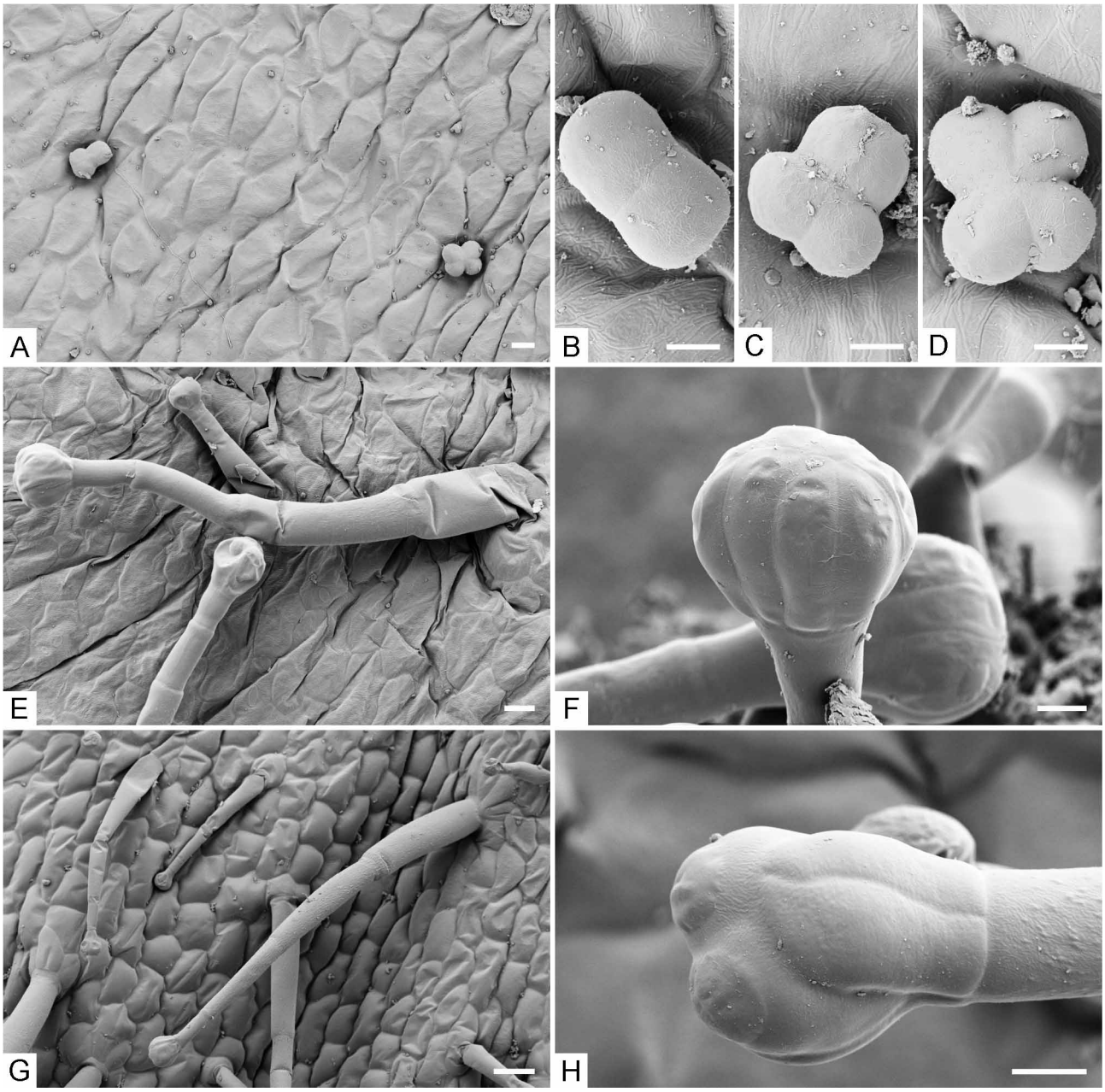

FIGURE 5: Scanning electron microscope images of micro-morphological features of the upper leaf surface (A, E, G) and details of indumentum (B–D, F, H): A–D) *Oreocharis argyreia* (CH-66); E, F) *O. magnidens* (CH-23); G, H) *O.* × *heterandra* (CH-70). Bars: A, E, 20μm; B–D, F, H, 10μm; G, 50μm.

The pollen of *O. argyreia* (CH-66) were on average 19.7μm in diameter (Table 5), spheroidal to oblate and tricolpate (Fig. 6A). The exine was reticulate with apertures of variable shape and size (Fig. 6B). A multitude of spherical orbicules ranging between 0.5 and 2μm in size were observed in the anther cavity and on the pollen grains (Fig. 6G).

Oreocharis magnidens (CH-23) had smaller pollen, with 14.6μm on average (Table 5; Fig. 6C). The exine morphology was comparable to that of the pollen of *O. argyreia* (Fig. 6D). The orbicules observed in the anther cavity were toroidal and c. 0.9μm in size (Fig. 6H).

Oreocharis heterandra (e.g. CH-70 Fig. 6E and below) had heterogeneous pollen grains: most were collapsed while those that stained with acetocarmine were with 21.9μm on average slightly larger than those of *O. argyreia* (Table 5). The putative hybrid pollen showed much more size variation compared to the parent species, and were mostly tri- and tetracolpate, with some uni- and bicolpate ones, with an exine similar to the parent species (Fig. 6F). No orbicules were seen in the anthers.

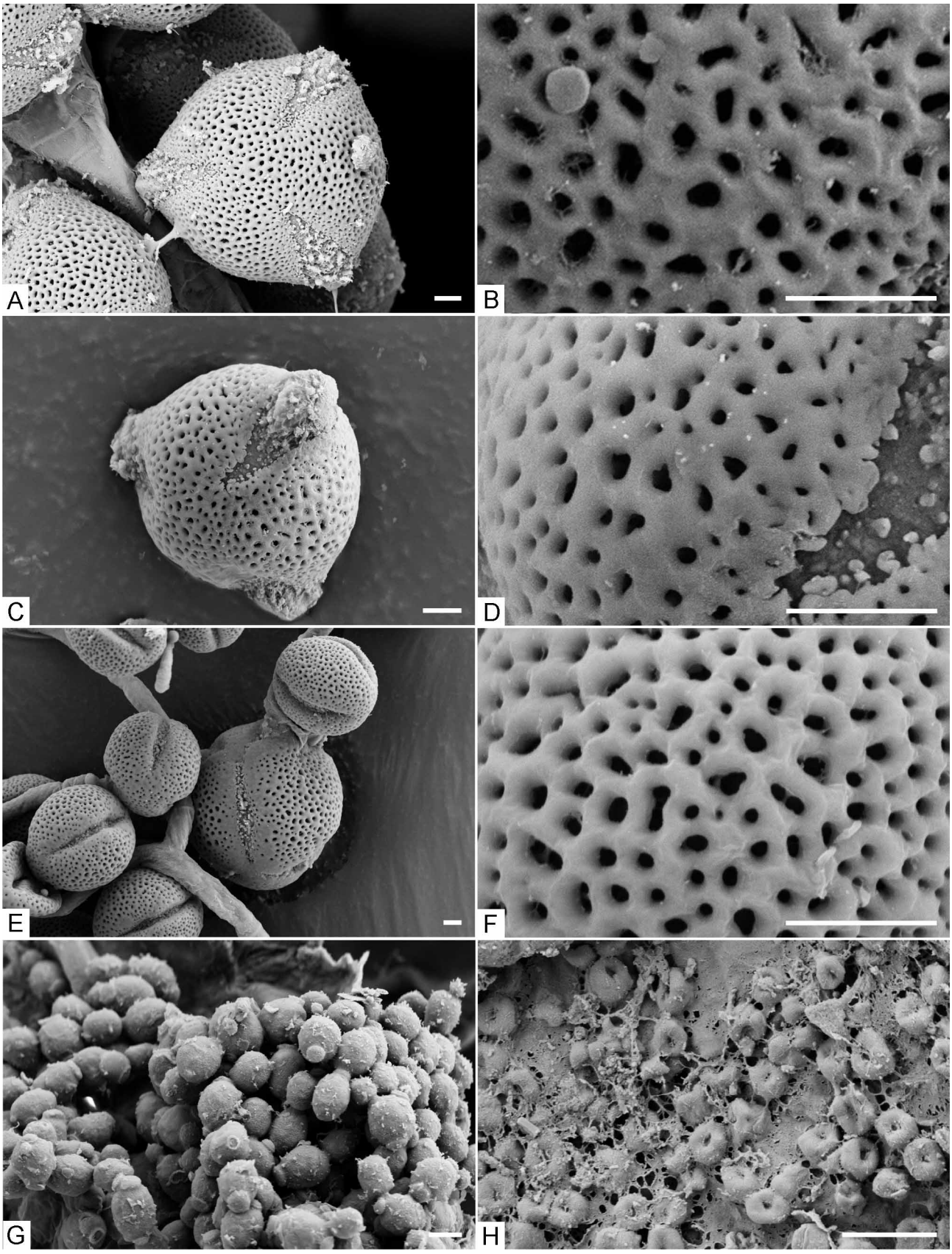

FIGURE 6: Scanning electron microscope images of pollen (A, C, E), details of the exine (B, D, F) and orbicules (G, H): A, B, G. *Oreocharis argyreia* (CH-66); C, D, H. *O. magnidens* (CH-23); E, F. *O.* × *heterandra* (CH-70). Bars: 2µm.

TABLE 5: Sizes of potentially viable pollen grains (stained in acetocarmine), fixed in the field (in Farmer's fluid) or from herbarium specimens of the three *Oreocharis* taxa analysed (*N*=14-40, means with standard errors in brackets).

taxon (identifier)	field-fixed	herbarium	**combined**	**% difference size/volume**
O. argyreia (CH-66)	19.9 (0.19)	19.2 (0.13)	**19.7 (0.14)**	**135 / 246**
O. magnidens (CH-23)	15.0 (0.11)	14.4 (0.06)	**14.6 (0.07)**	**100 / 100**
O. × *heterandra* (CH-69)	-[1]	21.6 (0.23)	21.6 (0.23)	148 / 325
O. × *heterandra* (CH-70)	22.4 (0.26)	21.0 (0.52)	22.0 (0.25)	151 / 345
O. × *heterandra* combined	22.4 (0.26)	21.5 (0.21)	**21.9 (0.17)**	**150 / 336**
all samples combined	*19.9 (0.30)*	*18.6 (0.32)*		

[1] – no field-fixed material available.

Pollen viability: Oreocharis argyreia (CH-66) – Freshly fixed samples: of the 48 pollen grains scored, 39 were stained (e.g. Fig. 7A) and 9 were not. From the anthers sampled from the herbarium specimen 140 grains were counted and only 2 of them were sterile, thus the percentage of potentially viable pollen was 98.6% (Table 6).

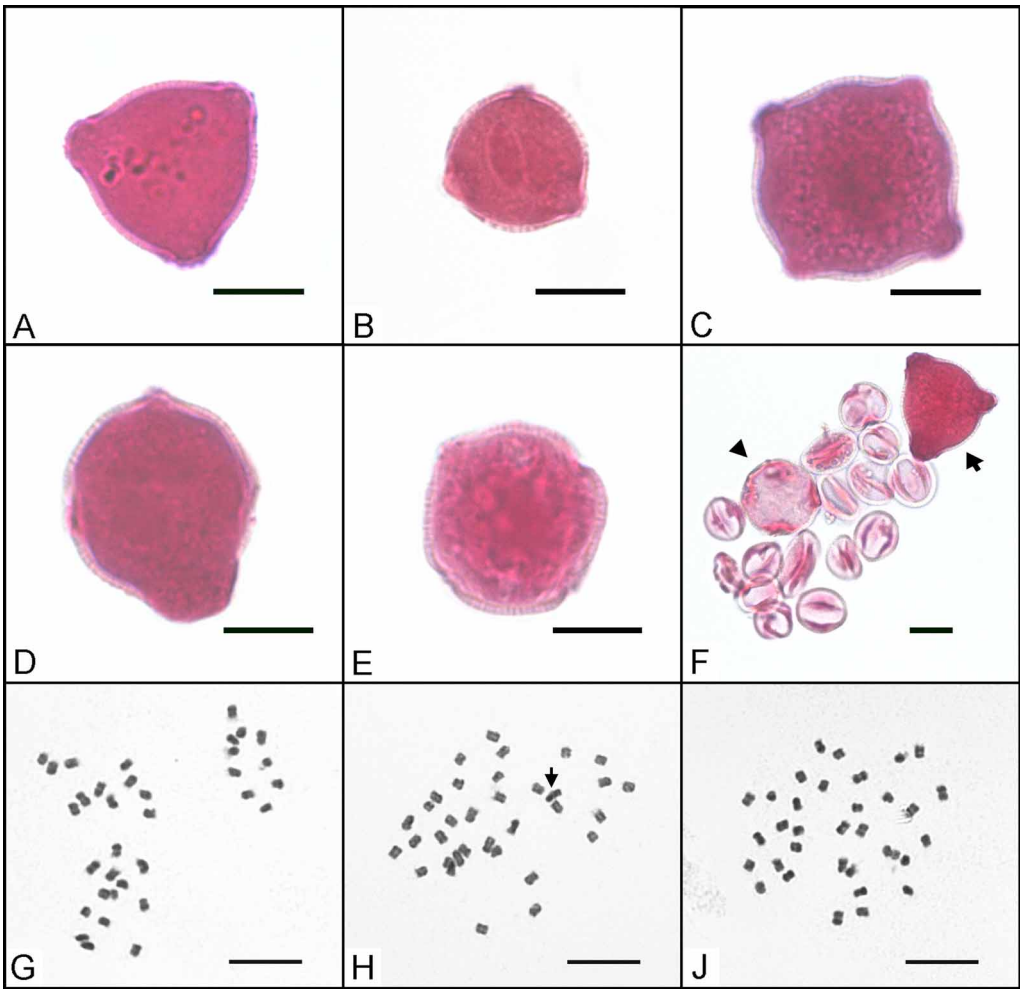

FIGURE 7: Pollen size (acetocarmine stained) and number of chromosomes (Feulgen stained): A. *Oreocharis argyreia* (CH-66), potentially viable pollen; B. *O. magnidens* (CH-23), potentially viable pollen; C. *O.* × *heterandra* (CH-69), stained pollen with four colpi; D. *O.* × *heterandra* (CH-69), stained pollen grains irregularly shaped; E. *O.* × *heterandra* (CH-69), stained pollen with granular cytoplasm; F. *O.* × *heterandra* (CH-70), stained tricolpate pollen (arrow), empty (arrowhead) and collapsed pollen. G. *O. argyreia* mitotic chromosome complement *2n*=34; H. *O. magnidens 2n*=34, arrow indicates two touching chromosomes; J. *O.* × *heterandra 2n*=34. Bars: 10µm.

Oreocharis magnidens (CH-23) – Both types of material contained prevalently viable pollen (e.g. Fig. 7B). In the freshly fixed anther, we found a viability of 98% and 96.1% for field-fixed and herbarium samples, respectively. As in *O. argyreia*, more pollen grains were recovered from anthers from the herbarium specimen (Table 6).

TABLE 6: Potential viability based on stainability in acetocarmine of pollen of samples of the three *Oreocharis* taxa analysed. Numbers are total pollen counts.

pollen source	field-fixed material				herbarium specimens				**combined**
taxon (identifier)	viable	sterile	sum	% viable	viable	sterile	sum	% viable	**% viable**
O. argyreia (CH-66)	39	9	48	81.3	138	2	140	98.6	**94.2**
O. magnidens (CH-23)	316	7	323	98.0	148	6	154	96.1	**97.3**
O. × *heterandra* (CH-69)	-[1]	-[1]	-[1]	-[1]	374	878	1252	29.9	29.9
O. × *heterandra* (CH-70)	45	712	757	5.9	20	387	407	4.9	5.6
O. × *heterandra* combined	45	712	757	5.9	394	1265	1659	23.7	**17.9**

[1] – no field-fixed material available.

Oreocharis heterandra (CH-69) – Pollen of this plant was available only from the herbarium specimen. The result of the count revealed that only 29.9% of the pollen grains were potentially viable (Table 6; Fig. 7C, D). Most were unstained and collapsed.

Oreocharis heterandra (CH-70) – Pollen from 70% ethanol preserved material was predominantly sterile (Fig. 7F). On average 5.6% of the pollen grains were stained by acetocarmine (Table 6). A large proportion of the stained ones, c. 18%, had a 'squareish' shape, remarkably different from the other, subspherical and tricolpate grains, due to the presence of four colpi (e.g. Fig. 7C).

Chromosome counts: A somatic count of *2n*=34 was determined for all three taxa (Fig. 7G–J). The chromosomes were between 1.6 and 2.3µm long and did not vary significantly between the parent species.

Seeds: Both species had a similar verruculose ornamentation of the seed testa cells (Fig. 8). The seeds of *O. argyreia* (CH-67) were 0.7 × 0.28mm in size, while those of *O. magnidens* (CH-69) were larger, with 1.1 × 0.4mm. No fully formed seeds were found in the capsules of *O. heterandra*.

Discussion

Natural hybridization is an important factor in plant evolution and is relatively common among vascular plants, but does not occur uniformly and is rather concentrated in certain families and genera (Ellstrand *et al.* 1996). This holds true for Gesneriaceae as well: the genus *Cyrtandra* in Hawaii would be an example (Ellstrand *et al*. 1996, Kiehn 2005), or the African genus *Streptocarpus* (Hilliard & Burtt 1971, deVilliers 2008), where numerous hybrids have been reported and species of hybrid origin described. In contrast, very few, if any, hybrids have been described in Chinese Gesneriaceae.

Here we have provided molecular and morphological evidence for a natural hybridisation scenario between two species of the recently redefined genus *Oreocharis* (Möller *et al*. 2011), both occurring sympatrically in the Shengtangshan Mountains, Dayao Shan, in Guangxi, China.

Molecular evidence

The molecular data showed that the two putative parent species differ greatly in both the ITS and *trn*L-F sequences, and that the three putative hybrid *Oreocharis heterandra* plants (including a plant found in

cultivation from wild collected seeds; see below) possess the additive nuclear ITS nucleotide signals of both its respective parents. Such additivity in these tandemly arranged multicopies has been reported previously for natural and artificial first or early generation hybrids in other plant families, such as Paeoniaceae [*Paeonia* Linnaeus (1753: 530), Sang *et al*. 1995], Asteraceae [× *Crepidiastrixeris* Kitamura (1937: 235), Saito *et al*. 2006; *Doellingeria*, Nees von Esenbeck (1833: 177), Saito *et al*. 2007], Begoniaceae [*Begonia* Linnaeus (1753: 1056), Peng & Chiang 2000, Peng & Ku 2009], and Gesneriaceae (*Streptocarpus*, Möller *et al*. 2004, Denduangboripant *et al*. 2007).

Additional molecular evidence for the hybrid origin of *O. heterandra* comes from the presence of both parental plastid sequences among the *O. heterandra* plants investigated. This is similar to the case of *Doellingeria* × *sekimotoi* (Makino) Nesom (1994: 457; Saito *et al*. 2007), but in contrast to *Begonia* × *taipeiensis* C.I.Peng in Peng & Sue (2000: 151) where the hybrid plants have only one parent chloroplast type, that of *B. formosana* (Hayata) Masamune (1961: pl. 41; Peng & Chiang 2000). The genetic make-up of the *Oreocharis* plants studied here with regard to both nuclear and plastid DNA sequences, is irrefutable evidence for a hybrid origin of *O. heterandra*.

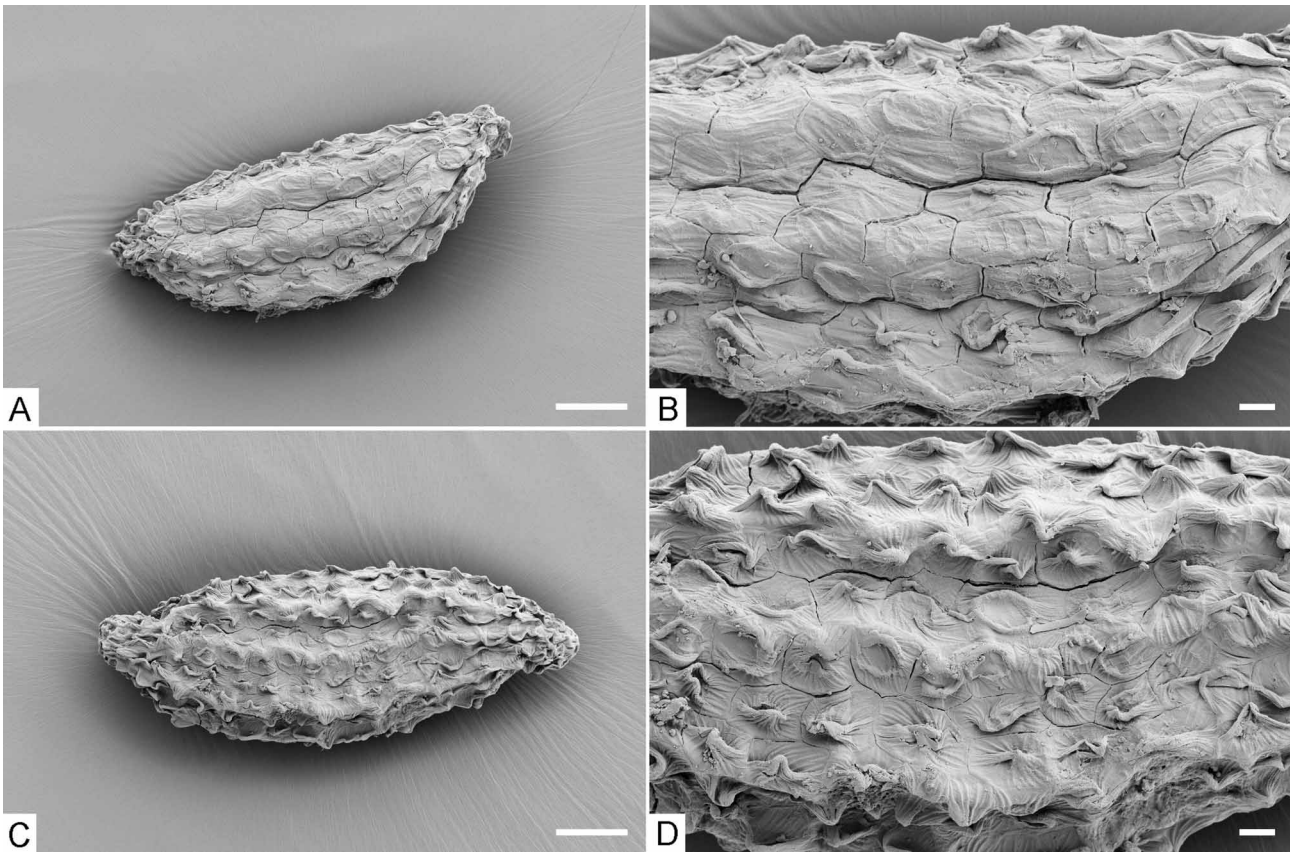

FIGURE 8: Scanning electron microscope images of seeds (A, C) and details of seed testa cells (B, D): A, B. *Oreocharis argyreia* (MMO 07-1131, Wuming, Guangxi, China); C, D. *O. magnidens* (CH-23). Bars: A, C, 100µm; B, D, 20µm.

Morphological evidence

Macro-morphological characters gave first indications of a possible hybrid status of *O. heterandra*. Our data suggest that the morphology of *O. heterandra* plants represents a predominant patchwork of characters of the two putative parents with little intermediacy (Table 4). It is interesting to note that the floral characters appear to follow a dominant / recessive inheritance, while the leaf characters appear to be intermediate between the parents. Closer inspection revealed that some micro-morphological leaf characters appear to be dominant (e.g. the indumentum on the lower leaf surface of *O. argyreia*) but most floral micro-morphological

characters appear to be inherited in a dominant / recessive fashion, with the exception of the disc. This appears not to be unique to *Oreocharis*, as has been observed in other Gesneriaceae hybrids, such as in *Streptocarpus* (Hughes *et al*. 2005) or in *Cyrtandra* (Kiehn 2005). In *Streptocarpus primulifolius* Gandoger (1919: 220) for example, all characters differentiating it from the very closely related species *S. rexii* Lindley (1828: pl. 1173; Hughes *et al*. 2005) are dominant in the first species (M.Möller, unpublished). Why such a strong dominant / recessive pattern for the floral characters would have evolved is not known, but may be linked to the expression of functional pollination syndromes.

Parentage of Oreocharis × heterandra

A recent molecular phylogenetic analysis including 43 samples, covering 40 species and one variety of a recently greatly enlarged genus *Oreocharis s. l.* (now >80 species) showed that *O. argyreia* and *O. magnidens* have unique ITS and *trn*L-F sequences (Möller *et al*. 2011). Since both parental *trn*L-F sequences and the combined ITS sequences were found in individual hybrid plants here, these *Oreocharis* species are unequivocally the parents of *O.* × *heterandra*.

Origin of the hybrid plants

Oreocharis argyreia has been collected widely across Guangxi (Wuming, Jinxiu, Jingxi, Mashan, Debao, Shangsi, Xiangzhou, Shanglin) and in Guangdong (Zhaoqing, Ruyuan), while *O. magnidens* is limited to Jinxiu and Xiangzhou in Guangxi, and Pingyuan and Ruyuan in Guangdong (Fig. 1). The two parent species occur together in three counties, in Jinxiu and Xiangzhou in Guangxi province and Ruyuan in Guangdong province. However, in Xiangzhou province they do not occur sympatrically. In Ruyuan County both occur in the Ruyuan Canyon, but are spatially separated with *O. magnidens* occupying habitats at the bottom of the canyon while *O. argyreia* grows at the top of the canyon. Only in Jinxiu, in the Shengtangshan Mountains in Guangxi, the two parents occur sympatrically in mixed populations and it is only here that the hybrid *O.* × *heterandra* is found.

From recent phylogenetic studies it is clear that this is most certainly a secondary contact zone; the two parent species are not closely related to each other; *O. magnidens* is, for example, closer allied to the very widespread *O. auricula* (S.Moore) Clarke (1883: 64; Möller *et al*. 2011). Our dating approaches indicated that gene flow between *O. argyreia* and *O. magnidens* ceased a long time ago (c. 6MY ago) at the end of the Miocene. Given the rarity of hybrid plants (we only found three individuals) compared to the thousands of parental plants of both species it is unlikely that they have coexisted in sympatry for such a long time without more occurrences of hybridisation or the formation of hybrid swarms.

Frequency and direction of hybridisation

As far as it is known from herbarium specimens, there have not been any collections, apart from our three specimens, since the type specimen of *O.* × *heterandra* was collected in 1981. This might suggest that these hybridisation events have a low frequency. However, among 20 plants grown from seed collected in the wild from an *O. magnidens* plant we found one hybrid plant in cultivation. This is significant in several ways; it suggests that hybridisation events might occur regularly (though the establishment success of the F_1 plants may be low), and that pollen mixtures are transferred. This hybrid plant also confirms a pollen transfer from an *O. argyreia* flower to an *O. magnidens* flower, and the presence of the *O. magnidens* chloroplast type in the hybrid (data not shown) confirms a maternal plastid inheritance in *Oreocharis*.

Among the three natural hybrid plants, we observed both cpDNA parental haplotypes, one from *O. magnidens* and two from *O. argyreia*. As shown above, the plastid inheritance in *Oreocharis* conforms to most angiosperms (Mogensen 1996) including the Gesneriaceae genus *Streptocarpus* (Möller *et al*. 2004), as being maternal, and this suggests that pollen-flow occurred in both directions. This points to a lax pollinator specificity. Nothing is known about the pollinators of *Oreocharis* species. The corolla mouth of *O. magnidens* flowers is narrow and constricted, allowing only minimal access with mouthparts, and the long corolla lobes provide a landing platform typical for butterfly- and moth-pollinated flowers. Furthermore, the flowers are

often resupinate (Fig. 3B, E) presenting the pollen variably either at the bottom or top of the flower opening. Flowers of *O. argyreia* have a wider opening that may allow either partial (head and long proboscis) or full access by pollinators. Long proboscid-pollinators are more likely, since these would be able to reach the nectar and pollinate flowers of both species. The presence of hybrids only in the locality where the two species co-exist closely, and their absence in localities with allopatric distributions of the parents (even if separated only by a short distance in Ruyuan), suggests that the pollinators do not disperse pollen very far and that they are locally active. On the other hand, because of their low frequency they may have been overlooked in other places, such as Ruyuan.

Persistence of the hybrid plants and lineages

The establishment of the F_1 generation is only the first step in a potential speciation process through hybridisation, and the reproductive success of this generation can make or break hybrid lineages and may determine the level of gene flow between the parental species in future generations (Barton & Hewitt 1985, Kirk *et al*. 2005). Often, hybrids fail to progress beyond the first generation due to a high incompatibility of the two parental genomes (e.g. Peng & Chiang 2000, Saito *et al*. 2007). In these cases the persistence of the 'species' as F_1 hybrid plants depends on recurrent hybridisation events of the parental species if these retain a contact or overlapping zone of distribution, which may not always be the case (e.g. *Paeonia*, Sang *et al*. 1995).

In the present study on *Oreocharis*, we appear to have found a case of recurring F_1 hybrids. The absence of a hybrid swarm in Jinxiu may suggest that even though hybridisation events may occur regularly, the frequency of hybrid plants is low (e.g. one among 20 plants in the cultivated material, although a single observation cannot be seen as representative) and the F_1 plants are unable to reproduce sexually. No seed set was observed in hybrid plants in the field or upon artificial self-pollination in the cultivated plant (M.Möller, pers. observ.). This is due to the high pollen sterility observed for the *O.* × *heterandra* plants (Table 6), which likely reflects a highly irregular meiosis between the two disparate parent genomes. This is not surprising given that they have ceased to exchange genes c. six million years ago, which would have allowed their genomes to diverge greatly. The pollen in *O. argyreia* is significantly larger than in *O. magnidens*, which could indicate chromatin differences, i.e. dysploid or polyploid variation, between the parental genomes. Although, different pollen sizes do not necessarily equate to differences in chromosome numbers as seen in the parents of *Begonia* × *chungii* Peng & Ku (2009: 241), both of which have *2n*=22 chromosomes, but possess greatly different pollen sizes [*B. palmata* Don (1825: 223) 16–23 × 9–13µm; *B. longifolia* Blume (1823: 102) 11–15 × 5–10µm] (Peng & Ku 2009). The same holds true for the parental species of *O.* × *heterandra* since both have been counted with *2n*=34 chromosomes. Since no apparent differences in chromosome sizes for the parents have been detected, chromosome arm rearrangements likely prevent meiosis to proceed normally, and result in a failure to produce functional pollen. This has been reported for example in the hybrid *Doellingeria* × *sekimotoi* where both parent species and hybrid plants have *2n*=18 chromosomes, but seemingly differ in their genome arrangement since one parent, *D. rugulosa* (Maxim.) Nesom (1994: 456) has four rDNA loci, while *D. scabra* (Thunb.) Nees von Esenbeck (1833: 183) has two (Saito *et al*. 2007).

Thus, genetic differences would very effectively isolate the two species reproductively, and prevent the establishment of consecutive generations. All further *O.* × *heterandra* plants must thus originate from recurrent hybridisation events to maintain the 'species'. This is similar to the situation found in × *Crepidiastrixeris* (Saito *et al*. 2006) and perhaps *Begonia* × *taipeiensis* (Peng & Chiang 2000).

Hybrids and taxonomy

Hybrids are frequently described and named in the taxonomic literature; this is nothing new or unusual. However, in the present case the hybrid was described as a species, *Oreocharis heterandra* D.Fang & D.H.Qin. It is interesting that the hybrid nature of *O.* × *heterandra* was not suspected earlier when it was

collected in 1981 or described in 1994 (Fang *et al*. 1994). Perhaps field notes on the distribution and abundance of the three taxa were incomplete. In its description by Fang *et al.* (1994), *O.* × *heterandra* plants were compared to *O. aurea*, although this species is not that similar to *O.* × *heterandra* with its yellow flowers and relatively short corolla lobes. Indeed *O.* × *heterandra* is more similar to *O. auricula* and even more so to *O. nemoralis* Chun (1946: 288) (see Wei *et al*. 2010).

In conclusion, in line with our results on the morphology and molecular data, *Oreocharis heterandra* D.Fang & D.H.Qin unequivocally represents a hybrid between *O. argyreia* and *O. magnidens* and this species is maintained by recurrent reciprocal hybridisation. Consequently, *Oreocharis heterandra* has to become *Oreocharis* × *heterandra* D.Fang & D.H.Qin.

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