

Cytological studies on the genus *Holcoglossum* (Orchidaceae)

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The karyotypes of ten species of *Holcoglossum* (Orchidaceae), a highly endangered and diversified genus from China, were investigated to study the infrageneric relationships, biogeography, and speciation patterns in the Hengduan Mountains. The karyotype formulae of the studied species are as follows: $2n = 38 = 20m + 18sm$ in *H. subulifolium*, $2n = 38 = 22m + 16sm$ in *H. amesianum*, $2n = 38 = 26m + 12sm$ (6 SAT) in *H. lingulatum*, $2n = 38 = 26m + 12sm$ in *H. wangii*, $2n = 38 = 10m + 28sm$ in *H. kimballianum*, $2n = 38 = 14m + 22sm + 2st$ in *H. flavescens*, $2n = 38 = 24m + 12sm + 2st$ in *H. rupestre*, $2n = 38 = 14m + 20sm + 4st$ in *H. sinicum*, $2n = 38 = 16m + 14sm + 8st$ in *H. weixiense*, and $2n = 76$ in *H. tsii*. The karyotypes of two tropical species, *H. amesianum* and *H. subulifolium*, are the most primitive in the genus, whereas those of four temperate alpine species, *H. sinicum*, *H. rupestre*, *H. weixiense*, and *H. flavescens*, are more advanced. *H. tsii* is a tetraploid and *H. rupestre* may be one of its ancestors. The low frequency of polyploidy in *Holcoglossum* in the Hengduan Mountains region supports the conclusion that chromosome stasis during rapid speciation is common there. © 2007 The Linnean Society of London. *Botanical Journal of the Linnean Society*, 2007, **154**, 283–288.

ADDITIONAL KEYWORDS: biogeography – chromosome stasis – Hengduan Mountains – karyotypes – tetraploid.

INTRODUCTION

The orchid genus *Holcoglossum* Schltr. comprises about 12 species extending from Thailand, Myanmar, Malaysia, and Vietnam to north-eastern Yunnan, with most species endemic in China (Tsi, 1982, 1999; Christenson, 1987, 1998a; Seidenfaden, 1988, 1992; Jin, 2005). It is one of the most endangered genera in China and south-eastern Asia (Averyanov & Averyanov, 2003), and some species, for example *H. rupestre* (Hand.-Maz.) Garay, *H. tsii* Yukawa, *H. omeiense* X.H. Jin and S.C. Chen, *H. weixiense* X.H. Jin and S.C. Chen, *H. lingulatum* (Aver.) Aver., and *H. wangii* Christenson, are very narrowly distributed. Although *Holcoglossum* is a small genus, it occupies an important position in the phylogeny of Aeridinae (Christenson, 1987; Seidenfaden, 1988), and displays an

interesting distribution pattern in Aeridinae by having two diversity centres (Jin, 2005). One centre, like those of most other genera in Aeridinae, is in the tropical border region between China, Vietnam, and Laos. The other is in the temperate alpine region comprising the Hengduan Mountains. This pattern has not been reported in other genera in Aeridinae.

The Aeridinae is a large subtribe which is important for horticulture, but there is little information available in the literature. Most existing information discusses intergeneric relationships, with few papers dealing with karyotype analysis. Shindo & Kamemoto (1963a) compared the karyotypes of 11 species in ten genera in Sarcanthinae, and concluded that karyotype analysis in this group helped to clarify its phylogenetic relationships. This conclusion was confirmed by Shindo & Kamemoto (1963b) and Tara & Kamemoto (1970). Polyploid species or populations have also been reported in Sarcanthinae (for example, Kamemoto, Sagarik & Kasemsap, 1964). The main purpose of this

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paper is to discuss the infrageneric relationships, biogeography, and speciation patterns of *Holcoglossum* in the Hengduan Mountains, based on classical cytological evidence.

MATERIAL AND METHODS

Accession data on each species are shown in Table 1, with voucher specimens and permanent slides deposited in the Herbaria of the Institute of Botany, Chinese Academy of Sciences (PE) and the Kunming Institute of Botany, Chinese Academy of Sciences (KUN). All cytological observations were made from root tips collected in the field or in a glasshouse. Fresh root tips about 0.2 cm in length were cut, pretreated in 0.002 M 8-hydroxyquinoline at 20 °C for 4 h, and then fixed with Carnoy (1 part glacial acetic acid to 3 parts absolute alcohol) at 4 °C for at least 2 h. They were rinsed in distilled water several times, placed in 70% ethanol for 10 min, macerated in a 1 : 1 mixture of 1 M HCl and 45% acetic acid at 60 °C for 1 min, and squashed and stained in 1% aceto-orcein. Permanent slides were made using the standard liquid nitrogen method. Observations were made on nuclei at the somatic mitotic metaphase, and measurements of chromosome arms were taken from at least three well-spread metaphases in three different root tips from each species. The designation of the centromeric position as median (m), submedian (sm), and subterminal (st) follows Levan, Fredga & Sandberg (1964). The terminology of karyotype symmetry follows Stebbins (1971) and the index of karyotype asymmetry follows Arano (1963). The types of nuclei in somatic cells follow Tanaka (1989).

RESULTS

The interphase nuclei in the somatic cells of the four species *H. subulifolium* (Rchb.f) Christenson, *H. ame-*

sianum (Rchb.f) Christenson, *H. rupestre*, and *H. tsii* are of the complex chromocentre type (Figs 1–4).

The chromosome numbers of *H. sinicum*, *H. rupestre*, *H. weixiense*, *H. wangii*, *H. flavescens* (Schltr.) Z.H. Tsi, *H. tsii*, *H. subulifolium*, and *H. lingulatum* are reported here for the first time. All have $2n = 38$, except *H. tsii*, which has $2n = 76$. The previously reported chromosome numbers of $2n = 38$ for *H. amesianum* and *H. kimballianum* (Rchb.f) Garay are confirmed here. The karyotype formulae of the studied species are as follows: $2n = 38 = 20m + 18sm$ in *H. subulifolium*, $2n = 38 = 22m + 16sm$ in *H. amesianum*, $2n = 38 = 26m + 12sm$ (6 SAT) in *H. lingulatum*, $2n = 38 = 26m + 12sm$ in *H. wangii*, $2n = 38 = 10m + 28sm$ in *H. kimballianum*, $2n = 38 = 14m + 22sm + 2st$ in *H. flavescens*, $2n = 38 = 24m + 12sm + 2st$ in *H. rupestre*, $2n = 38 = 14m + 20sm + 4st$ in *H. sinicum*, and $2n = 38 = 16m + 14sm + 8st$ in *H. weixiense*. The karyotype of the tetraploid *H. tsii* was not determined (Figs 5–14). *H. lingulatum* has three pairs of satellite chromosomes. No aneuploid was found in these species.

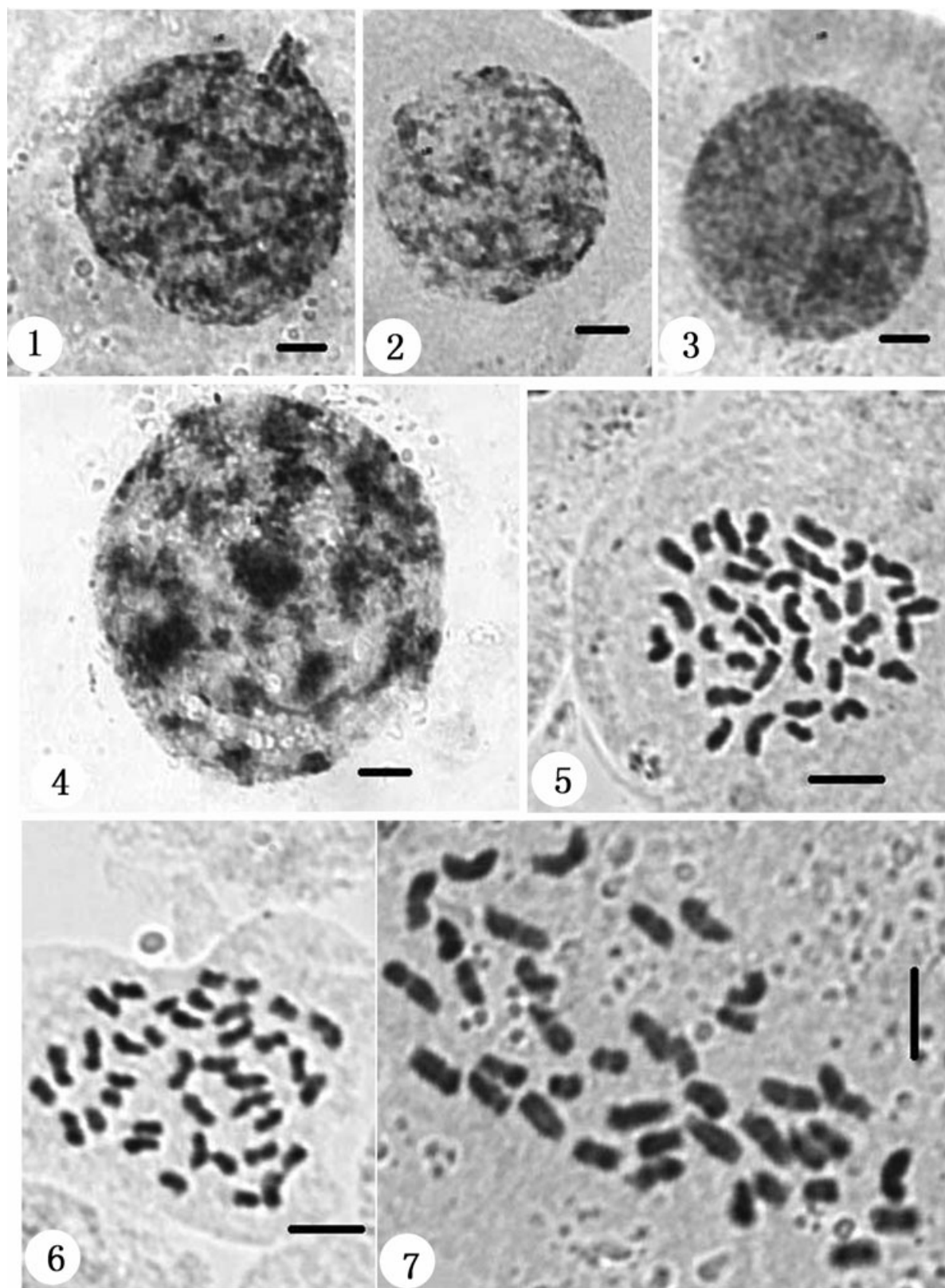
The karyotype structures were analysed and the results are shown in Table 2. The karyotype of each species is highly symmetrical and with a large proportion of metacentric and submetacentric chromosomes, especially in *H. amesianum* and *H. subulifolium*. According to the classification of Stebbins (1971), the karyotypes of most species belong to type 2A or 2B.

DISCUSSION

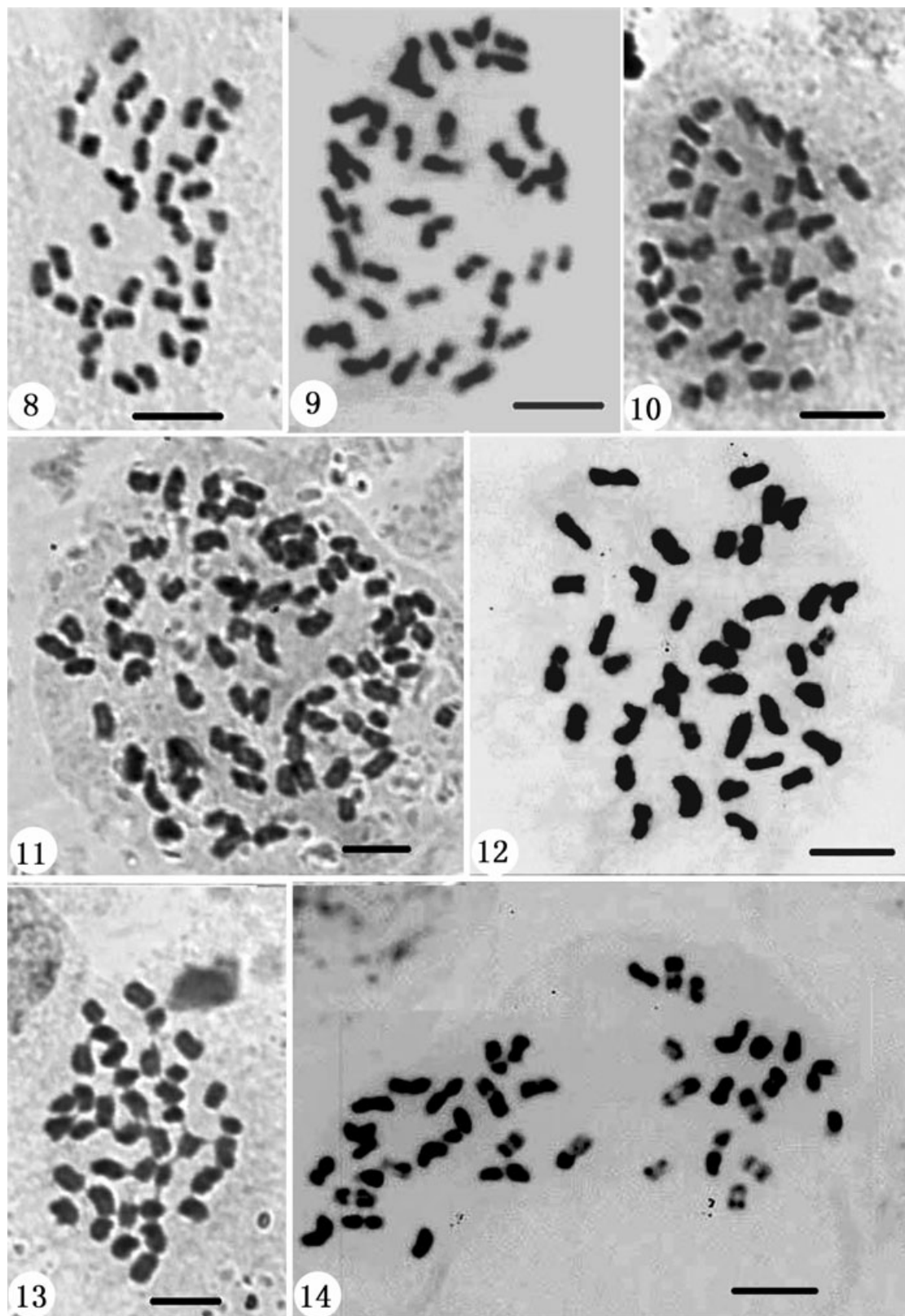
Holcoglossum is a diverse genus and Seidenfaden (1988) suggested that a new genus was required to contain some species. However, the karyotypes of the species investigated here, representing two different infrageneric systems (Christenson, 1987, 1998a; Jin, 2005), are very similar, and no distinct differences in asymmetry index were discovered, supporting the

Table 1. Sources of material of *Holcoglossum* studied (all voucher specimens kept in PE)

Species	Locality	Voucher
<i>H. wangii</i>	Yunnan, Malipo	HK Kadoorie PT 3353
<i>H. sinicum</i>	Yunnan, Bingchuan	HK Kadoorie PT 1042
<i>H. flavescens</i>	Yunnan, Bingchuan	HK Kadoorie PT 1041
<i>H. weixiense</i>	Yunnan, Weixi	HK Kadoorie PT 3490
<i>H. amesianum</i>	Yunnan, Menghai	HK Kadoorie PT 1051
<i>H. rupestre</i>	Yunnan, Zhongdian	HK Kadoorie PT 3496
<i>H. kimballianum</i>	Yunnan, Simao	HK Kadoorie PT 658
<i>H. subulifolium</i>	Hainan, Ledong	HK Kadoorie PT 3053
<i>H. tsii</i>	Yunnan, Yuanmou	XH Jin 6915
<i>H. lingulatum</i>	Yunnan, Malipo	HK Kadoorie PT 3333



Figures 1–7. Interphase nuclei and metaphase chromosomes of *Holcoglossum* species. Figs 1–4. Interphase nuclei. Fig. 1. *H. subulifolium*. Fig. 2. *H. amesianum*. Fig. 3. *H. rupestre*. Fig. 4. *H. tsii*. Figs 5–7. Metaphase chromosomes. Fig. 5. *H. amesianum* ($2n = 38$). Fig. 6. *H. subulifolium* ($2n = 38$). Fig. 7. *H. lingulatum* ($2n = 38$). Scale bars, 5 μm .



Figures 8–14. Metaphase chromosomes of *Holcoglossum* species. Fig. 8. *H. kimballianum* ($2n = 38$). Fig. 9. *H. wangii* ($2n = 38$). Fig. 10. *H. rupestre* ($2n = 38$). Fig. 11. *H. tsii* ($2n = 76$). Fig. 12. *H. flavescens* ($2n = 38$). Fig. 13. *H. weixiense* ($2n = 38$). Fig. 14. *H. sinicum* ($2n = 38$). Scale bars, 5 μ m.

Table 2. Karyomorphological parameters of species of *Holcoglossum*

Species	Chromosome number (2n) and karyotype	Asymmetry		
		T	A1	A2
<i>H. subulifolium</i>	38 = 26m + 12sm	2A	1.87	0.13
<i>H. amesianum</i>	38 = 20m + 18sm	2A	1.78	0.21
<i>H. wangii</i>	38 = 26m + 12sm	2B	2.28	0.18
<i>H. kimballianum</i>	38 = 10m + 28sm	2A	1.74	0.45
<i>H. lingulatum</i>	38 = 26m + 12sm	2A	1.92	0.24
<i>H. sinicum</i>	38 = 14m + 20sm + 4st	2B	2.36	0.34
<i>H. rupestre</i>	38 = 24m + 12sm + 2st	2B	2.13	0.16
<i>H. flavescens</i>	38 = 14m + 22sm + 2st	2A	1.65	0.34
<i>H. weixiense</i>	38 = 16m + 14sm + 8st	3A	1.92	0.53
<i>H. tsii</i>	76, karyotype not analysed			

A1, ratio between the longest and shortest chromosome; A2, percentage of arm ratio (long/short, L/S) larger than two; T, karyotype.

Table 3. Plant form and floral characters of *Holcoglossum tsii*, *H. rupestre*, and *H. flavescens*

	<i>H. tsii</i>	<i>H. rupestre</i>	<i>H. flavescens</i>
Plant form	Erect	Erect	Slanting
Leaves	c. 20–30 cm long	c. 20–30 cm long	Less than 8 cm long
Dorsal sepal	Entire, 21 × 13 mm	Entire, 10–12 × 6–7 mm	Entire, 9–12 × 4.5–6 mm
Lateral sepals	Entire, 21 × 14.5 mm	Entire, 10–12 × 7 mm	Entire, 9–12 × 4.5–6 mm
Petals	Entire, 20 × 12 mm	Entire, 10–12 × 6–7 mm	Entire, 9–12 × 4–5.5 mm
Middle lobe	Orbicular, 19 × 1.5 mm	Orbicular, 9–12 × 8–11 mm	Rhombic, 6–8 × 7 mm
Spur	Funnel-shaped, 23 mm	Funnel-shaped, 8–12 mm	Conical, 7 mm
Scent	Buttered tea	Buttered tea	None

retention of two tropical species, *H. amesianum* and *H. subulifolium*, in *Holcoglossum*.

The karyotypes of most of the species in two diversity centres of *Holcoglossum* have been investigated here. The asymmetry indices indicate that the karyotypes of two tropical species, *H. amesianum* and *H. subulifolium*, are the most primitive in *Holcoglossum*, whereas those of four temperate alpine species are more advanced, all having subterminal chromosomes. At the same time, *H. amesianum* and *H. subulifolium* share many floral characters with *Vanda*, for example, ridged calli, saccate spur, and near-rectangular stipe. These observations indicate that these two species may represent a transitional state between *Vanda* and *Holcoglossum*.

Of the ten investigated species, all except *H. tsii* are diploid with $2n = 38$, which is the chromosome number typical of Aeridinae in Vandeae. Christenson (1998b) considered *H. tsii* (now known to be tetraploid with $2n = 76$) as *H. rupestre*, whereas Yukawa (2000) treated it as a distinct species. Our observations show that these two species are very similar in plant form

and the shape of the floral parts, but *H. tsii* displays typical 'gigas' characteristics, with flowers nearly double the size of those of *H. rupestre* (Table 3). These two species can be separated from other species of *Holcoglossum* by having an erect plant form, orbicular middle lobe, and scented flowers. Although they are allopatric, they grow by different parts of the Yangtze River. These observations suggest that they may have very close affinity, with *H. rupestre* being one of the ancestors of *H. tsii*, or even with *H. tsii* being an autopolyploid of *H. rupestre*. However, this remains to be tested.

Recently, increasing evidence has shown that chromosome stasis is one of the features of rapid speciation of many endemic taxa on islands (Stuessy & Crawford, 1998). The investigation of chromosome numbers in several archipelagos, such as Hawaii and the Galapagos, has shown that very little change in chromosome number occurs during the evolution of endemic taxa, even though these species can have great morphological diversity. Nie *et al.* (2005) drew a similar conclusion that polyploidy may have played only a minor role

in the evolutionary diversification in the Hengduan Mountains, one of the biodiversity hotspots of the world. Our work on *Holcoglossum* supports this finding, with evidence from an endemic orchid species in this region.

Although the Hengduan Mountains mark the northern distribution limit of Aeridinae, there are at least four species of *Holcoglossum* (*H. flavescens*, *H. rupestre*, *H. sinicum*, and *H. weixiense*) distributed there at elevations from 2000 to 3300 m, and the last three are restricted to this area. These four species replace each other from west to east, but differ in growth habit and ecological environment. *H. weixiense* is pendulous and grows in *Quercus* forest along the Mekong River. *H. rupestre* is erect, growing at elevations from 2000 to 2200 m in mixed *Quercus* and *Pinus* forest along the hot, dry valley of the Upper Yangtze River. *H. sinicum* is again pendulous and grows in *Quercus* and *Rhododendron* forest between 2600 and 3600 m in the Cangshan Mountains. *H. flavescens* has a slanting habit and grows in *Quercus* forest between 2000 and 2400 m along the valley of the Middle Yangtze River. They form a natural subunit of *Holcoglossum*, together with *H. tsii*, sharing many floral character states (Jin, 2005). They have karyotypes that are more similar to each other than those of other species of *Holcoglossum*. Wide divergences in plant habit and ecological environment, but similarities in floral morphology and karyotype, indicate that these species may be the result of adaptive radiation during the uplift of the Qinghai-Xizang (Tibet) Plateau, rather than immigration from other regions.

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