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A new species and new section of *Viola* (Violaceae) from Guangdong, China

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Abstract

Viola hybanthoides W. B. Liao et Q. Fan (Violaceae), a new species from Mount Danxia of Guangdong province in China, is described and illustrated. The most distinctive characters of *Viola hybanthoides* are the subshrub habit, the anterior petal with a long stalk-like claw (up to 3.5 mm, ca. 1/3 of the petal's length), and the very short upper petals (only 2.5–3 mm long, less than 1/2 of the lateral petals' length), all of which are characters shared with some *Hybanthus* species. These characters of this species are distinguishable from all other *Viola* species, which are usually herbs, and have the anterior petal without a long stalk-like claw and much longer upper petals (usually more than 8 mm long). Based on the new species, a new *Viola* section *Danxiaviola* W. B. Liao et Q. Fan is described. To distinguish *Viola* sections in China, an identification key is provided.

Key words: *Viola hybanthoides*, section *Danxiaviola*, Violaceae, new species, new section

Introduction

Mount Danxia is famous for its Danxia landform, a term that refers to various landscapes formed from red-colored sandstones and conglomerates, largely of Cretaceous age (http://www.worldheritagesite.org/sites/danxia.html). Because the entire hilly area looks like a red stone sculpture park, Mt. Danxia is also known as Red Stone Park of China (Peng 2001), and has now become a national park and a natural World Heritage Site. Since 2004, we have conducted continuous biological surveys on Mt. Danxia in order to elucidate the biodiversity patterns of Danxia landforms (Peng *et al.* 2011). During the expeditions, a distinct new *Viola* species, *Viola hybanthoides* W. B. Liao et Q. Fan, was collected on Mt. Danxia.

Viola L. is the largest genus of Violaceae, with approximately 525–600 species and an extensive north-temperate distribution (Ballard *et al.* 1999). Becker (1925) recognized 14 sections, 28 subsections, and 7 series based on ultrastructure of the pistil (reviewed in Yoo & Jang 2010). After that, many researchers made taxonomic revisions to Becker's classification. However, the infrageneric classification still remains controversial today (reviewed in Ballard *et al.* 1999, Yoo & Jang 2010; Fig. 1 in Ballard *et al.* 1999). There are 96–111 *Viola* species in China which belong to five sections according to Becker's classification (or five subgenera according to Yuzepchuk and Klokov's (1949) classification), i.e., *Erpetion*, *Melanium*, *Chamaemelanium*, *Dischidium* and *Nomimium.* Among them, *Nomimium* is the largest section, with 78–95 species in China (Wang 1991, Chen *et al.* 2007).

2. Materials and Methods

2.1 Molecular Methods

Leaf materials of the putative new species, *Viola hybanthoides*, were collected from three randomly selected adult individuals on Mt. Danxia in May 2012. All the leaves for DNA extraction were stored with silica gel in zip-lock plastic bags until use. A voucher specimen (*Q. Fan 11605*) is deposited in the Herbarium (SYS) of Sun Yat-sen University.

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Total genomic DNA was isolated from leaves using the modified CTAB method (Doyle & Doyle 1987).

The entire ITS-1, 5.8S, and ITS-2 region, and three chloroplast regions, *mat*K, *rpl*16, and the *atp*B–*rbc*L intergenic spacer, were amplfied using the primers by White *et al.* (1990), Johnson & Soltis (1994), Chanderbali *et al.* (2001), and Setoguchi *et al.* (1997), respectively. PCR reactions were conducted using the protocols of Fan *et al.* (2014). The PCR products were then sequenced on an ABI 3730 DNA sequencer (Applied Biosystems, Invitrogen, Foster City, CA, USA). The ITS and the three chloroplast sequences (*mat*K, *rpl*16 and *atp*B–*rbc*L) of *V. hybanthoides* were submitted to Genbank with accession numbers KF011244, KF011245, KF011246, and KF011247. For phylogenetic analysis, 95 additional ITS sequences and 33 sequences for each chloroplast region were downloaded from GenBank. The taxa sampled and corresponding GenBank accession numbers are listed in the Appendix. The ITS sequences and the combined chloroplast sequences were aligned by Clustal X (Thompson *et al.* 1997). Manual alignment was subsequently carried out with Bioedit (Hall 1999).

Using MrModeltest 2.2 (Nylander 2004), the GTR+I+G and GTR+G models were selected as the best models for ITS sequences and the GTR+G model was best for the combined chloroplast sequences. Phylogenetic analyses based on Bayesian inference were carried out using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001). Bayesian analyse was run with four chains for 10,000 generations with the first 25% of sampled trees discarded as burn-in.

2.2 Pollen morphology

Pollen samples were affixed to specimen tabs with double-sided tape and sputter-coated with gold-palladium. Observations were conducted using a JSM-6330F (JEOL Ltd., Tokyo, Japan) scanning electron microscope (SEM) with 10 KV at Sun Yat-sen University, Guangzhou, China. Pollen terminology follows Punt *et al.* (2007). A pollen voucher specimen (*Q. Fan 11605*) is deposited in the Herbarium of Sun Yat-sen University.

2.3 Chromosome number

Seven living individuals were collected from Mt. Danxia and cultivated in the greenhouse of our lab in May 2012. After two months, roots tips were sampled and pretreated with 0.002mol/L 8-Hydroxyquinoline for about 4.5 hours before being fixed in Carnoy Ⅰ (glacial acetic acid : absolute ethanol = 1 : 3), then macerated in a 1: 1 mixture of 45% acetic acid and 1 mol/L HCl at 60 ℃ for 15 minutes, stained and squashed in carbol fuchsin.

3. Results

3.1 Molecular Phylogenetics

The sequenced ITS region of *V. hybanthoides* was 655 bp with a GC content of 57.7%, and no variations were found among the three individuals. A total of 96 ITS sequences, including three outgroups, were aligned, resulting in an alignment length of 695 bp. The 50% majority-rule consensus tree based on Bayesian analysis of the ITS sequences showed that the *Viola* species were divided into two well-supported subclades (bootstrap support 98%). Among them, sections *Chamaemelanium* and *Dischidium* clustered first, and *V. hybanthoides* was added with strong bootstrap support (81%), then together with one member of section *Nomimium* (*V. mucronulifera*), they formed a distinct cluster with a bootstrap support of 60% (Fig. 1) .

The combined chloroplast sequences of *V. hybanthoides* is 1907 bp with a GC content of 28.3%, and no variations were found among the three individuals. A total of 34 combined chloroplast sequences, including one outgroup, were aligned, resulting in an alignment length of 2114 bp. The 50% majority-rule consensus tree based on Bayesian analysis of the combined chloroplast sequences showed that the section *Nomimium* is divided into two well-supported clusters (bootstrap support 100% and 92% respectively), while *V. hybanthoides* is clustered with one of *Nomimium* clusters, yet with a low bootstrap support (50%) (Fig. 2).

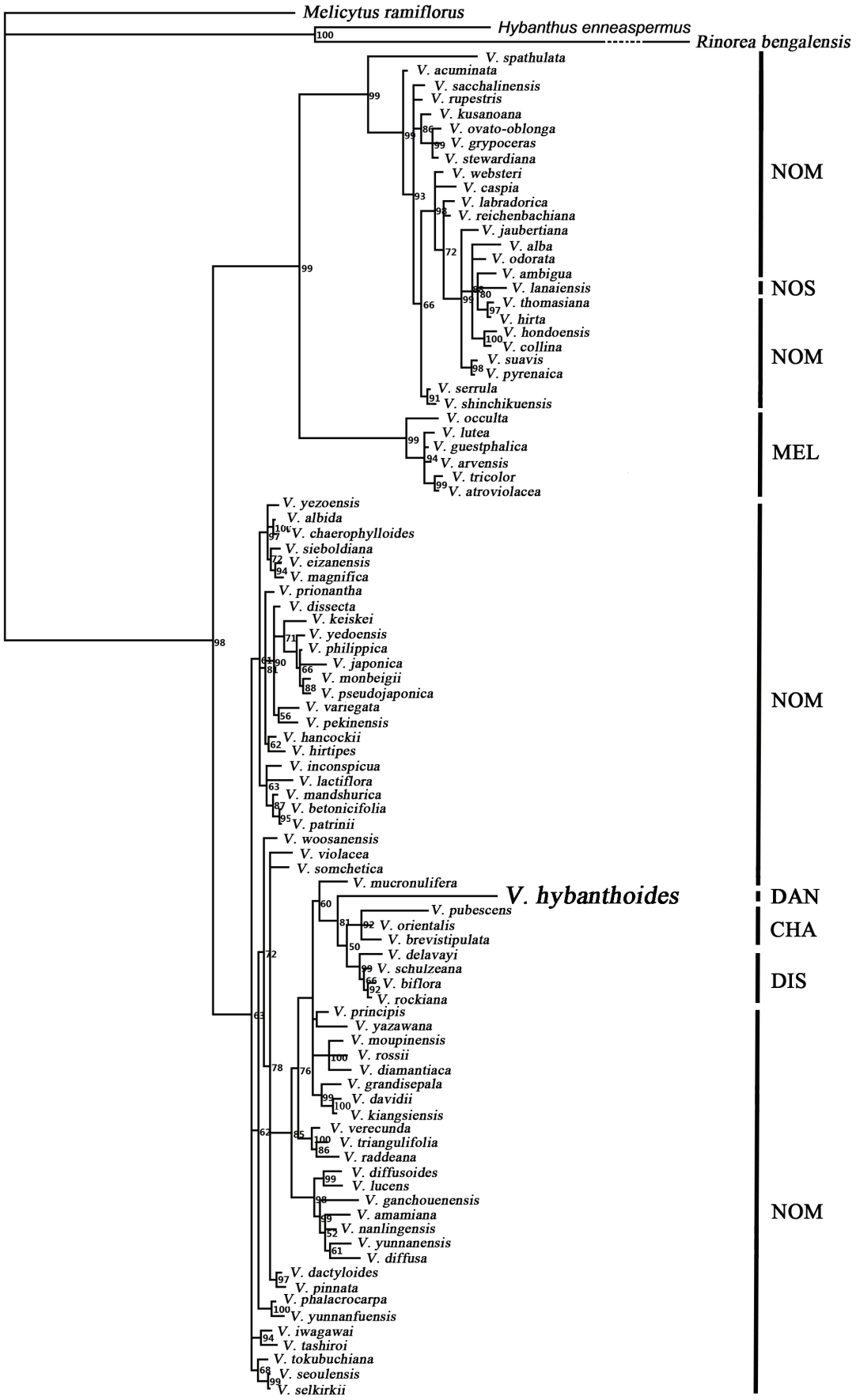

FIGURE 1. Strict consensus tree derived from ITS sequence with bayesian posterior probabilities (>50) in the nodes. outgroups: *Melicytus ramiflorus*, *Hybanthus enneaspermus* and *Rinorea bengalensis*. The species in bold face is the new one described in this study. Acronyms at the right are first three letters of sections *Chamaemelanium*, *Dischidium*, *Melanium*, *Nomimium* and *Danxiaviola*.

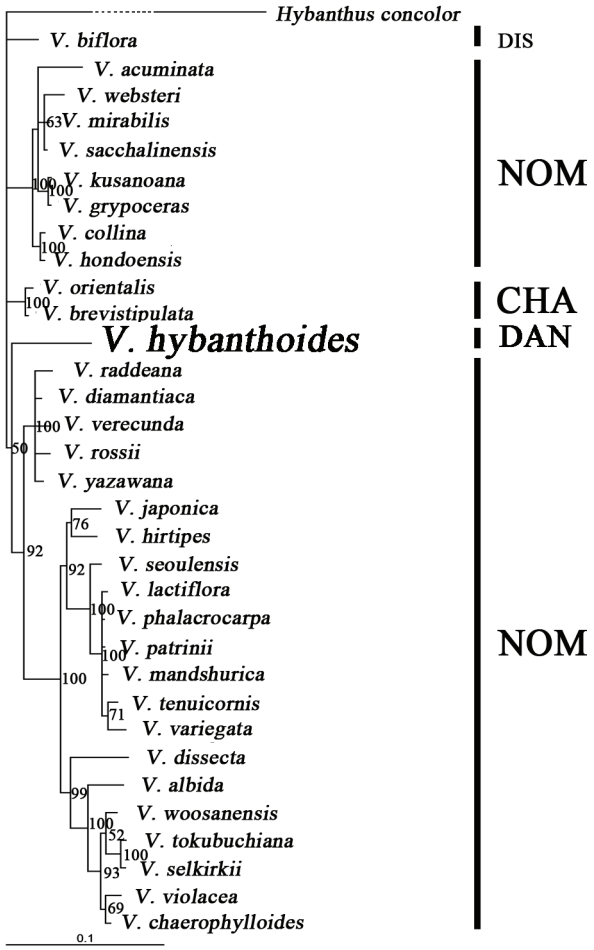

FIGURE 2. Strict consensus tree derived from a combined analysis of *mat*K, *rpl*16 and *atp*B-*rbc*L, with bayesian posterior probabilities (>50) in the nodes. Outgroup: *Hybanthus concolor*. The species in bold face is the new one described in this study. acronyms at the right are first three letters of sections *Chamaemelanium*, *Dischidium*, *Nomimium* and *Danxiaviola*.

3.2 Pollen Morphology

The pollen grains of *Viola hybanthoides* are tricolpate. Polar axis (P) = 37.77 ± 5.08 um, equatorial axils (E) = 24.86 ± 5.90 um (n = 20). The exine is almost smooth, dotted with small punctures (Fig. 3).

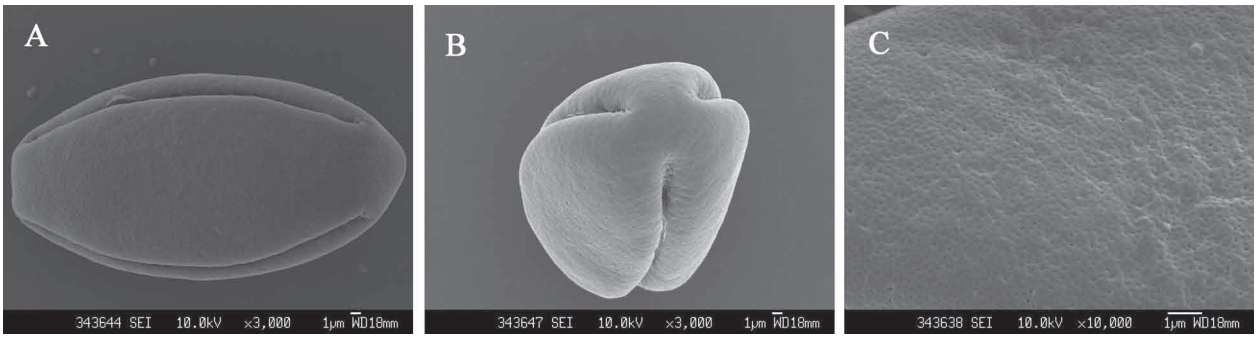

FIGURE 3. SEM photomicrographs of pollen grain of *Viola hybanthoides*. A. Equatorial view. **B**. Pollar view. **C**. Exine sculpture.

3.3 Chromosome Number

Seven individuals of the new species were studied. The metaphase chromosomes were counted to be $2n = 20$ in all individuals (Fig. 4). The chromosomes showed a gradation in length from the longest to the shortest, with no evidence of bimodality. The chromosomes were so tiny (less than 2.84 μm) that the karyotype analysis was difficult to do.

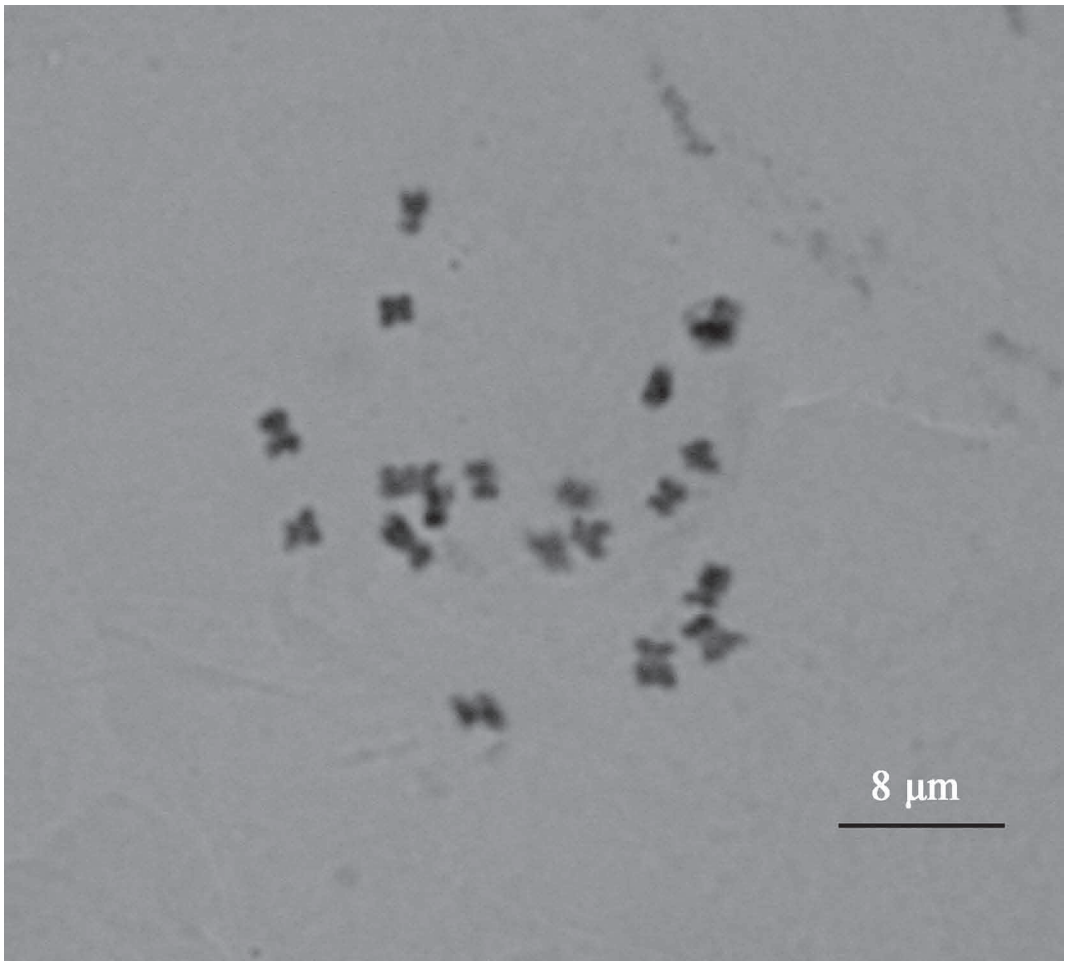

FIGURE 4. Somatic chromosomes at mitotic metaphase of *Viola hybanthoides* (2*n* = 20).

3.4 Taxonomic Treatment

Viola* section *Danxiaviola* W. B. Liao et Q. Fan, *sect. nov.

Type:—*Viola hybanthoides* W. B. Liao et Q. Fan

Subfrutex perennis. Rhizoma praesente vel abscente. Caulis aeria evoluta. Sepala subaequalia, basi producta auriculata. Petalum inferius maximum, ungue elongato, basi saccato. Stylus clavatus inferne curvatus, stigmate non antice rostro, lamellato processu infra cavum stigmatis.

Subshrubs perennial. Rhizomes present or absent. Aerial stems developed. Sepals subaequalia, basally auriculate. Petals unequal, upper ones smallest, anterior one biggest, with a narrow and long claw and short saccate spur at base. Style clavate, curved at base; stigma capitate, bifid slightly, not beaked and with a stigmatic hole in front and with a lamellar processus below the hole.

Etymology:—We named this new section "*Danxiaviola*" because the type species is endemic to Mt. Danxia.

This new section contains only one species. To distinguish the sections of *Viola* in China, an identification key is provided (based on Chen *et al*. 2007).

Key to Viola sect. Danxiaviola and related sections in China

1. Subshrubs; anterior petal with a long stalk-like claw; upper petals smallest, less than 1/2 of lateral petals' length..... *Viola* sect. *Danxiaviola*
- Herbs; anterior petal without a long stalk-like claw; upper petals smallest or not, usually more than 1/2 of lateral petals' length .....2.
2. Anterior petal shallowly saccate but not spurred; stipules free and small; petals white to violet..... *V.* sect. *Erpetion*
- Anterior petal spurred; stipules adnate or free; petals violet, purple, rose, yellow, or white.....3.

3. Stipules large, free, leaflike, deeply lobed; anterior petal broadly cuneate, with aerial stem.....*V.* sect. *Melanium*
- Stipules small, entire or fimbriate; anterior petal narrowly ovate to linear-oblong, with or without aerial stem.....4.
4. Petals purple or white, never yellow; style beaked at apex; stipules adnate or free; plant with or without aerial stem ..... *V.* sect. *Nomimium*
- Petals yellow, never purple; style not beaked at apex; stipules free; plant with aerial stem .....5.
5. Lateral petals and stigmas beardless; stigmas bilobed; spur short or long .....*V.* sect. *Dischidium*
- Lateral petals and stigmas bearded; stigmas capitate; spur very short .....*V.* sect. *Chamaemelanium*

Viola hybanthoides W. B. Liao et Q. Fan, *sp. nov.* (Fig. 5, 6)

Species Violae tricolori Linn. similis, sed subfruticibus, *floribus minoribus, 8–11 mm diam., petalis inferioribus unguibus, stigmatibus leviter bifidis differt.*

TYPE:—CHINA. Guangdong: Renhua County, Mt. Danxia, on the top of a small hill, 197 m, 25°01′05.92″N, 113°44′41.23″E, 23 May 2012, *Q. Fan 11605* (holotype SYS!, barcoding number 190591; isotype IBSC!) .

Subshrubs, perennial, without basal leaves. Rhizomes erect, rather stout, sometimes absent. Stems erect or somewhat ascending, much-branched, 20–45 cm tall, glabrous. Leaves alternate; stipules leaflike, free, glabrous, oblong-lanceolate, 5–9 × 1–2 mm acuminate, fimbriate; petioles glabrous, 0.6–2.1 cm long, narrowly decurrent-alate; blades elliptic or ovate-lanceolate, 1.8–2.8 × 0.8–1.4 cm, thinly chartaceous or membranous, glabrous, densely dotlike brown glandular on both sides, 2 to 3 veins on each side of midrib, margin coarsely serrate, base cuneate-decurrent. Flowers 8–11 mm diam; pedicels slender, 1.6–4.2 cm long, exceeding leaves, glabrous, with two opposite bracteoles above middle or near flower; bracteoles glabrous, linear, 1.5–2.2 × 0.3–0.6 mm, margin scarious, apex acuminate. Sepals green, glabrous, linear-lanceolate, 2.7–3.7 × 0.5–1 mm, margin entire, apex acuminate, base truncate or rounded, sepal appendages very short, c. 0.3 mm long. Petals whitish to pale purple, with apparent violet lines, anterior one with a green patch at base, glabrous; upper petals, oblong to linear-lanceolate, 2.5–3 × 0.5–0.8 mm, margin erose basally, apex obtuse or erose; lateral petals, oblong, 4.5–5 × c. 1.5 mm, margin erose basally, apex obtuse or erose; anterior petal with a long claw and a short saccate spur at base, broadly spathulate or flabellate, margin slightly undulate, blade 5–6 × 7–8 mm, claw 3–3.5 mm long (spur included), margin reflexed, apex rounded, truncate or emarginate, spur straight and saccate, very short, c. 1.5 mm, apex obtuse. Stamens 5, unequal, with very short filaments, the anther thecae c. 0.6 mm long, terminal appendages c. 0.5 mm long, the posterior appendages (nectar spurs) of two anterior stamens 0.7–1 mm long. Ovary ovoid to ellipsoid, c. 0.8 mm diam., glabrous; style c. 0.8 mm long, conspicuous geniculate at base; stigma capitate, slightly 2-lobed, not beaked in front, with a stigmatic hole between the lobes and with a membranaceous lamellar processus below the hole. Capsule brownish at maturity, ovoid, 3–4 mm long. Seeds brown, ovoid, 1.2–1.5 mm long.

Phenology:—Flowers from March to July and fruits from April to August (observation by Zaixiong Chen in the field from March, 2012 to December, 2013).

Distribution and Habitat:—*Viola hybanthoides* is currently known only from two localities in on Mt. Danxia, Renhua County, northern Guangdong, China. The species grows in sunny thickets on dry red-colored sandy soil at the top of a small hill and on a steep slope near the summit. The associated species include *Osteomeles subrotunda*, *Firmiana danxiaensis*, *Lagerstroemia indica*, *Rhamnus brachypoda* and *Pinus massoniana*.

Conservation Status:—Only two populations were found with no more than 1000 mature individuals in an total area of about 1 km^2. It's about 10 km away between the two populations. Thus the species could be considered as CR (Critically Endangered) status according to IUCN Red List criteria (B2a; IUCN 2001).

Etymology:—The anterior petal with a long stalk-like claw and the inconspicuous upper petals of the new species are very similar to those of some *Hybanthus* species, therefore we used the specific epithet "*hybanthoides*".

Paratype: CHINA. Guangdong: Renhua County, Bazai in Mt. Danxia, on the sunny slope, 400 m, 25°00′29.03″N, 113°39′41.82″E, 26 April 2013, Z. X. Chen 401 (SYS).

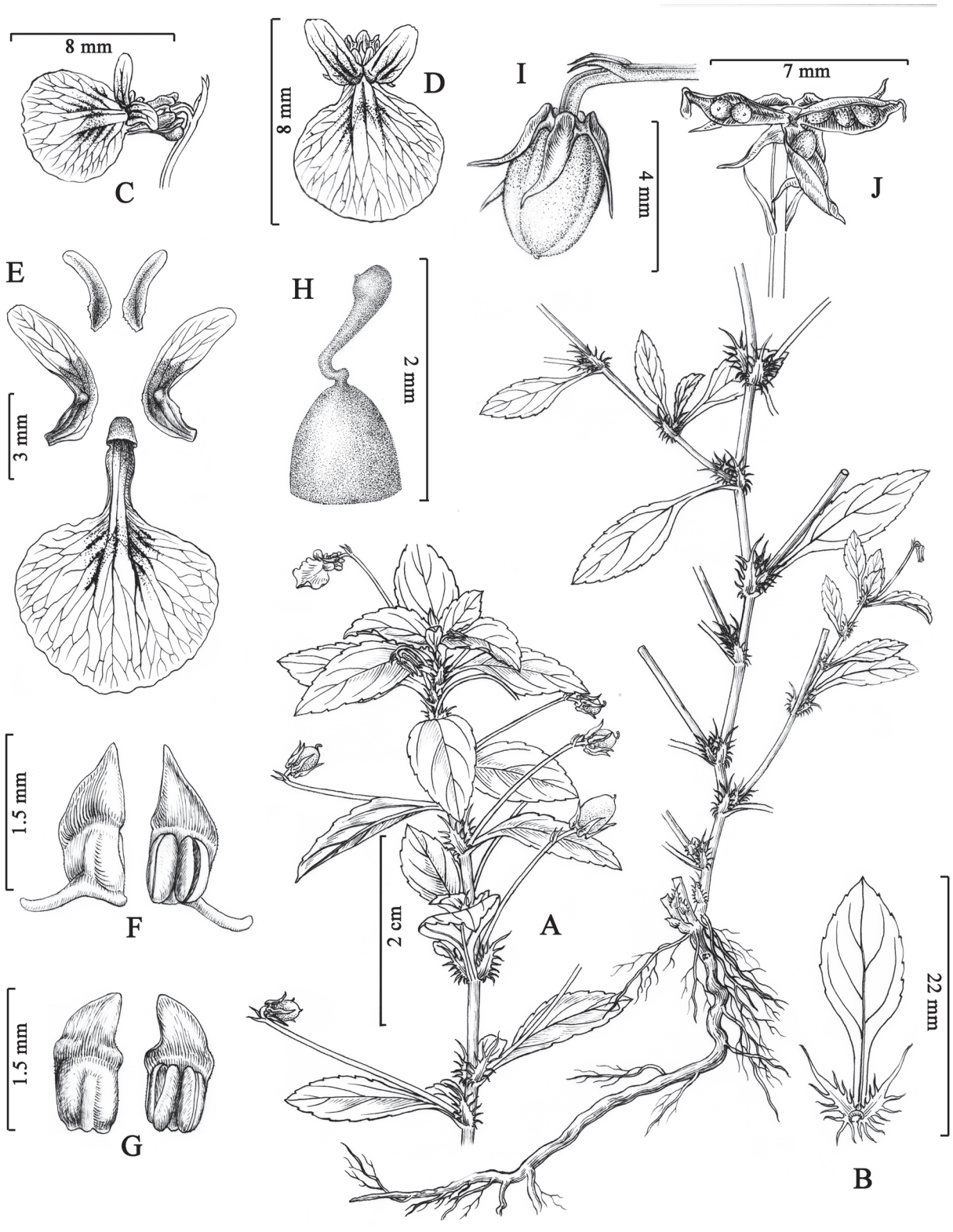

FIGURE 5. *Viola hybanthoides* W. B. Liao et Q. Fan. **A**. Habit and flowering branch. **B**. Leaf and stipules. **C**. Flower, lateral view. **D**. Flower, front view. **E**. Petals. **F**. Ventral stamen in adaxial (right) and abaxial (left) view. **G**. Posterior stamen in adaxial (right) and abaxial (left) view. **H**. Pistil. **I**. Young capsule with sepals and bracteoles. **J**. Dehiscent capsule and seeds. Drawn by Yunxiao Liu from the holotype.

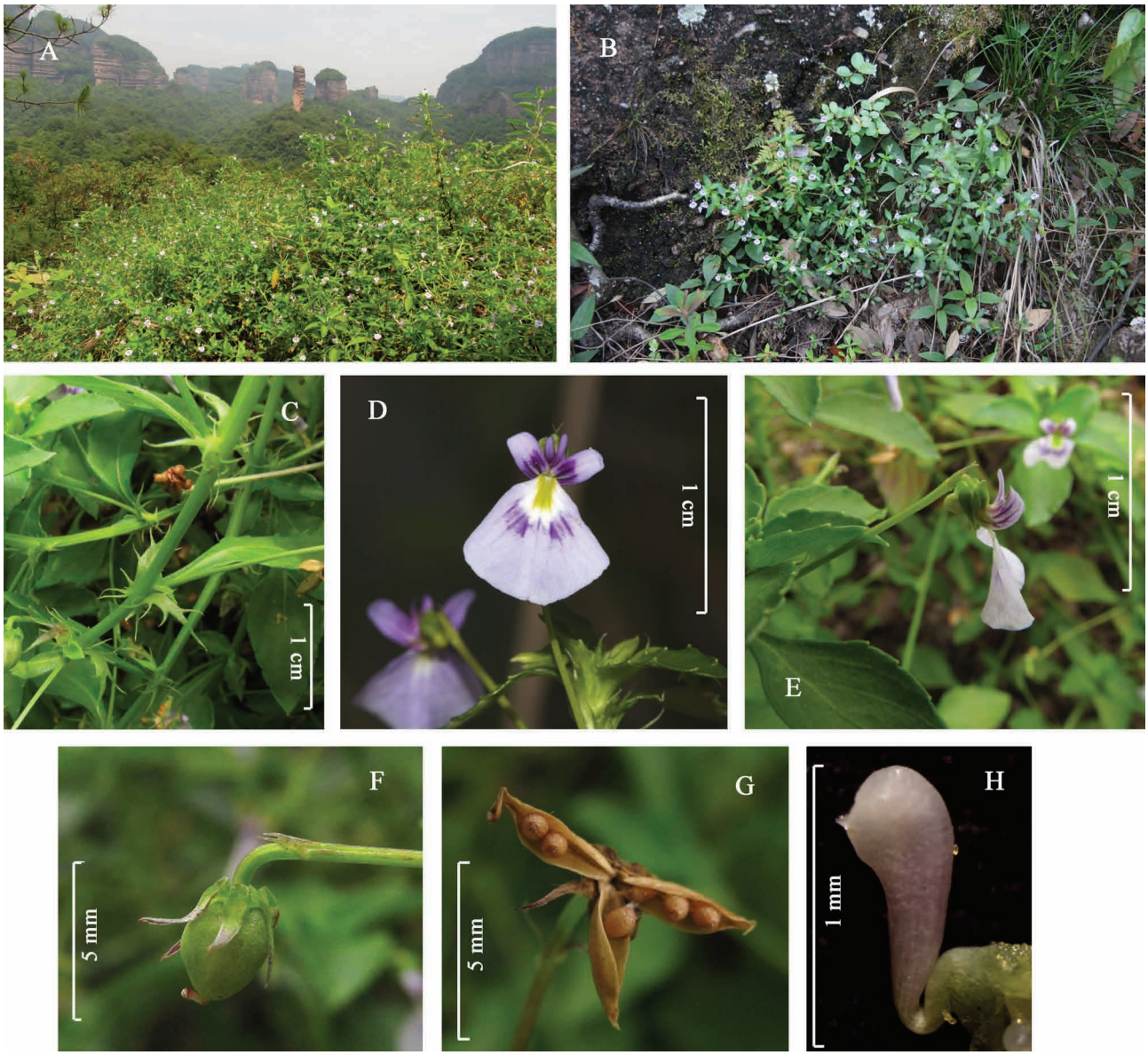

FIGURE 6. *Viola hybanthoides* W. B. Liao et Q. Fan. **A**. Habitat. **B**. Habit. **C**. Stem with stipules. **D**. Flower, front view. **E**. Flower, lateral view. **F**. Young capsule with sepals and bracteoles. **G**. Dehiscent capsule and seeds. Photos taken by Sufang Chen.

4. Discussion

The most distinctive characters of *Viola hybanthoides* are the subshrub habit, the anterior petal with a long stalk-like claw (up to 3.5 mm, ca. 1/3 of the petal's length) and the very short upper petals (only 2.5–3 mm long, less than 1/2 of the lateral petals' length), all of which are characters shared with some *Hybanthus* species. These characters of this species are distinguishable from all other *Viola* species, which are usually herbs, and have the anterior petal without a long claw and much longer upper petals (usually more than 8 mm long). Regardless of these differences, the new species exhibits some morphological similarities to *V. tricolor*, such as the large and leaflike stipules and a lamellar process below the stigmatic hole. However, the present phylogenetic results do not support that *V. hybanthoides* has s close relationship with *V. tricolor* or any other *Melanium* species.

Based only on the morphology of the corolla, the species might be mistaken as *Hybanthus* Jacq. However, the sepals of *V. hybanthoides* are auriculate basally, which is different from all *Hybanthus* species. Moreover, the phylogenetic trees show that *V. hybanthoides* is grouped with *Viola*. Therefore, the new species should belong to the genus *Viola*.

Neither the ITS tree nor the cpDNA tree support that *V. hybanthoides* belongs to any known section. On the cpDNA tree, the systematic position of *V. hybanthoides* is unresolved. On the ITS tree, the new species forms a well-supported

clade (BS = 81%) with sections *Chamaemelanium* and *Dischidium*. They share the character that the stigmas are not beaked in front. However, it has whitish to pale purple petals, while all species of the two sections mentioned have yellow petals. Furthermore, the chromosome number of *V. hybanthoides* is $2n = 20$, while the two sections possess the chromosome number of $2n = 12$ (Clausen 1929; Yoo and Jang 2010). Therefore, our new species belongs to neither of them. Morphologically, we could separate the new species from section *Nomimium* easily though they have the similar flower color. The styles of section *Nomimium* are beaked at the apex, while that of *V. hybanthoides* is not beaked. In addition, neither of the two phylogenetic trees support that *V. hybanthoides* is grouped in section *Nomimium*.

In conclusion, the morphological differences and the molecular phylogenetic results provide sufficient evidence for treating *V. hybanthoides* as a distinct new species, and we have also described a new section for this species.

5. Acknowledgements

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Appendix: species name and GenBank accessions for phylogenetic analysis

Taxon	ITS	matK	rpl16	atpB-rbcL
V. acuminata	AY928273	DQ842573	GQ262683	DQ834750
V. alba	EU413913			
V. albida	AY928292	DQ842589	GQ262702	DQ834766
V. amamiana	JF830899			
V. ambigua	EU413933			
V. arvensis	DQ055340			
V. atroviolacea	FJ002878			
V. betonicifolia	JF830902			
V. biflora	AY928309	DQ842607	GQ262678	DQ834784
V. brevistipulata	AY928275	DQ842570	GQ262680	DQ834747
V. caspia	HM486500			
V. chaerophylloides	AY928290	AB038188	GQ262699	DQ834764
V. collina	EU413938	DQ842571	GQ262681	DQ834748
V. dactyloides	JQ950561			
V. davidii	FJ002902			
V. delavayi	FJ002908			
V. diamantiaca	AY928288	DQ842585	GQ262697	DQ834762
V. diffusa	FJ002917			
V. diffusoides	FJ002914			
V. dissecta	DQ787774	DQ842609	GQ262700	DQ834786
V. eizanensis	DQ787773			
V. ganchouenensis	FJ002918			
V. grandisepala	FJ002903			
V. grypoceras	AY928280	DQ842577	GQ262689	DQ834754
V. guestphalica	DQ055378			
V. hancockii	FJ002890			
V. hirta	EU413945			
V. hirtipes	AY928297	DQ842595	GQ262706	DQ834772
V. hondoensis	AY928272	DQ842572	GQ262682	DQ834749

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Appendix (Continued)

Taxon	ITS	matK	rpl16	atpB-rbcL
V. inconspicua	FJ002897			
V. iwagawai	JF830854			
V. japonica	AY928295	DQ842592	GQ262705	DQ834769
V. jaubertiana	EU430659			
V. keiskei	AY928303			
V. kiangsiensis	FJ002901			
V. kusanoana	AY928278	DQ842576	GQ262687	DQ834753
V. labradorica	FJ002889			
V. lactiflora	AY928299	DQ842597	GQ262708	DQ834774
V. lanaiensis	JN682058			
V. lucens	FJ002913			
V. lutea	DQ055354			
V. magnifica	FJ002899			
V. mandshurica	AY928300	DQ842598	GQ262709	DQ834775
V. mirabilis		GQ262544	GQ262688	GQ262539
V. monbeigii	FJ002894			
V. moupinensis	FJ002900			
V. mucronulifera	FJ002910			
V. nanlingensis	FJ002916			
V. occulta	HM851452			
V. odorata	EU413918			
V. orientalis	AY928271	DQ842569	GQ262679	DQ834746
V. ovato-oblonga	AY928277			
V. patrinii	AY928298	DQ842596	GQ262707	DQ834773
V. pekinensis	FJ002892			
V. phalacrocarpa	AY928294	DQ842591	GQ262704	DQ834768
V. philippica	FJ002895			
V. pinnata	JQ950571			
V. principis	FJ002904			
V. prionantha	FJ002893			
V. pseudojaponica	JF830901			
V. pubescens	DQ006044			
V. pyrenaica	JF683821			
V. raddeana	AY928279	DQ842582	GQ262694	DQ834759
V. reichenbachiana	DQ055382			
V. rockiana	FJ002906			
V. rossii	AY928286	DQ842583	GQ262695	DQ834760
V. rupestris	FJ002888			
V. sacchalinensis	AY928276	DQ842608	GQ262684	DQ834785
V. schulzeana	FJ002907			
V. selkirkii	AY928307	DQ842605	GQ262714	DQ834782
V. seoulensis	AY928301	DQ842599	GQ262710	DQ834776
V. serrula	FJ002887			
V. shinchikuensis	FJ002885			
V. sieboldiana	DQ787772			

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Appendix (Continued)

Taxon	ITS	matK	rpl16	atpB-rbcL
V. somchetica	HM851457			
V. spathulata	HM851456			
V. stewardiana	FJ002883			
V. suavis	EU413923			
V. tashiroi	JF830881			
V. tenuicornis		DQ842604	GQ262713	DQ834781
V. thomasiana	JF683842			
V. tokubuchiana	KC330745	GQ262545	GQ262715	GQ262540
V. triangulifolia	FJ002912			
V. tricolor	DQ055390			
V. variegata	AY928305	DQ842603	GQ262712	DQ834780
V. verecunda	AY928283	DQ842580	GQ262692	DQ834757
V. violacea	AY928308	DQ842606	GQ262717	DQ834783
V. websteri	AY928274	DQ842574	GQ262685	DQ834751
V. woosanensis	AY928291	DQ842588	GQ262701	DQ834765
V. yazawana	AY928289	DQ842586	GQ262698	DQ834763
V. yedoensis	AY928302			
V. yezoensis	JQ950573			
V. yunnanensis	FJ002915			
V. yunnanfuensis	HM483597			
Hybanthus concolor		EF135550	GQ262720	GQ262543
Hybanthus enneaspermus	HM483598			
Melicytus ramiflorus	EF635449			