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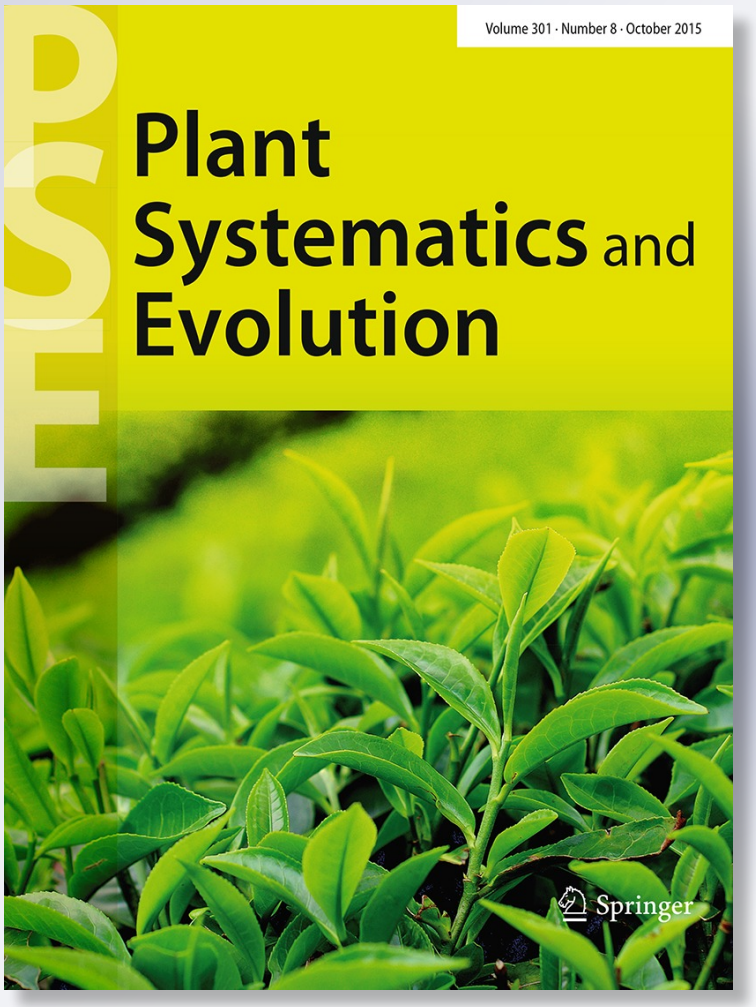

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ORIGINAL ARTICLE

Molecular systematics and morphometrics in *Veronica* subsect. *Canae* (Plantaginaceae)

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Abstract *Veronica* subsect. *Canae* constitutes a group of approximately 22 species, mostly endemic to China. The clade is an early branching taxon in the earliest branching subgenus of *Veronica* and its phylogenetic relationships are therefore relevant for understanding evolutionary patterns in the genus as a whole. In the present study, we aim to confirm the circumscription of the subsection, estimate phylogenetic relationships within the subsection, and compare patterns based on morphological similarities with this phylogenetic hypothesis. Using 80 individuals representing ten species for the molecular and 16 for the morphometric analyses, we confirm the monophyly of the subsection but present results that will require further testing using more intense intraspecific sampling. For example, *V. szechuanica* was shown to be polyphyletic in molecular analyses and morphologically heterogeneous. The distinction of *V. cana* and *V. henryi* requires further investigation. Finally, two migration events from China to Japan are inferred by the phylogenetic analyses.

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Introduction

The flora of China comprises approximately 1/8th of all flowering plant species of the world (Flora of China project 2008). This is partly attributed to the sheer size of the country and the corresponding habitat diversity ranging from boreal to tropical and from desertic to coastal habitats. However, another part of the story is that the area has also been an active center of speciation for a long time and has also been a refuge for many plant species (López-Pujol et al. 2011). These processes have been most actively investigated in the Qinghai–Tibet plateau (QTP) (Qiu et al. 2011), where the uplift ca. 15 mya and glaciations in the Pleistocene have often lead to extinction, fragmentation and speciation in marginal refugia (Liu et al. 2012; Qiu et al. 2011). Other regions of China have also started to be investigated in more detail biogeographically (Qiu et al. 2011). However, insufficient knowledge of species circumscriptions and phylogenetic relationships makes biogeographical investigations and inferences difficult (Liu et al. 2012; Avise 2000).

The tribe Veroniceae with more than 500 species is a group having likely originated in the QTP–Himalayan area before the uplift based on the current occurrence there of its early diverged members, such as *Lagotis* and *Wulfeniopsis* (Surina et al. 2014). Specifically, four of the nine genera in Veroniceae are restricted to that area (*Picrorhiza, Scrofella, Wulfeniopsis, Kashmiria*). Two have their center of origin in that region (*Lagotis, Veronicastrum*; Li et al. 2014; Albach unpubl.) and two occur exclusively in regions farther to the west of QTP but likely originated from

more eastern relatives (*Wulfenia, Paederota*) (Surina et al. 2014).

The biggest genus of the tribe, *Veronica* with 450 species, however, has been underinvestigated in this respect. Whereas European, southwest Asian and Australasian species have been intensively analyzed phylogenetically (Albach and Briggs 2012; Albach et al. 2004a, 2005; Wagstaff and Garnock-Jones 1998), Chinese species were scarcely represented in these analyses. The flora of China includes 53 species of *Veronica* with five of them introduced (Hong and Fischer 1998). Of the remaining species, 19 are endemic. Most notably, these endemics and most of the ones restricted to China and adjacent regions belong to just two subgenera. One is subgenus *Stenocarpon* with 25–30 species in alpine areas across Eurasia, a rather young group in the genus (Albach et al. 2009). The other, the focus of the study here, is *V.* subsection *Canae* sensu Albach et al. (2008) in subgenus *Veronica* (Albach et al. 2004b; Müller and Albach 2010). The first species of the subsection discovered were *V. cana* and *V. deltigera* described by Wallich from Nepal (Bentham 1835). These species were considered closely related to European species *V. scutellata* and *V. montana* based on long pedicels and capsules being wider than long (Bentham 1846). The group was first recognized as distinct by Yamazaki (1957) as *V.* series *Canae* based on the flabellate, compressed capsule with subacute edges and put close to series *Scutellatae* at that time. Elenevskij (1977) later placed the respective species in four separate subsections, subsections *Canae* [*V. cana* (incl. *V. henryi* and *V. miqueliana* as subspecies) and *V. oligosperma*], *Piroliformis* (*V. piroliformis*, *V. fargesii*, *V. riae*), *Szechuanicae* (*V. szechuanica* incl. *V. sutchuensis*) and *Montanae* (*V. vandellioides* together with *V. montana*). Work on the flora of China later increased the number of species significantly by seven species most of them by Hong in Flora Reipublica Sinensis (Hong 1979). Subsection *Canae* now includes 22 species (Albach et al. 2008), 14 of which are endemic to mainland China and occur at least partly in Xizang, Sichuan and Yunnan. Two species occur in Taiwan and three species in Japan. Phylogenetically, it is an early branching clade in its subgenus. Therefore, an increased knowledge of phylogenetic relationships within the subsection is important for inferences about biogeographic scenarios and character evolution in the subgenus as a whole.

Until now, only three or four species of *V.* subsect. *Canae* were included in phylogenetic analyses (Albach et al. 2006; Müller and Albach 2010). These studies supported the close relationship of Chinese and Japanese species with *V. abyssinica, V. scutellata* and especially *V. montana* as suggested by earlier authors (Bentham 1846; Römpp 1928). Within the clade, *V. vandellioides* was shown to be sister to *V. piroliformis* and *V. miqueliana*. Thus, Albach et al. (2008) recognized *V.* subsect. *Canae* in *V.* sect. *Montanae*.

The purpose of the present analysis is therefore, to confirm its circumscription with wider taxon sampling, provide a first estimate for the phylogenetic relationships within the subsection, compare patterns based on morphological similarities with this phylogenetic hypothesis, and infer some biogeographic patterns in the group.

Materials and methods

Plant material

A total of 80 individuals were sampled for this study, representing 16 (morphometric) or 10 (molecular) of the 22 currently accepted species of *V.* subsect. *Canae*. Plant material was collected specifically for this study (vouchers deposited at OLD and HSNU), and plant material from herbarium specimens from E, GH, K, M/MSB, MB, MO, WU and WUK was also included (Table 1). However, since most of the specimens used in the morphometric analysis came from herbaria and DNA extraction was not feasible or allowed, only five specimens were used in molecular and morphometric analyses. Geographical localities of specimens are depicted in the map (Fig. 1) using DIVA-GIS (Hijmans et al. 2001) and modified in Pixelmator 3.1 (Pixelmator Team Ltd., Vilnius, Lithuania).

Morphological analyses

Initially, morphological characters were chosen based on prior use in the original description of the species and in more recent floras (e.g., Hong and Fischer 1998). Thirty characters from fruits, inflorescence and leaves were observed. Those with an intraspecific standard deviation of less than two were omitted to avoid confusion by large intraspecific variation analyzed (Table 2). Flowers were not used because they were often missing or withered, and have been little used in previous studies to differentiate species in the subsection. Character states of herbarium specimens were recorded using a binocular microscope (Zoom stereo microscope SZX16, Olympus Corporation) and the Software CellSens Dimensions (Olympus Corporation).

To avoid the problem of different age and size of the individuals, ratios were used in the analyses, e.g., the width of a leaf in relation to the length. The data were analyzed in R (R-Development-Core-Team 2011) using the packages ape (Paradis et al. 2004), vegan (Oksanen et al. 2011) and pvclust (Suzuki and Shimodaira 2006). A Principal Coordinates Analysis (PCoA) was computed with a Euclidean

Table 1 Information on voucher specimens used for morphometric and molecular analyses including GenBank accession numbers

Species	Number	Voucher	Country, province	Genbank ITS	Genbank *trnL*	Genbank ndhF-rpl32
V. cana Wall.	1	Heng et al. 26724, GH 00255636	China, Yunnan			
V. cana Wall.	2	Henry 5688, MO 102729	China, Sichuan			
V. cana Wall.	3	?, M-0177304	China, Sichuan			
V. cana Wall.	4	Williams 611, M-0177269	Eastern Nepal			
V. cana Wall.	5	Hooker s.n., M-0177265	India, Sikkim			
V. deltigera Wall.	6	Wakabayashi et al. 9715071, MO 5939985	Eastern Nepal			
V. deltigera Wall.	7	Duthie 5864, WU-065338	India, Uttarakhand			
V. deltigera Wall.	8	Duthie 13754, WU-065340	Pakistan, Gilgit-Baltistan			
V. fargesii Franch.	9	Chen et al. 2273, WUK 0492655	China, Shaanxi	KM277633	KM277648	KP844866
V. forrestii Diels	10	Forrest 7227, K H2011/01752 12	China, Yunnan			
V. forrestii Diels	11	Podlech 54327, MSB-004260	China, Yunnan	KM277634	KM277649	
V. forrestii Diels	12	Forrest 7227, E 00416733	China, Yunnan			
V. henryi T.Yamaz.	14	Ye 7125, MO 04563988	China, Jiangxi			
V. henryi T.Yamaz.	15	Xu 1994210, MO 04699312	China, Sichuan			
V. henryi T.Yamaz.	16	Liu 16978, MO 04701459	China, Chongqing			
V. henryi T.Yamaz.	17	Teng 90150, GH 00311112	China, Guizhou			
V. henryi T.Yamaz.	18	Chen et al. 1877, WUK 0493229	China, Shaanxi	KM277632	KM277646	KP844867
V. henryi T.Yamaz.	19	Ying et al. 675, WUK 0478867	China, Hubei	KM277637	KM277652	KP844868
V. henryi T.Yamaz.	20	Ying et al. 451, WUK 0478834	China, Shaanxi		KM277647	KP844869
V. henryi T.Yamaz.	21	Li 153, MO 04513964	China, Sichuan			
V. henryi T.Yamaz.	22	Zheng 1423, MO 04520358	China, Hubei			
V. henryi T.Yamaz.	23	Ye 1401, MO 04506219	China, Jiangxi			
V. henryi T.Yamaz.	24	Ying et al. 918, WUK 0478345	China, Shaanxi	KM277631	KM277645	KP844870
V. miqueliana Nakai	25	Stuessy et al., 17270, WU	Japan	AF509807	AF486392	
V. miqueliana Nakai	26	Furuse 19241, K H2011/01752 23	Honsu, Japan			
V. miqueliana Nakai	27	Furuse 42335, K H2011/01752 24	Shizuoka, Japan			
V. miqueliana Nakai	28	Furuse 45405, K H2011/01752 25	Kanagawa, Japan			
V. miqueliana Nakai	29	Furuse 27402, K H2011/01752 26	Tochigi, Japan			
V. miqueliana Nakai	30	Stuessy et al., 17225, WU	Japan	EU224207	AY486444	KP867620
V. miqueliana var. takedana (Makino) Nemoto	31	Murata 705, K H2011/01752 20	Shizuoka, Japan			
V. muratae T. Yamaz.	32	Setoguchi J20051, WU	Japan	KM277635	KM277650	
V. oligosperma Hayata	33	Leu 1211, GH 00311110	Taiwan			
V. piroliformis Franch.	34	Forrest 21542, E 00416768	China, Yunnan			

Table 1 continued

Species	Number	Voucher	Country, province	Genbank ITS	Genbank *trnL*	Genbank ndhF-rpl32
V. piroliformis Franch.	35	Dickoree 14146, GOET	China, Yunnan		AF486390	
V. piroliformis Franch.	36	Dickore 14146, MSB-143735	China, Yunnan			
V. piroliformis Franch.	37	Ohba 1042, GH 00129472	China, Yunnan			
V. piroliformis Franch.	38	Forrest 21542, K H2011/01752 7	China, Yunnan			
V. piroliformis Franch.	39	Schneider 1765, E 00416773	China, Yunnan			
V. riae H.J.P.Winkl.	40	Wilson 893a, K H2011/01752 4	China, Hubei			
V. riae H.J.P.Winkl.	41	Limpricht 1566, WU-060243	China, Hubei			
V. robusta (Prain) T.Yamaz.	42	Wakabayashi et al. 9715284, MO 5939981	Eastern Nepal			
V. robusta (Prain) T.Yamaz.	43	Ohba et al. 8530115, MO 4379460	Central Nepal			
V. sutchuenensis Franch.	44	Sun and Chang 190, GH 00129511	China, Sichuan			
V. sutchuenensis Franch.	45	Wilson 5087, K H2011/01752 2	China, Sichuan			
V. szechuanica Batalin	46	H.-Q. Li 2008120, HSNU	China, Xizang	KM277636	KM277651	KP844871
V. szechuanica Batalin	47	Yu 7699, BM	China, Sichuan	AY741527		
V. szechuanica Batalin	48	Boufford et al. 27642, GH 00285584	China, Sichuan			
V. szechuanica Batalin	49	Boufford et al. 27933, GH 00146614	China, Sichuan			
V. szechuanica Batalin	50	Wu 90-734, WUK 0476235	China, Shaanxi	KM277638	KM277653	KP844872
V. szechuanica Batalin	51	Hu 1362, GH 00129520	China, Hubei			
V. szechuanica Batalin	52	Chen et al. 3080, WUK 0496283	China, Shaanxi		KM277654	KP844873
V. szechuanica Batalin	53	Fu 5232, MO 04712510	China, Shaanxi			
V. szechuanica Batalin	54	Wu 92-1274, MO 04764825	China, Shaanxi			
V. szechuanica ssp. sikkimensis (Hook. f.) D.Y.Hong	56	Dickore 10541, MSB-143734	China, Xizang			
V. szechuanica ssp. sikkimensis (Hook. f.) D.Y.Hong	57	Dickore 11845, MSB-143730	China, Xizang			
V. szechuanica ssp. sikkimensis (Hook. f.) D.Y.Hong	58	Dickore 5476, MSB-143728	China, Xizang			
V. szechuanica ssp. sikkimensis (Hook. f.) D.Y.Hong	59	Dickore 5382, MSB-143731	China, Xizang			
V. tsinglingensis D.Y.Hong	60	Henry 6843, GH 00311107	China, Hubei			
V. vandellioides Maxim.	61	Boufford et al. 31527, GH 00288599	China, Xizang	KM277640	KM277656	KP844874
V. vandellioides Maxim.	62	Dickore 8417, MSB-143727	China, Sichuan			
V. vandellioides Maxim.	63	Boufford et al. 36921, GH 00288598	China, Sichuan			
V. vandellioides Maxim.	64	EG-11-094-01, MB	China, Qinghai			
V. vandellioides Maxim.	65	EG-11-098-15, MB	China, Qinghai	KM277641	KM277657	KP844875
V. vandellioides Maxim.	66	Ho et al. 163, GH 00311106	China, Qinghai	KM277639	KM277655	KP844876
V. vandellioides Maxim.	67	Ho et al. 163, MO 04648077	China, Qinghai			
V. vandellioides Maxim.	68	Smith 10964, MO 5958161	China, Sichuan			
V. vandellioides Maxim.	69	Potanin s.n., K 738586	China, Sichuan			
V. vandellioides Maxim.	70	Dickoree 8417, GOET	China, Sichuan	AF509806	AY776287	

Table 1 continued

Species	Number	Voucher	Country, province	Genbank ITS	Genbank *trnL*	Genbank ndhF-rpl32
V. vandellioides Maxim.	71	Lian 93-58, MO 04664551	China, Gansu			
V. vandellioides Maxim.	72	Albach 1182 specimen 1, OLD	China, Shaanxi			
V. vandellioides Maxim.	73	Albach 1182 specimen 2, OLD	China, Shaanxi	KP867617	KP867618	KP867819
V. vandellioides Maxim.	74	Smith 11422, MO 5958912	China, Xizang			
V. vandellioides Maxim.	75	Smith 11422, MO 5964422	China, Xizang			
V. yunnanensis D.Y.Hong	76	H.-Q. Li 2008121, HSNU	China, Xizang	KM277642	KM277658	KP844877
V. yunnanensis D.Y.Hong	77	H.-Q. Li 2008114, HSNU	China, Xizang	KM277643	KM277659	KP844878
V. yunnanensis D.Y.Hong	78	Gaoligong Shan Exp. 12732, E 00246645	Myanmar			
V. yunnanensis D.Y.Hong	79	Sino-American Bot. Exp. 588, E 00416737	China, Yunnan			
V. yunnanensis D.Y.Hong	80	Collector illegible, GH 00311105	China, Yunnan	KM277644	KM277660	
Outgroups						
V. abyssinica Fresen.	81	Fischer 728/98, BONN	Kenya	AF313009		KP867639
V. abyssinica Fresen.	82	Fischer 8060, BONN	Ruanda		AF513350	
V. allionii Vill.	83	Chase 8911, K	Italy	AF509809	AF513348	KP867632
V. alpina L.	84	Albach 184, WU	France	AF313013	AF486387	KP867628
V. aphylla L.	85	Zhang s.n, WU	Italy	AF515211/2	AF513349	KP867635
V. baumgartenii L.	86	Albach 542, WU	Bulgaria	AY144464	AY780808	KP867636
V. bellidioides L.	87	Albach 118, K	Austria	AF313010	AF513345	KP867629
V. copelandii Eastw.	88	Janeway 6557, WU	USA, California	AF515213	AF513344	KP867626
V. cusickii A.Gray	89	Albach 288, WU	USA, Oregon		AY486443	KP867627
V. glandulosa Hochst.	90	Fischer 713/98, WU	Kenya	AF313008	AF486394	KP867638
V. montana L.	91	Albach 151, WU	Germany	AF313014	AF486388	KP867621
V. morrisonicola Hayata	92	Albach and Chase 112,K	Japan	AF509808	AF513347	KP867633
V. nipponica Makino	93	Horii 20401, WU	Japan		AY776286	KP867625
V. officinalis L.	94	Albach and Chase 114,K	United Kingdom	AF313012	AF486391	KP867634
V. ponae Gouan.	95	Martínez Ortega 769/1, SALA	Spain			KP867630
V. scutellata L.	96	Dobes 7026, WU	Austria	AF509805	AF486393	KP867637
V. stelleri Pall. Ex Link	97	Gage 4627, WTU	USA, Alaska		AY847149	KP867624
V. urticifolia Jacq.	98	Albach 73, BONN	Austria	AF313011	AF486389	KP867631
V. wormskjoldii Roem. & Schult.	99	Olmstead 99-180, WTU	USA, Washington	AF509811	AF511481/2	KP867623
V. wormskjoldii Roem. & Schult.	100	Albach 217, WU	USA, New Hampshire		AY847151	KP867622

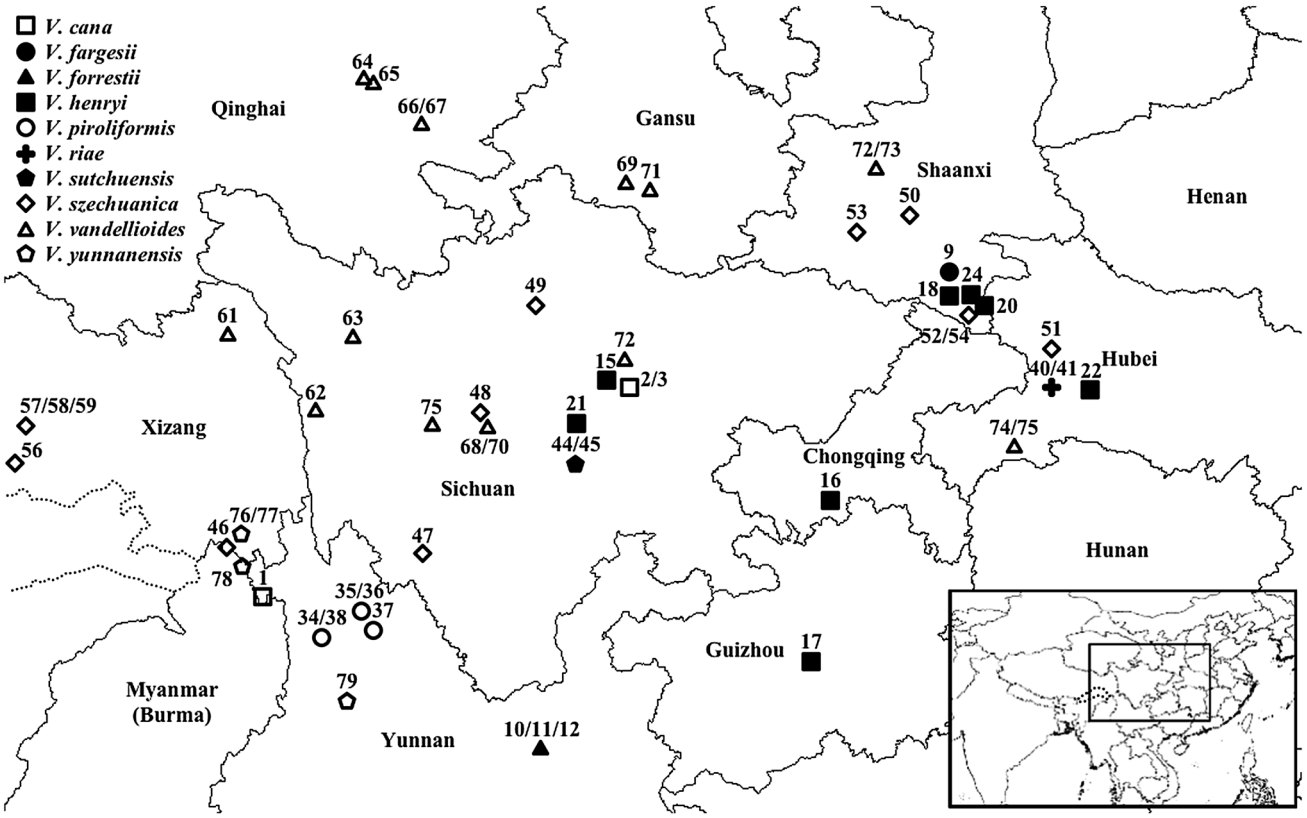

Fig. 1 Map of the localities from which the Chinese samples in the analyses have been collected. *Numbers* refer to individuals as given in Table 1

distance matrix, based on standardized data using the scale command in R. A hierarchical cluster analysis was conducted using the Ward method on the same distance matrix and support estimated by assessing approximately unbiased *p* values estimated by 1000 multiscale bootstrap replicates.

DNA extraction, amplification and sequencing

About 20 mg of dried plant material of the samples was homogenized in a swing-mill (MM200, Retsch). DNA was extracted using a Plant-DNA-kit (innuPREP plant DNA kit, Analytik Jena) following manufacturer's instructions with minor modifications. To remove RNA, 10 µl RNase (3 mg/ml) was added, the amount of elution buffer was reduced to 100 µl and incubation time for the elution buffer increased from 1 to 5 min.

DNA regions amplified and sequenced for this study were the *ndh*F-*rpl*32-intron and the *trn*L-F cpDNA-region, which includes the *trn*L-intron, the 3′*trn*L-exon as well as the *trn*L-*F*-spacer, and the nuclear ITS region. The primers ITS-A and ITS-4 (Sang et al. 1995; White et al. 1990) were used for the ITS region, primers ndhF and rpl32F (Shaw et al. 2007) for the *ndh*F-*rpl*32-intron and primers c and f for the *trn*L-F-region (Taberlet et al. 1991). In some cases of old herbarium material, the regions were amplified in two pieces using internal primers ITS-C and ITS-D (Sang et al. 1995) for ITS and primers d and e for the *trnL-F*-region (Taberlet et al. 1991). The amplifications were conducted with either a TProfessional Standard 96 Thermocycler (Biometra) or a Mastercycler Gradient thermocycler (Eppendorf) using mostly programs with 1- or 5-min denaturing at 94 °C followed by 35–36 cycles of 18 or 30 s at 94 °C, 30 s at 54 °C or 55 °C and 60–80 s at 72 °C with a final extension step of 8–10 min at 72 °C. Alternatively, we employed the program "rpl16" of Shaw et al. (2007) if reactions failed. Sequencing was carried out by GATC Biotech Konstanz (www.gatc-biotech.com).

Phylogenetic analyses

Using the software Sequencher (Gene Codes Corporation; Ann Arbor, MI, USA), forward and reverse sequences were combined and inspected by eye to make a consensus sequence. All sequences were aligned using the muscle algorithm in SeaView v.4.3.4 (Gouy et al. 2010) and manually corrected in PhyDE (Müller et al. 2010). Indels were coded using SeqState (Müller 2005) with simple indel coding (Simmons and Ochoterena 2000). All analyses were conducted separately for ITS and the cpDNA-regions and for the combined data set.

Table 2 Morphological traits used in the morphometric analysis with abbreviations used in Fig. 2

Trait abbreviation	Explanation
Traits of the shoot	
S1	Length of the shoot, crossing shoot–rhizome to upper node
S2	Length of the first internode from above (apical)
S3	Length of the second internode from above
S4	Length from the first internode from below (basal)
S5	Length of the shoot from the lowest node to the highest node with fruits
S6	Length of the shoot from the lowest node to the lowest node with fruits
Traits of the leaf	
B1	Length of the leaf of the second node from above (without shoot)
B2	Length of a leaf of the lowest node
B3	Length of the biggest leaf that is at any node except the highest and the lowest
B4	Length of the leaf–shoot of the highest node
B5	Length of the leaf–shoot of the lowest node
B6	Width if the biggest leaf at 25 % of its length, measured from the shoot
B7	Width if the biggest leaf at 50 % of its length, measured from the shoot
B8	Width if the biggest leaf at 75 % of its length, measured from the shoot
B9	Depth of the toothing, measured from the highest end of the tooth (from the tip)
Traits of the fruit	
F1	Length of the fruit (without style and stipe)
F2	Width at widest point (of one half of a fruit)
F3	Maximum length towards the style (of one half of a fruit) measured from the axis of maximum width
F4	Maximum width toward the style (of one half of a fruit) measured from the axis of the maximum width
F5	Distance of the axis of maximum length to the middle axis of the fruit
F6	Distance of the axis of the fruits maximum width to the spite
F7	Length of the spite
F8	Width of the fruit 25 % length, measured from the spite
F9	Width of the fruit 50 % length, measured from the spite
F10	Width of the fruit 75 % length, measured from the spite
F11	Width of the fruit 100 % length, measured from the spite
Other traits	
A1	Number of nodes
A2	Number of nodes that carry fruits
A3	Number of fruits per node
A4	Number of teeth per side

Rapid-Bootstrap-Analyses were conducted with RaxML-HPC (Stamatakis 2006) using the GTR + Γ Model with 1000 bootstraps. The used optimal model of substitution was initially determined using Modeltest server v1.0 (Posada 2006).

The same model (GTR + Γ) was identified also for the Bayesian analyses with mrModeltest (Nylander et al. 2008) and the Akaike Information Criterion (AIC). The HKY + Γ model was assigned to the indels. The analyses were conducted in MrBayes v.3.2.4 (Huelsenbeck and Ronquist 2001) with four chains and 25 million generations sampling every 1000th tree. Stationarity was checked in Tracer v. 1.5 (Rambaut and Drummond 2009). The first 20 % of the trees were discarded as burn-in. Biogeographical patterns were tested in RASP 3.0 using S-DIVA for the likelihood tree and the Bayesian Binary Method (Yu et al. 2015) using trees of the Bayesian analysis, default settings and restricting the number of ancestral areas to either one or two. Since the emphasis was on investigating differences between Sino-Himalayan and Sino-Japanese species, we coded four areas: Japan (*V. muratae, V. miqueliana*), east of the Tanaka-Kaiyong-line (*V. fargesii*, *V. forrestii*, *V. henryi*, *V. szechuanica* (46, 47)), west of that line (*V. piroliformis*, *V. szechuanica* (50, 52), *V.*

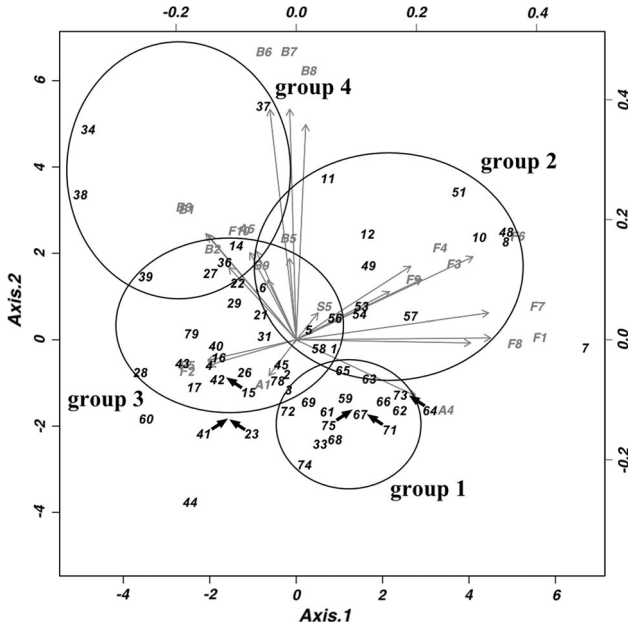

Fig. 2 Principal coordinate analysis of 30 characters in 62 individuals of *V.* subsect. *Canae*. The first and second axis explain 24.4 and 15.9 % of the variation, respectively. *Numbers* refer to individuals as given in Table 1, whereas *letter–number combinations* refer to characters as given in Table 2

vandellioides (all except 73), *V. yunnanensis*) and those outside Asia. All data sets and likelihood-derived phylogenetic trees are deposited in Treebase (http://www.treebase.org, study number 17173).

Results

Morphological analysis

For the morphometric analysis, 62 specimens including three specimens from one herbarium sheet that showed dissimilar specimens were included in the analysis (Online Resource 1). Only specimens with fruits were included in the analysis. The principal coordinate analysis (Fig. 2) shows the individuals clustered in four groups: *vandellioides*-group, *szechuanica*-group, *cana*-group and *piroliformis*-group. The first three axes explain 24.4 and 15.9 % of the variation, respectively. The *cana*-group is the largest group and contains mainly individuals of *V. cana*, *V. miqueliana* and *V. henryi* together with species with a smaller number of individuals in the analysis (*V. yunnanensis, V. robusta, V. tsinglingensis*). In the *vandellioides*-group, only individuals of *V. oligosperma* and *V. vandellioides* are found. The positioning of one individual of *V. szechuanica ssp. sikkimensis* (59) in this group is an artifact of the two-dimensional representation based on the analysis of the third axis (11.6 % of the variation; Online Resource 2). The *szechuanica*-group, which is best separated based on the third axis (Online Resource 2), contains, in addition to *V. szechuanica* (both subspecies), individuals of *V. deltigera* (not based on third axis) and *V. forrestii* (partly based on third axis). The *piroliformis*-group contains only the specimens of *V. piroliformis* but is scattered among the *cana*-group in the cluster dendrogram (Online Resource 3).

Leaf characters (ratio of leaf length to width, characters B6, B7, B8) and infructescence density (A4) are the most prominent characters differentiating groups along the *y*-axis. The *cana*- and the *szechuanica*-groups are separated by characters regarding the shape of the fruits, e.g., the ratio of fruit length to width at different positions (F1, F3, F4, F7, F8, F9).

Phylogenetic analyses

The ITS data set included 691 aligned base pairs (98 of them potentially parsimony-informative). The cpDNA data set consists of 910 aligned base pairs (54 of them potentially parsimony-informative) with 35 coded indels for the *trn*L-F-region and 830 aligned base pairs (58 of them potentially parsimony-informative) and 39 coded indels for the *ndh*F-*rpl*32-intron region. Likelihood and Bayesian analyses agree except for differences in resolution in otherwise unsupported relationships. Since there is no supported incongruence between ITS- and cpDNA-analyses, we concentrate on the combined analysis. Results from separate analyses are provided as supplementary material (Online Resource 4, 5). All analyses (Fig. 3) support the European species, *V. montana*, as the sister group to the Asian species (likelihood bootstrap (BS 99/63) and posterior probability (PP 1.0/0.96) for the monophyly of *V.* sect. *Canae* and *V.* subsect. *Canae*, respectively). The next node separates *V. henryi*, *V. fargesii* and *V. muratae* (BS 100, PP 1.0) from the rest (BS 56, PP < 0.9). The remaining taxa are found in five well-supported clades with no support for relationships between these clades and differing topologies between the likelihood and Bayesian analysis (Fig. 3, Online Resource 6). These clades are (1) Japanese *V. miqueliana*, (2) *V. yunnanensis,* (3) eastern specimens of *V. szechuanica*, (4) a clade of western individuals of *V. szechuanica* (individuals 46, 47) plus *V. forrestii* without support by either analysis and (5) a clade of *V. vandellioides* being the sister group to *V. piroliformis*, although with low support (BS 53, PP 0.88). Despite the differing topologies, biogeographical analyses suggest an Eastern Chinese origin with two dispersals to Japan and two or three dispersals from Eastern to Western China and one dispersal back (Online Resource 6).

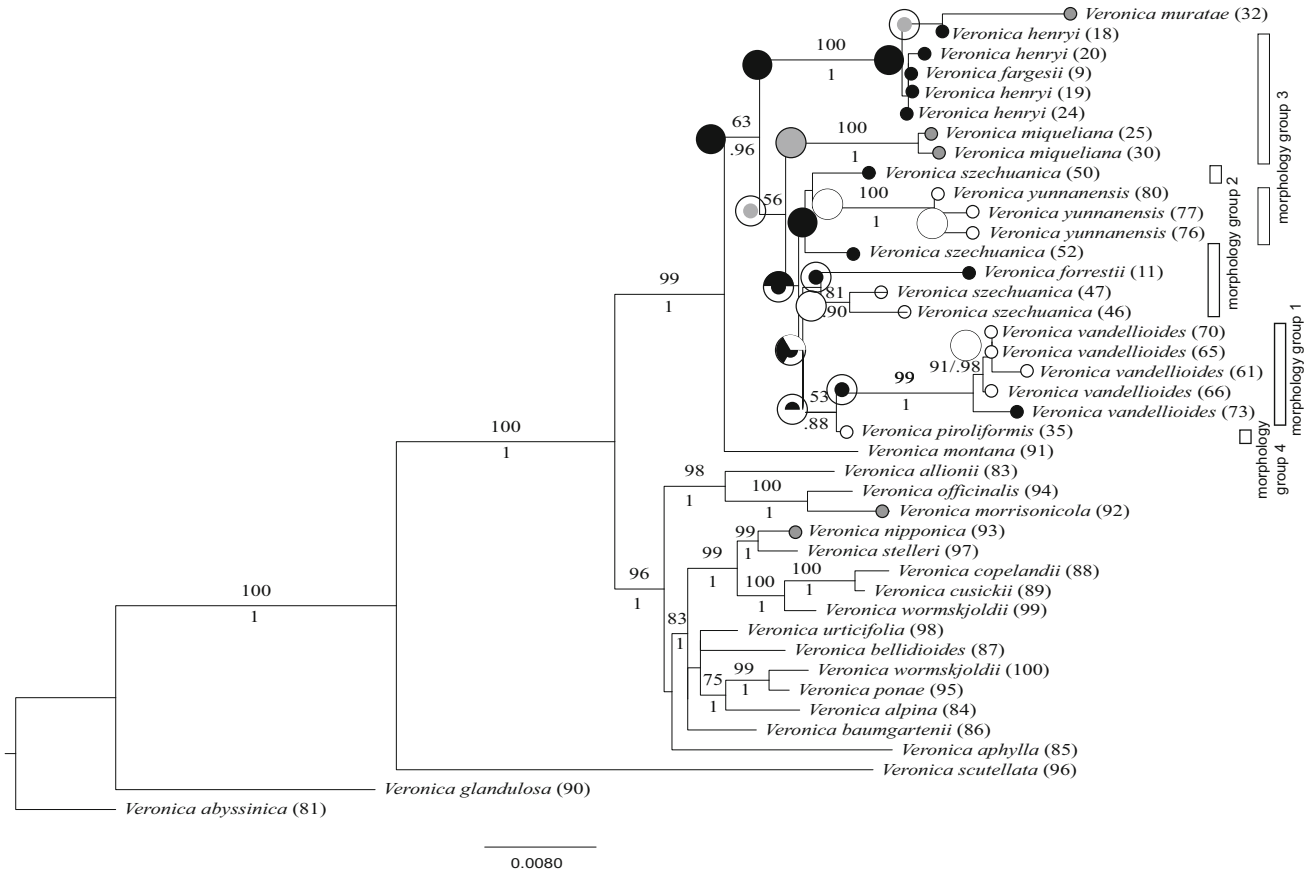

Fig. 3 Maximum likelihood tree of the combined data set with likelihood bootstrap values (above 70, plus additional values discussed in the text) above the branch and posterior probabilities (above 0.8) below the branch. *Numbers in brackets* after species name refer to individual number in Table 1. *Circles* before the taxa indicate geographic origin (*black* eastern China, *gray* Japan, *white* western China). *Circles* with *different inner* and *outer color* indicate that both regions have been inferred as ancestral area

Discussion

The aims of this study were to test the monophyly of *Veronica* subsection *Canae*, identify phylogenetic relationships among species using ITS and cpDNA and to compare these DNA-based results with a morphometric analysis of 30 morphological characters. Inter- and to some extent intraspecific variation of 22 individuals from ten species out of 22 recognized species of *V.* subsect. *Canae* were investigated using DNA sequences and 62 individuals representing 16 species morphometrically. Our phylogenetic analyses confirm the monophyly of the subsection and its sister group relationship to *V. montana* (Fig. 3) found in previous phylogenetic analyses using fewer sequences. A synapomorphy of the group is the wide capsule (≤5 mm long, ≥6 mm wide), which is mostly flabellate or rhomboid (except in *V. chayuensis*, *V. sutchuensis*, *V. vandellioides*). Species not included in the analyses are unlikely to change this conclusion since all species not included are morphologically close to included species as will be discussed below.

In contrast to the monophyly of the section, increasing taxon sampling changed considerably the conclusions regarding intrasectional relationships as found in previous analyses. Most importantly, the sister relationship between *V. vandellioides* and the rest of the subsection as suggested in a previous study (Albach et al. 2006) is not supported. Thus, the suggested trend in capsule shape from rounded to flabellate-deltoid with angled margins is not supported by our data but rather a reversal seems to have occurred in capsule shape. Although separate data sets have different topologies, the combined analysis suggests an Eastern Chinese origin of the subsection. An Eastern rather than a Western Chinese origin is further supported by the distribution of *Fagus* forests in Asia (Huang et al. 1999) since *V. montana*, the sister to *V.* subsect. *Canae*, is restricted to *Fagus* forests in Europe and suggests that *Fagus* forests are the ancestral habitat from which the group spread westwards and to other habitats.

The present results offer the possibility to clarify additional issues regarding the evolution and taxonomy of this group since taxonomic errors abound since the first

description of the Chinese specimens. These were first considered conspecific with *V. capitata* (Hooker 1885), a species now considered to be only distantly related (Hong and Fischer 1998) to *V.* subsect. *Canae*, but have subsequently been split in more and more species with some additional realignment apparently becoming necessary according to our results. In the following, we discuss our phylogenetic results with a focus on the taxonomic problems in the group, compare those with the morphometric results and indicate taxonomic problems requiring further collecting and analyses that are necessary to achieve a robust taxonomic treatment in the future.

The first branching group in *V.* subsect. *Canae* (Fig. 3) based on ITS (80 BS, 56 BS in combined analysis) and not refuted by cpDNA due to low support of the backbone consists of *V. henryi* and *V. fargesii*. The latter species was not analyzed in the morphological analysis, although a similarity can be seen between the two. They mainly differ from *V. fargesii* in having larger flowers, subwhorled leaves below the inflorescence and later flowering time (Hong and Fischer 1998). Geographically, the distribution area of *V. fargesii* is completely encompassed by that of *V. henryi*. So, an origin of the former from a *V. henryi*-like ancestor is likely. The close relationship of *V. fargesii* to *V. piroliformis* referred to by Li (1952) is not confirmed by our study. *Veronica henryi* has also been shown to be similar in the morphometric analysis (Fig. 2, *cana*-group) to a number of species not investigated in the molecular analyses (*V. cana*, *V. robusta*, *V. riae*, *V. sutchuensis, V. japonensis*) or only distantly related in the molecular analyses (*V. yunnanensis*, *V. miqueliana*). Most of these species have been considered to be closely related by previous authors and often subsumed under *V. cana* (e.g., Yamazaki 1957). *Veronica cana* and *V. henryi* are the two most widespread species of the group and occur parapatrically with the border in Sichuan, although specimens of *V. cana* from Sichuan need closer analysis to detect the exact position. Morphological characters used to distinguish the two species (leaf shape, petiole length) are only quantitative with a strong overlap in several specimens (Hong and Fischer 1998). This is indicated, for example, by the close similarity of two Sichuan samples to typical *V. cana* from Xizang in the morphometric analysis (Fig. 2) despite being geographically closer to *V. henryi* (Fig. 1). Including *V. cana* in future DNA-based studies seems essential in understanding the relationships among these species.

Chromosome numbers are known from just five species in subsect. *Canae*, ranging from $2n = 16$ to 52 (Albach et al. 2008; Malik and Gupta 2013; Malik et al. in Marhold and Breitwieser 2011). Since aneuploidy is rare in *Veronica* (Albach et al. 2008), estimates of $n = 21$ for *V. robusta* and $n = 23$, 25, and 26 for *V. cana* could easily be incorrect counts of $n = 24$. Unfortunately, only Malik et al. in Marhold and Breitwieser (2011) reported a photo for his count of *V. cana* ($n = 23$) and this also allows counting 24 chromosomes. Thus, the base chromosome number for the group would be $x = 8$ with *V. deltigera* being diploid, *V. henryi* tetraploid and *V. cana*, *V. robusta* and *V. miqueliana* hexaploids. Therefore, chromosome numbers and geography may be more useful to differentiate *V. henryi* and *V. cana* than morphology.

Other species related to *V. henryi* and *V. cana* (group 1 sensu Fig. 2) based on our morphological analysis are *V. riae*, *V. tsinglingensis* and *V. sutchuensis*. Elenevskij (1977) considered *V. sutchuensis* a synonym of *V. szechuanica*. This hypothesis can be clearly refuted by our morphometric analysis (Fig. 2). Other related taxa may be the local endemic *V. laxissima* from eastern Sichuan (Hong 1996), *V. longipetiolata* from south-central Xizang (Hong 1979), *V. taiwanica* from Taiwan (Yamazaki 1956) and *V. tibetica* from southern Xizang.

The sister group to *V. henryi* and related species and the branch following this clade (Fig. 3) comprise *V. muratae* from Japan and the two samples of the likewise Japanese species *V. miqueliana*, respectively. The Japanese species of *V.* subsection *Canae* were first described by Miquel (1865) and Makino (1907) as varieties of *V. cana* and only later elevated to species status (Makino 1912; Nakai 1918). Two separate migrations to Japan are revealed in the biogeographic analysis (Online Resource 6) and are consistent with analyses of other taxa disjunctly distributed between the two countries. The floristic exchange across the East China Sea has potentially been possible during the Pleistocene glacials with a much lower sea level and a possibly continuous warm-temperate deciduous forest spanning across the East China Sea. Subsequent sea level rises then led to fragmentation and possible allopatric speciation in many genera (Qian and Ricklefs 2000). With regard to the second dispersal event to Japan, the phylogenetic trees show a close genetic relationship of *V. muratae* and *V. henryi* (Fig. 3) making the latter a likely ancestor of *V. muratae* and likely also *V. japonensis* (S. Nemoto, pers. comm.). *Veronica muratae and V. japonensis* differ from *V. miqueliana*, first, in distribution area, the latter occurring further south and on the Pacific side of Japan (Yamazaki 1957). Second, they differ in morphology with the most prominent character to distinguish the three species being capsule shape, which is deltoid in *V. miqueliana* and rhomboid in the other two. The close relationship between *V. muratae* and *V. henryi* in the molecular analyses compared with the more isolated position of *V. miqueliana* suggests a later spread and—based on the distribution area of *V. muratae*—along a more northern route, whereas *V. miqueliana* likely spread along a more southern route to Kyushu and the Pacific Ocean side of Japan similar to that of ecologically similar species (Qi et al. 2014).

The remaining species in the molecular analyses form a well-supported clade in the ITS analysis (BS 89 %) but not the combined analysis. The genetic similarities between *V. yunnanensis*, *V. forrestii*, *V. piroliformis*, *V. szechuanica* and *V. vandellioides* are not supported by the morphological analysis since they correspond to three different groups in the morphometric analysis (Fig. 2). A close similarity between *V. yunnanensis* and *V. cana* was noted by Hong (1979) and is demonstrated by the morphometric analysis (Fig. 2) but rejected by the molecular analyses (Fig. 3).

V. szechuanica (incl. *ssp. sikkimensis*) seems to be a heterogeneous but distinct group based on the morphometric analysis (Fig. 2) which is not surprising given its wide geographic range from Western Hubei to NW India. The molecular analysis supports this conclusion by showing that *V. szechuanica* is polyphyletic. Reasons for this could be that *V. szechuanica* hybridizes with other species, that it is the common ancestor of these species or that it needs to be split into distinct species. Whereas *V. szechuanica* individuals 46 and 47, both collected in the southwestern part of China, branch basally or show a genetic similarity to *V. forrestii*, *Veronica szechuanica* individuals 50 and 52 from Shaanxi are found as sister to *V. yunnanensis* (Fig. 3). The close geographic proximity (Fig. 1) of *V. szechuanica* individual 46 and *V. forrestii* combined with the morphological similarity (Fig. 2) and genetic relatedness (Fig. 3) suggests that *V. forrestii* may have separated from *V. szechuanica* at its southern range margin east and west of the Tanaka-Kaiyong line (see below). In contrast, specimens from Shaanxi are geographically distant from *V. yunnanensis* (Fig. 1) and have no morphological similarity with it (Fig. 2). Future study in *V.* subsect. *Canae* will especially need to focus on *V. szechuanica* and analyze morphology in parallel to molecular data, which was not possible in the present study.

A species also possibly related to *V. szechuanica* based on the morphometric analysis (Fig. 2) is *V. deltigera* from eastern Nepal and adjacent China, the western distribution edge of *V. szechuanica*. *Veronica deltigera* differs from *V. szechuanica* in inflorescence morphology. Unfortunately, no recent collection was available for DNA extraction despite being an essential taxon for a biogeographical analysis. Another possibly related taxon is the unsampled *V. chayuensis*, an alpine form from southern Xizang and adjacent Yunnan (Hong 1979).

Veronica piroliformis forms a separated group in the morphometric analysis (Fig. 2). The analysis of DNA sequence data retrieves *V. piroliformis* as sister to *V. vandellioides* but without support and no morphological or biogeographic argument favoring such a relationship. The only support for such a relationship is found in the analysis of seed coat structure (Munoz-Centeno et al. 2006) since both species are unique in the subsection in having a reticulate seed coat.

Veronica vandellioides occupies a similar isolated position in all analyses. The morphometric analysis does not support similarity to *V. piroliformis* nor to *V. szechuanica* (Fig. 2). In previous classifications, *V. vandellioides* was suggested to be either close to *V. montana* (Elenevskij 1977) or to *V. cana* (Li 1950). Neither position is supported by our data. The main characters that separate *V. vandellioides* from the remaining groups in the morphometric analysis are characters related to leaf shape with *V. vandellioides* having more ovate-orbicular leaves than other species in the section. Comparing genetic similarity of the individuals of *V. vandellioides* with their geographic origin, a separation between the eastern sample (73) from the western samples is apparent (Fig. 3). The Taiwanese *V. oligosperma* is morphologically similar (Fig. 2).

A multitude of analyses has focused on biogeographic patterns in the flora of China (for reviews see (Hou 1983; Xie et al. 2004). In recent times, these have been tested by molecular data, which also allowed inferences on intraspecific level. Such phylogeographic studies have especially focused on the Tibet–Qinghai plateau (e.g., Chen et al. 2008; Zhang et al. 2005) but also beyond that (Qiu et al. 2011). Our data allow testing some of these patterns. For example, Li and Li (1992) discussed the Tanaka-Kaiyong line, a north–south spanning line in southern China separating the Sino-Himalayan floristic region in the west from the Sino-Japanese region in the east. More specifically, it separates the Hengduan Mountain region in the west from the Yunnan–Guizhou and Qinling Mountains in the east. This line has since been inferred to be a strong barrier for gene flow in a number of species (e.g., Fan et al. 2013). The geographic distribution of species from *V.* subsect. *Canae* broadly coincides with this line with *V. cana*, *V. yunnanensis* and *V. piroliformis* west of the line and *V. henryi*, *V. forrestii*, *V. fargesii* and *V. riae* east of the line. *Veronica vandellioides* spreads north of the line from west to east but still shows this separation (Fig. 3) suggesting a single migration across the Tanaka-Kaiyong line in central China. As discussed above, the geographic separation in east and west of the line is also found in *V. szechuanica*, the other species previously thought to stretch across the line. This leaves *V. cana* as the only species spanning this biogeographic line uninvestigated with molecular tools. However, we have two individuals (individuals 2/3), both clearly *V. cana* based on fruit characters, that correspond to eastern *V. cana* but future molecular analyses will need to be conducted to check for corresponding molecular divergence across the distribution of the species. Fan et al. (2013) dated the separation of eastern and western groups in *Sophora davidii* to 1.3 my, which is broadly consistent with age

estimates for *Veronica* (Meudt et al., submitted). However, a rigorous dating analysis for *V.* subsect. *Canae* must await a denser species sampling and a better resolved phylogeny.

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Conflict of interest The authors declare that they have no conflict of interest.

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