

# Phylogeographic insights on the evolutionary breakdown of heterostyly

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## Summary

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- The breakdown of heterostyly to homostyly is a classic system for the investigation of evolutionary transitions from outcrossing to selfing. Loss of sexual polymorphism is characterized by changes to population morph structure and floral morphology. Here, we used molecular phylogeography to investigate the geographical context for the breakdown process in *Primula chungensis*, a species with distylous and homostylous populations.
- We genotyped plants from 20 populations throughout the entire range in south-west China using the chloroplast intergenic spacer (*trnL-trnF*), nuclear internal transcribed spacer (ITS) and 10 nuclear microsatellite loci, and determined the genetic relationships among populations and the variation in floral traits associated with homostyle evolution.
- The marker data identified two multi-population lineages (Tibet and Sichuan) and one single-population lineage (Yunnan), a pattern consistent with at least two independent origins of homostyly. Evidence from flower and pollen size variation is consistent with the hypothesis that transitions to selfing have arisen by the same genetic mechanism involving recombination and/or mutation at the distyly linkage group. Nevertheless, flowers of homostylous lineages have followed divergent evolutionary trajectories following their origin, resulting in populations with both approach and reverse herkogamy.
- Our study illustrates a rare example of the near-complete replacement of sexual polymorphism by floral monomorphism in a heterostylous species.

## Introduction

The evolutionary pathway from outcrossing to high levels of self-fertilization has been followed in numerous taxa of herbaceous flowering plants. Because of the profound influence of this change in mating system on the biology of populations, there has been sustained interest in the determination of the selective mechanisms and evolutionary consequences of the transition (Stebbins, 1974; Igic & Busch, 2013; Wright *et al.*, 2013; Barrett *et al.*, 2014). Of particular value for studies of mating system transitions are species in which outcrossing and selfing populations are maintained across the geographical range (e.g. Busch, 2005; Moeller & Geber, 2005; Barrett *et al.*, 2009). Species with intraspecific variation in mating system provide opportunities to determine the genetic and evolutionary relationships among populations and the geographical context in which selfing evolves. Molecular phylogeography provides a powerful tool for the investigation of the evolutionary history of mating system evolution, although such approaches have seldom been employed to analyze intraspecific variation in species with outcrossing and selfing populations.

A classic example of the transition from outcrossing to selfing involves the breakdown of the sexual polymorphism heterostyly to a monomorphic condition. This transition often has a relatively simple genetic basis and is usually followed by a characteristic sequence of changes to floral morphology and the morph structure of populations (Barrett, 1989; Weller, 1992). Heterostylous populations are composed of two (distyly) or three (tristyly) morphs that differ reciprocally in stigma and anther position, and possess a suite of ancillary polymorphisms of stigmas and pollen (Darwin, 1877; Barrett, 1992). Heterostylous populations commonly exhibit a heteromorphic incompatibility system that prevents self- and intramorph mating, thus enforcing phenotypic disassortative mating and resulting in equal morph ratios. In most heterostylous families, species also occur that are monomorphic for style length, with populations comprising a single floral form with anthers and stigmas close together within a flower. These plants are generally self-compatible and predominantly selfing as a result of autonomous self-pollination (Ganders, 1979). Following Darwin (1877), these forms are referred to as homostyles and are evolutionarily derived from heterostylous ancestors.

Investigations of the genetic basis of homostyly indicate that this condition can arise by rare crossovers or mutations in the linkage group (supergene) that governs the distylous syndrome (Ernst, 1955; Dowrick, 1956; Baker, 1966; Charlesworth & Charlesworth, 1979; Lewis & Jones, 1992; Barrett & Shore, 2008; Huu *et al.*, 2016; Li *et al.*, 2016). However, an alternative pathway to homostyly involves gradual changes to sex organ position as a result of modifier genes unlinked to the distyly linkage group (Ganders, 1979), and unlinked modifiers altering style length and stamen height have been reported in cultivated lines of *Primula sinensis* (Mather & de Winton, 1941). The patterns of phenotypic variation in natural populations of several distylous species are suggestive of this mechanism of homostyle origin (Ganders, 1975; Brys & Jacquemyn, 2015), although genetic analyses have not been conducted. Homostyles can either have long styles and long-level stamens ('long homostyles') or short styles and short-level stamens ('short homostyles'). Long homostyles are more commonly encountered, either as variants in distylous populations (Crosby, 1949; Bodmer, 1960) or as the phenotypes of derived monomorphic species in distylous genera (Ernst, 1955; Ray & Chisaki, 1957; Barrett & Shore, 1987; de Vos *et al.*, 2014). Theoretical analyses by Dowrick (1956) and Charlesworth & Charlesworth (1979) have provided an explanation for the preferential spread and fixation of long homostyly.

In common with transitions from outcrossing to selfing in non-heterostylous groups, the breakdown of heterostyly is frequently associated with striking alterations to floral morphology and sex allocation. Autogamous species usually possess a suite of floral traits including smaller, less showy flowers, reduced investment in pollen and nectar production, and the loss of herkogamy (spatial separation of anthers and stigmas). These changes to floral biology comprise the selfing syndrome (Lloyd, 1965; Sicard & Lenhard, 2011). However, the evolution of the selfing syndrome is not an inevitable consequence of the evolution of homostyly and depends on a variety of factors, including the history of selfing and the costs, benefits and intensity of selfing in lineages (Barrett & Shore, 1987; Li & Johnston, 2001; de Vos *et al.*, 2014).

*Primula* provides rich opportunities to investigate the evolution of selfing from outcrossing. Beginning with Darwin's seminal work on heterostyly in *Primula* (summarized in Darwin, 1877), the genus has been a focus of attention for over a century and is probably the most well-studied heterostylous taxon (Mast & Conti, 2006; Gilmartin, 2015). Of the *c.* 430 species of *Primula*, 92% are distylous with 45 species monomorphic for stylar condition (Richards, 2003; Mast *et al.*, 2006; de Vos *et al.*, 2014). Monomorphism is scattered among 19 of the 38 sections in the genus, 18 of which contain distylous species. Ancestral state reconstructions indicate that stylar monomorphism in *Primula* has been repeatedly derived from distyly (Mast *et al.*, 2006). A comparative analysis of floral traits of 124 *Primula* species (de Vos *et al.*, 2014), including nine independent transitions from distyly to homostyly, found that, although many species have evolved smaller flowers and features of the selfing syndrome, other homostylous species

possess large flowers despite reduced levels of herkogamy. In most *Primula* species, populations are either exclusively distylous or homostylous. *Primula chungensis*, a diploid, short-lived, self-compatible species endemic to south-west China, was formerly thought to include only homostylous populations (Ernst, 1955); however, more recent work has reported distyly (Richards, 2003). Our preliminary observations of *P. chungensis* revealed three distinct types of population morph structure: distylous populations, homostylous populations and mixed populations containing both distylous and homostylous forms. These patterns of variation motivated the present study and provided us with an unusual opportunity to address several questions of general relevance to the breakdown of heterostyly to homostyly.

Here, we use a broad geographical sampling of populations throughout the range of *P. chungensis* to examine the evolutionary history of distyly and homostyly. By investigating range-wide phylogeographic patterns inferred from plastid and nuclear data, in concert with an analysis of variation in floral traits, we address the following questions. (1) Is there evidence that homostylous populations are derived from distylous populations and, if so, has the transition occurred more than once in different parts of the range? (2) Are homostyles of *P. chungensis* long homostyles? If so, have they originated by recombination and/or mutation in the distylous linkage group, or by an alternative mechanism involving direct descent from the long-styled morph through gradual modifications to stamen height governed by unlinked modifiers? (3) Do homostyles in distylous populations differ in floral morphology from those that occur in monomorphic populations? We predicted that the degree of floral modification from heterostylous ancestors in homostylous forms would be greatest in monomorphic populations and less so where they coexist with distylous morphs. Our study provides the first examination of the historical processes governing the breakdown of distyly to homostyly and reveals unexpected and contrasting trajectories of floral evolution in selfing populations.

## Materials and Methods

### Study species

*Primula chungensis* Balf. f. et Ward (Primulaceae; section *Proliferae*) is a perennial herb restricted to China and distributed from the south-east margin of the Tibetan plateau to the Hengduan mountains of Yunnan and Sichuan between 2900 and 3200 m (Hu & Kelso, 1996; Richards, 2003). It occurs in wet meadows, forest edges, open slopes and roadsides. Flowering occurs from the middle of May to the end of June, with each flower lasting up to 5 d. Flowers are visited primarily by bees, butterflies and moths. Controlled pollinations of *P. chungensis* indicate that distylous and homostylous plants are uniformly self- and intramorph compatible (X. F. Jiang *et al.*, unpublished). Thus, the species does not possess the typical heteromorphic incompatibility system found in heterostylous plants, including many *Primula* species (Richards, 2003).

## Population sampling and morph structure

To investigate population morph structure and variation in floral morph frequencies (long-styled, short-styled, homostyled; hereafter L-, S- and H-morphs, respectively), we sampled 20 populations covering the entire range of *P. chungensis* (Supporting Information Table S1). Nineteen of the 20 populations were either on the southern portion of the Tibetan plateau or in the mountains of south-west Sichuan. The only known population of *P. chungensis* in Yunnan province was also included in our sample. We recorded the representation and frequencies of morphs by inspecting all flowering plants in populations with  $\leq 100$  individuals at peak flowering. In larger populations, we sampled at 0.5–1 m intervals along transects positioned 1–2 m apart. We determined whether the frequencies of the L- and S-morphs in polymorphic populations deviated from the expected equilibrium ratio of 1 : 1 using *G*-tests (Sokal & Rohlf, 1995). In each population, we also sampled leaf tissue from 15 individuals that was dried in silica gel for sequencing and genotyping.

## Molecular procedures

We extracted total genomic DNA from dried leaf tissue using a modified cetyl trimethyl ammonium (CTAB) protocol (Doyle, 1991). Quantification of DNA was checked by electrophoresis on 0.8% agarose gels, and the concentration was determined using a SmartSpec™ Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). We conducted an initial screen for DNA sequence variability at various chloroplast markers using universal primers (Shaw *et al.*, 2007) on 20 samples comprising one individual from each population. We chose the chloroplast DNA (*cpDNA*) *trnL-trnF* intergenic spacer and nuclear internal transcribed spacer (ITS) for the full survey, because they contained the most polymorphic sites. We sequenced purified DNA in both directions by standard methods on an ABI Prism 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). We used 10 primer pairs (PC15519, PC29976, PC34870, PC39450, PC46911, PC14297, PC21731, PC39361, PC109 and PC50689) to assess population genetic structure at microsatellite loci following Zhou *et al.* (2016). Further details of the molecular procedures, including PCR amplification, are given in Methods S1.

## Data analysis of sequences

We assessed the phylogenetic relationships between haplotypes of *P. chungensis* with data partitioned by *cpDNA* and nuclear ribosomal DNA (nrDNA) using maximum parsimony (MP) and Bayesian inference, with *P. chrysochlora* and *P. prenantha* (section *Proliferae*) as outgroups. The MP analysis was conducted in PAUP v.4.10b (Swofford, 2002), employing heuristic tree searches with 100 replications of random sequence entries, using TBR (tree bisection–reconnection) branch swapping, with MULTREES and COLLAPSE options selected. We generated bootstrap values for all nodes by 1000 iterations of the original dataset with the above settings, and then a majority rule consensus tree was created. We

used MRMODELTEST v.2.2 (Nylander, 2004) with the Akaike information criterion (AIC) (Posada & Buckley, 2004) to determine the most appropriate model of sequence evolution for each dataset. We used MRBAYES v.3.1.2 (Ronquist & Huelsenbeck, 2003) to find the optimal tree topology and to calculate Bayesian posterior probabilities (PPs) for the dataset. Four runs were made, each to 10 million generations, with trees sampled every 1000 generations. Trees generated before the four Markov chains reached stationarity were discarded. The remaining trees were used to construct a majority rule consensus tree. In addition, we determined the relatedness among haplotypes by constructing median-joining networks using NETWORK v.4.6 (Bandelt *et al.*, 1999) with inserts and indels within all *cpDNA* and ITS sequences treated as a single character resulting from one mutation.

## Data analysis of simple sequence repeat (SSR) markers

We tested for Hardy–Weinberg equilibrium (HWE) for each locus and population using GENEPOP v.1.2 (Raymond & Rousset, 1995). We calculated allele frequencies at microsatellite loci, as well as the number of alleles per locus ( $N_A$ ), observed and expected heterozygosity ( $H_O$ ,  $H_E$ ), effective number of alleles ( $N_E$ ) and allelic richness ( $A_R$ ), using the program FSTAT v.2.9.3 (Goudet, 1995). We compared the patterns of genetic diversity and morph ratios of populations using regression models, in which the relation between homostyle frequency and genetic parameters was analyzed using generalized least squares (GLS) to estimate regression parameters. We predicted that, because homostyles have a greater tendency to self-pollinate than distylous forms, their frequency within populations should reduce genetic diversity.

To assess the most probable origin(s) of monomorphic populations and to determine the relationships between dimorphic and mixed populations, we used three approaches to investigate the patterns of differentiation among populations with different morph structure. First, we used the original genotypic data to calculate a Euclidean distance matrix between all samples, and created a visual representation of the genetic relationships among populations using principal coordinates analysis (PCoA) in the program MVSP v.1.3 (Kovach, 1999). Second, we calculated Nei's (1987) unbiased genetic distance ( $D$ ) among all possible pairs of populations from allele frequencies estimated in the program microsatellite analyzer (MSA) (Dieringer & Schlötterer, 2003). We then constructed a consensus neighbor-joining (NJ) tree based on pairwise estimates of genetic distance, using 2000 bootstrap trees and random input order in PHYLIP v.3.63 (Felsenstein, 2005). Finally, we used the program STRUCTURE v.2.2 (Pritchard *et al.*, 2000) to infer the number of genetic clusters ( $K$ ) in our entire dataset under the 'no admixture' and 'uncorrelated allele frequencies' model. Clusters were set from 1 to 8, and 10 independent runs were performed at  $10^5$  Markov Chain Monte Carlo simulations after a burn-in of  $5 \times 10^4$  iterations. We estimated the most likely number of clusters based on the log probability of the data between successive  $K$  values (Evanno *et al.*, 2005).

## Floral morphology

To investigate the differentiation of floral traits among distylous, mixed and homostylous populations, we obtained a random sample of flowers from each of the 20 populations during peak flowering in 2014. The total sample contained 1183 flowers; the average numbers of plants and flowers sampled in each population were 29.57 and 59.15, respectively (range: plants, 20–72; flowers, 40–150). Only flowers in which the style was fully elongated were sampled. Flowers were classified according to floral morph before being preserved in 70% ethanol for subsequent measurement. Our samples comprised 200, 199 and 784 flowers for the L-, S- and H-morphs, respectively. The following five traits were measured on each flower: stigma and anther height, herkogamy, corolla tube length and corolla diameter. All measurements were taken to the nearest 0.01 mm using digital calipers. To assess the overall variation in each trait, we calculated the mean, standard deviation and coefficients of variation. We explored the relations among floral trait variation, region (or lineage) and floral morph using generalized linear mixed models (GLMMs) (Pinheiro & Bates, 2000; Zuur *et al.*, 2009) with a Gaussian distribution and restricted maximum likelihood (REML). The five traits were summarized using principal components analysis (PCA). Scores of the PCA were then used as the dependent variable, with region and floral morph as fixed effects and population as random effect (packages 'lme4', Bates *et al.*, 2012).

To examine whether monomorphic populations were composed of long homostyles or L-morph plants with modified 'short-level' stamens, we used pollen size to discriminate between these alternatives. The pollen size of long homostyles should be significantly larger than pollen from the L-morph because the stamens of this morph are of a fundamentally different origin than short-level anthers of the L-morph (see Barrett & Shore, 2008). We sampled 20 buds from each morph in each population, and the stigma and anther heights of mature flowers on the same plant were recorded. We digitally photographed pollen grains (L-morph,  $n=561$  flowers; S-morph,  $n=559$  flowers; H-morph,  $n=1547$  flowers) with an Olympus optical microscope, and used ImageJ software to measure the equatorial axis of pollen grains to the nearest 0.01  $\mu\text{m}$ . We then employed normal family functions to fit pollen size distributions and estimated parameters using the maximum likelihood (ML) method in R v.3.1.1 (R Development Core Team, 2014).

## Results

### Population morph structure

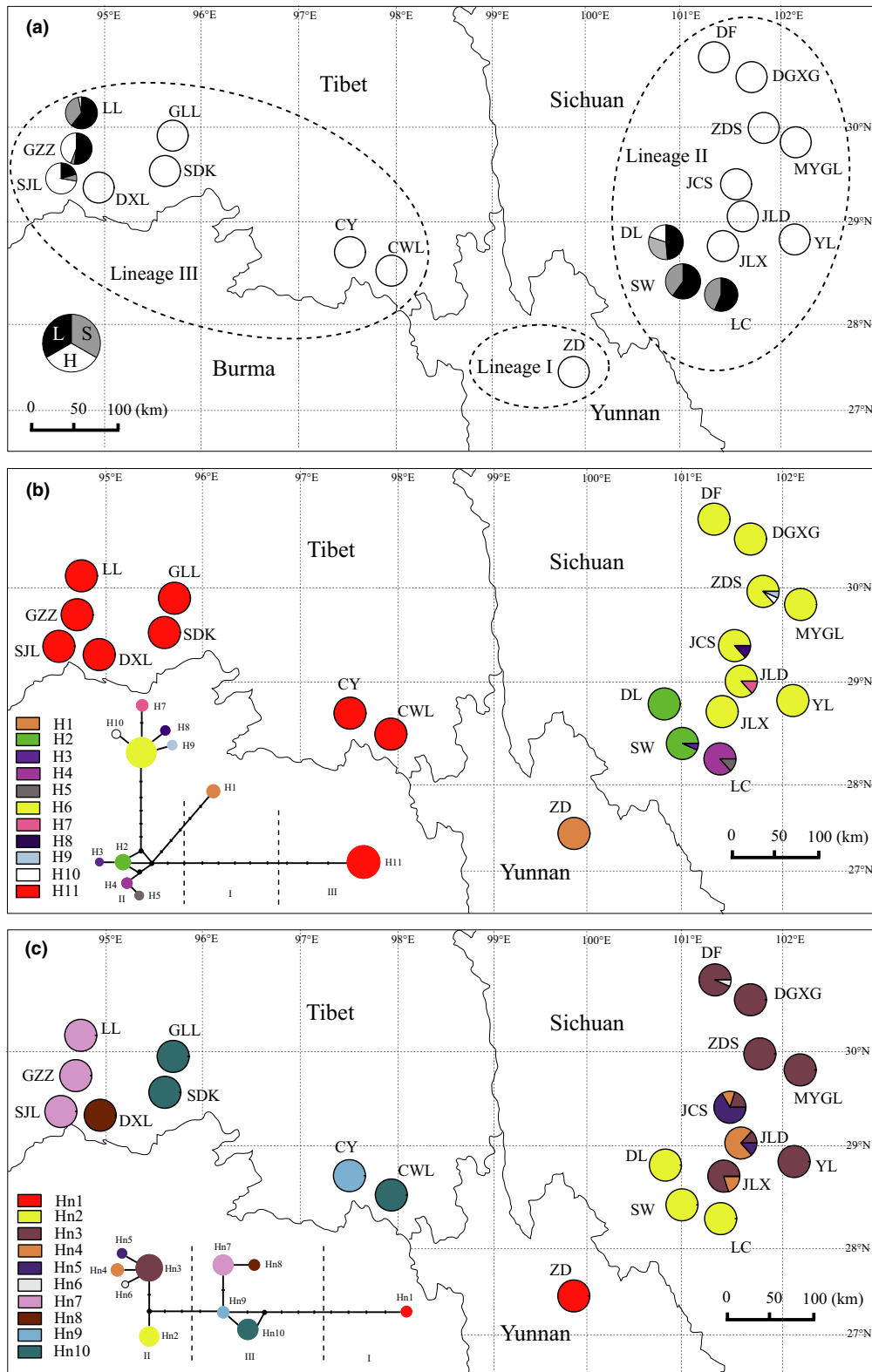
Of the 20 sampled populations of *P. chungensis*, 14 were monomorphic, containing only homostylous plants, and the remaining six populations were either distylous (two populations) or contained a mixture of distylous and homostylous morphs (four populations). Homostylous populations were located in the western (DXL, GLL, SDK, CY and CWL), central (ZD) and

eastern (JLX, JLD, YL, JCS, MYGL, ZDS, DGXG and DF) portions of the geographical range. Two distylous populations (SW and LC) and one mixed population (DL) occurred at the south-eastern periphery of the eastern concentration of homostylous populations in Sichuan. The other three mixed populations (LL, GZZ and SJL) were located at the western margin of the range on the Tibetan plateau (Fig. 1a). The morph ratios of the two dimorphic populations were weakly L-morph biased, but not significantly different from 1 : 1 (SW:  $G=1.45$ ,  $P=0.227$ ; LC:  $G=1.86$ ,  $P=0.172$ ). By contrast, in mixed populations, distylous morph frequencies were significantly L-morph biased in three of the four populations (DL:  $G=3.06$ ,  $P=0.08$ ; LL:  $G=17.8$ ,  $P=0.04$ ; GZZ:  $G=88.1$ ,  $P<0.01$ ; SJL:  $G=13.0$ ,  $P<0.01$ ). There was considerable variation in the frequencies of homostyles in mixed populations, ranging from 3.0% in population LL to 71.9% in population SJL (Table 1).

### Patterns of variation in *trnL-trnF* and ITS

From the 300 individuals sampled, we recovered 11 and 10 unique sequences for the *trnL-trnF* and ITS fragments, respectively. The *trnL-trnF* intergenic spacer varied from 448 to 456 bp and exhibited considerable polymorphism, with eight substitutions and 11 indels (1–6 bp). These polymorphisms comprised a total of 11 haplotypes (H1–H11) and their geographical distribution is illustrated in Fig. 1(b). The eight populations located on the south-eastern Tibetan plateau were fixed for the unique haplotype (H11); the only population (ZD) known from Yunnan was fixed for another haplotype (H1). The remaining haplotypes occurred in Sichuan, of which the most common (H6) was restricted to the eight homostylous populations in this region. Haplotype H2 was shared by two distylous populations, DL and SW, and was inferred to be the ancestral haplotype of *P. chungensis* according to the results of network analysis (Fig. 1b). Phylogenetic trees constructed using MP and Bayesian methods were largely congruent in topology (Fig. 2). All *cpDNA* haplotypes clustered into three lineages: Tibetan, Yunnan and Sichuan. In the strict consensus tree (Fig. 2a), with PPs and bootstrap values assigned, haplotypes H2–H10 formed a clade (Sichuan lineage) with 0.96 PP and 100% bootstrap support (BS), whereas the other two haplotypes formed the Yunnan lineage (H1) and Tibetan lineage (H11), respectively.

The ITS sequences had a length of 640 bp, in which 11 sites (substitutions) were variable, and 10 haplotypes (Hn1–Hn10) were identified. Haplotypes Hn1, Hn8 and Hn9 were unique to populations ZD, DXL and CWL, respectively; haplotypes Hn2 and Hn7 were restricted to the three distylous and/or mixed populations from Sichuan (DL, SW and LC) and the Tibetan plateau (LL, GZZ and SJL), respectively. Haplotype Hn3 was shared by all homostylous populations in Sichuan. Consistent with the observed variation in chloroplast haplotypes, the ITS haplotypes for *P. chungensis* also clustered into the same three lineages with high PP and BS: Sichuan lineage containing Hn2–Hn6 (PP 1; BS 84%); Tibetan lineage containing Hn7–Hn10 (PP 1; BS 83%); and Yunnan lineage containing Hn1 (Fig. 2b).



**Fig. 1** Geographical distribution of the 20 populations of *Primula chungensis* from south-west China investigated in this study. (a) Floral morph frequencies in populations; black, gray and white segments indicate the frequencies of the long-styled, short-styled and homostyled morphs, respectively. (b) Distribution and median-joining networks of 11 chloroplast DNA (*cpDNA*, *trnL-trnF*) haplotypes. (c) Distribution and median-joining networks of 10 internal transcribed spacer (ITS) haplotypes. In the median-joining networks, the size of circles is proportional to the overall frequency of each haplotype in the entire sample of the species. Each solid line represents one mutational step that interconnects two haplotypes for which parsimony is supported at the 95% confidence level. The small black circles indicate hypothetical missing haplotypes. I, Yunnan lineage; II, Sichuan lineage; III, Tibetan lineage. Population codes are identified in Table 1.

**Table 1** Summary data for *Primula chungensis* populations sampled for morph ratios, chloroplast DNA (*cpDNA*, *trnL-trnF*) and internal transcribed spacer (ITS) variation from south-west China: population code; floral morph ratio; and frequencies of chloroplast (H1–H11) and nuclear (Hn1–Hn10) haplotypes

Region/locality (population code)	Population size	Morph frequency			Sample size	<i>cpDNA</i> haplotypes (individuals sampled)	ITS haplotypes (individuals sampled)
		L	S	H			
Yunnan							
Shangri-La (ZD)	~500	0	0	1.00	257	H1(15)	Hn1(15)
Sichuan							
Donglang (DL)	~350	0.49	0.31	0.20	160	H2(15)	Hn2(15)
Shawang (SW)	~300	0.57	0.43	0	166	H2(14), H3(1)	Hn2(15)
Linchang (LC)	~200	0.55	0.46	0	155	H4(13), H5(2)	Hn2(15)
Jiulongxi (JLX)	>500	0	0	1.00	380	H6(15)	Hn3(12), Hn4(3)
Jiulongdong (JLD)	>500	0	0	1.00	407	H6(13), H7(2)	Hn3(2), Hn4(11), Hn5(2)
Jichoushan (JCS)	>1000	0	0	1.00	300	H6(13), H8(2)	Hn3(3), Hn4(2), Hn5(10)
Yele (YL)	>1000	0	0	1.00	300	H6(15)	Hn3(15)
Moyugonglu (MYGL)	~500	0	0	1.00	180	H6(15)	Hn3(15)
Zheduoshan (ZDS)	~100	0	0	1.00	80	H6(13), H9(1), H10(1)	Hn3(15)
Dongguxiagu (DGXG)	~500	0	0	1.00	270	H6(15)	Hn3(15)
Daofu (DF)	>1000	0	0	1.00	500	H6(15)	Hn3(14), Hn6(1)
Tibet							
Lulang (LL)	~300	0.61	0.36	0.03	292	H11(15)	Hn7(15)
Gongzuoan (GZZ)	~400	0.52	0.04	0.44	211	H11(15)	Hn7(15)
Sejila (SJL)	~300	0.21	0.07	0.72	214	H11(15)	Hn7(15)
Chawalong (CWL)	~200	0	0	1.00	122	H11(15)	Hn10(15)
Chayu (CY)	~100	0	0	1.00	83	H11(15)	Hn9(15)
Suidaokou (SDK)	~100	0	0	1.00	68	H11(15)	Hn10(15)
Galongla (GLL)	>1000	0	0	1.00	435	H11(15)	Hn10(15)
Duoxiongla (DXL)	~200	0	0	1.00	120	H11(15)	Hn8(15)

L, S and H refer to the long-styled, short-styled and homostyled morphs, respectively.

### Population structure inferred from SSR

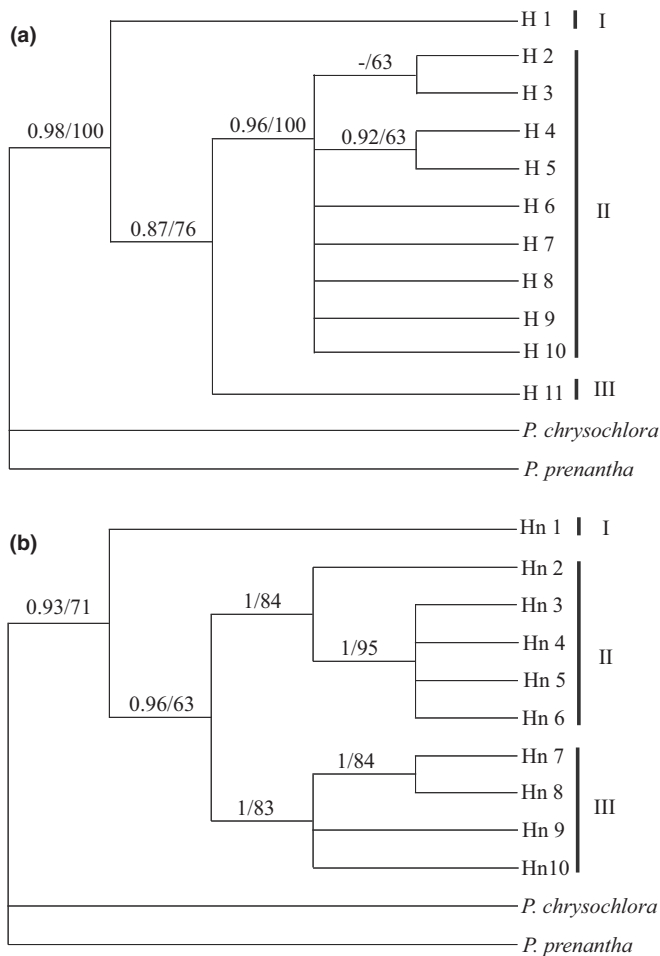
The total number of alleles per locus in our sample of 300 individuals ranged from four (PC109) to 22 (PC29450), with a total of 102 alleles distributed among the 10 microsatellite loci. Departures from HWE varied among loci as a result of an excess number of homozygotes. Genetic diversity estimates also varied among populations:  $N_A$  ranged from 1.70 to 3.20 (mean  $\pm$  SE,  $2.43 \pm 0.08$ ),  $N_E$  from 1.52 to 2.16 ( $1.86 \pm 0.04$ ),  $H_O$  from 0.41 to 0.71 ( $0.64 \pm 0.03$ ) and  $H_E$  from 0.25 to 0.49 ( $0.39 \pm 0.02$ ).  $A_R$  estimates among all populations ranged from 3.40 to 10.54, with an average of 6.27. As predicted, there was a significant negative relation between homostyle frequency and genetic diversity, as indexed by  $N_A$  ( $r = -0.0062$ ,  $P = 0.014$ ) and  $N_E$  ( $r = -0.0018$ ,  $P = 0.039$ ), although both relations were weak and populations fixed for homostyles exhibited a wide range of values.

Two-dimensional PCoA of SSR phenotypes (Fig. S1) separated all individuals of the Sichuan lineage from the other two lineages along the first axis, with PCoA1 explaining 81.75% of the total variance. The Yunnan lineage was separated from the Tibet samples along PCoA2 (10.63%). Individuals of the Sichuan lineage clearly clustered by population morph structures along PCoA2, but this pattern was not evident in the Tibetan lineage (Fig. S1). The population-based NJ tree (Fig. 3) confirmed the strong pattern of regional differentiation separating the species into two multi-population lineages (i.e. Tibet and Sichuan) and one single-population lineage (i.e. Yunnan). In the

SSR admixture analysis using STRUCTURE, the values of the log-likelihood of the data ( $\log_e \Pr(K)$ ) increased progressively with cluster number ( $K$ ), but delta  $K$  indicated that the best fit for  $K$  was 3. At  $K = 3$ , all populations from the Tibetan plateau clustered into one group (Tibetan lineage), and populations from Sichuan clustered into another group (Sichuan lineage), with the last remaining population (ZD) forming an independent group (Yunnan lineage). Using  $K = 2$ , population ZD merged with the Sichuan lineage.

### Variation in floral traits

Data on the mean, standard deviation and coefficients of variation of floral traits for all populations are presented in Table S2. In general, floral traits in populations of the Tibetan and Yunnan lineages were larger in size compared with populations in the Sichuan lineage. This pattern was also associated with larger vegetative traits (data not shown). These results were confirmed by GLMMs, which indicated overall geographic differentiation in flower size between the two main lineages ( $F_{1,13} = 17.41$ ,  $P < 0.01$ ) and morphs ( $F_{3,38} = 236.82$ ,  $P < 0.001$ ). Corolla width was significantly larger in flowers of the L-morph compared with the S-morph (Fig. 4a), whereas corolla tube length was larger in the S-morph (Fig. 4b). Significantly, the flowers of homostyles in mixed populations were of similar size to the S-morph, but differed significantly from homostyles in monomorphic populations (Fig. 4a,b). Within the Tibetan lineage, flowers of homostyles in monomorphic populations had significantly shorter and narrower



**Fig. 2** Strict consensus trees obtained by analysis of (a) 11 chloroplast DNA (*cpDNA*, *trnL-trnF*) and (b) 10 internal transcribed spacer (ITS) haplotypes of *Primula chungensis*, with *P. chrysochlora* and *P. prenantha* as outgroups. Lineage codes: I, Yunnan; II, Sichuan; III, Tibet. The numbers on the left of the branches indicate posterior probabilities from the Bayesian analysis; the numbers on the right indicate bootstrap values (> 50%).

corolla tubes and corolla lobes compared with homostyles in mixed populations, but the opposite pattern was evident in the Sichuan lineage.

Measurements of flowers pooled across all three population morph structures indicated that the heights of stigmas and anthers in the L- and S-morphs exhibited a clear bimodal distribution, whereas these organs showed a unimodal distribution in homostyles (Figs S2, S3). Average stigma–anther separation was significantly smaller in the L- compared with the S-morph (L-morph = 5.547 mm, SD = 1.107; S-morph = 3.163 mm, SD = 0.776;  $t = 24.892$ ,  $P < 0.001$ ) (Fig. 5). As expected, stigma–anther separation in homostyles was significantly reduced in comparison with the distylous morphs (mean = 1.187 mm, SD = 1.530;  $F = 1543$ ,  $P < 0.001$ ). However, the relative positions of sexual organs exhibited wide variation (Fig. 5), ranging from flowers with approach herkogamy to flowers with reverse herkogamy (stigmas below anthers). All homostyles in monomorphic populations in Sichuan and Yunnan, except population

ZDS, had stigmas either at the same level as the anthers or above the stigmas (Fig. 5b). By contrast, in the Tibetan lineage population, CY and CWL exhibited approach herkogamy, whereas flowers in populations DXL, GLL and SDK more commonly displayed reverse herkogamy (Fig. 5a). The most prominent feature of homostyles in mixed populations (GZZ, SJL and DL) was that they retained higher overall levels of stigma–anther separation involving reverse herkogamy than was evident in monomorphic populations, with the exception of population DXL.

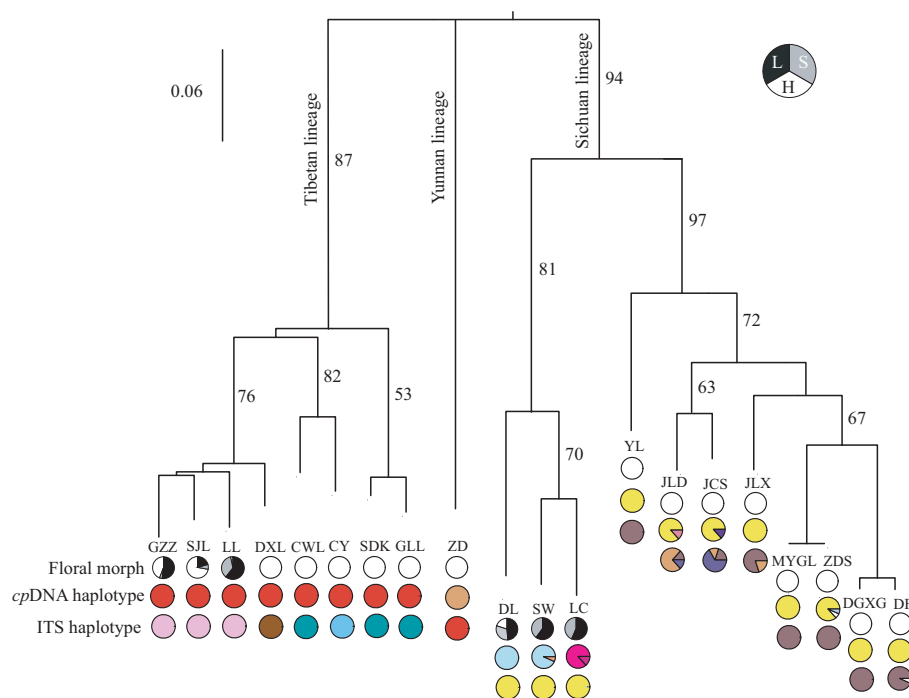
In common with most heterostylous species, pollen grains from the L- and S-morphs of *P. chungensis* differed markedly in size (Fig. S4). Pollen grains of the S-morph were *c.* 1.5 times larger than those of the L-morph with respect to the equatorial axis (L-morph:  $n = 561$ , mean = 7.93  $\mu\text{m}$ ; S-morph:  $n = 559$ , mean = 11.76  $\mu\text{m}$ ;  $t = 91.96$ ,  $df = 1103.1$ ,  $P < 0.001$ ). Variation in pollen size was normally distributed (Tibetan lineage:  $\mu = 7.77$ ,  $\sigma = 0.59$  for L-morph;  $\mu = 11.74$ ,  $\sigma = 0.68$  for S-morph; Sichuan lineage:  $\mu = 8.18$ ,  $\sigma = 0.66$  for L-morph;  $\mu = 11.79$ ,  $\sigma = 0.79$  for S-morph), and showed a clear bimodal distribution (Fig. 6). The mean pollen size of homostylous plants pooling across all monomorphic and mixed populations ranged from 11.32  $\mu\text{m}$  (in population GZZ) to 12.82  $\mu\text{m}$  (in population JLD), with an overall average size of 12.11  $\mu\text{m}$  ( $n = 1547$ ). Significantly, the distributions of homostylous pollen size within each population largely overlapped with the pollen size of the S-morph, but not the L-morph (Figs 6, S4).

## Discussion

Our analysis of intraspecific variation in *P. chungensis* populations revealed several key findings. (1) The patterns of molecular variation indicated that the species was initially split into two major lineages in Tibet and Sichuan. Both lineages are dominated by homostylous populations today, with a few distylous and/or mixed populations restricted to small areas within the range of each lineage (Fig. 1a). The patterns of molecular differentiation are consistent with at least two independent transitions from distyly to homostyly followed by homostyle spread. (2) Homostylous populations exhibited different degrees of herkogamy, including populations from Sichuan with mainly approach herkogamy and populations from Tibet that more commonly possessed reverse herkogamy (Fig. 5). (3) Data on pollen size variation (Fig. 6) indicated that homostyles have a similar range of sizes to pollen from long-level anthers of the S-morph, a pattern consistent with the hypothesis that these forms are long homostyles that have arisen by recombination and/or mutation at the distyly linkage group, although unlinked modifiers may also contribute to variation in sex organ heights and pollen size variation. We now examine the historical and contemporary ecological and demographic factors that may explain the novel patterns of floral variation revealed by our study.

## Origins of homostyly

In contrast with the relatively gradual evolution of selfing from outcrossing characteristic of many angiosperm lineages, high



**Fig. 3** Neighbor-joining tree illustrating the genetic relationships among 20 populations of *Primula chungensis*, based on Nei's (1987) unbiased genetic distance calculated from simple sequence repeat (SSR) data. Circles at the terminal end of the branches indicate the floral morph frequencies and distribution of chloroplast DNA (*cpDNA*, *trnL-trnF*) and internal transcribed spacer (ITS) haplotypes in each population. Floral morphs and haplotypes are color coded as in Fig. 1. Numbers by the nodes are bootstrap values (> 50%) from 1000 replicates. Population codes and positions are identified in Table 1 and Fig. 1, respectively.

selfing in distylous groups can arise in a single generation by recombination and/or mutation in the linkage group governing heterostyly. Rare crossover events give rise to homostyles that combine the female and male sex organs of the L- and S-morphs in a single phenotype. Thus, long homostylous plants possess the female sex organs of the L-morph and the male sex organs of the S-morph. Crossing studies have demonstrated that this condition is inherited as an allele at the distyly linkage group (Dowrick, 1956; Shore & Barrett, 1985). The positions of sex organs in homostyles usually guarantee autonomous self-pollination, and high selfing rates have been reported in long homostyles of *P. vulgaris* co-occurring with outcrossing distylous morphs (Piper *et al.*, 1984). High selfing rates have also been reported in homostyles in unrelated heterostylous groups (e.g. *Amsinckia* – Ganders *et al.*, 1985; *Eichhornia* – Barrett & Husband, 1990; *Turnera* – Belaussoff & Shore, 1995). Although we have not quantified the outcrossing rates of populations in this study, we assume that homostyles, because of reduced herkogamy (Fig. 5), experience higher rates of selfing than distylous morphs owing to their greater facility for autonomous self-pollination.

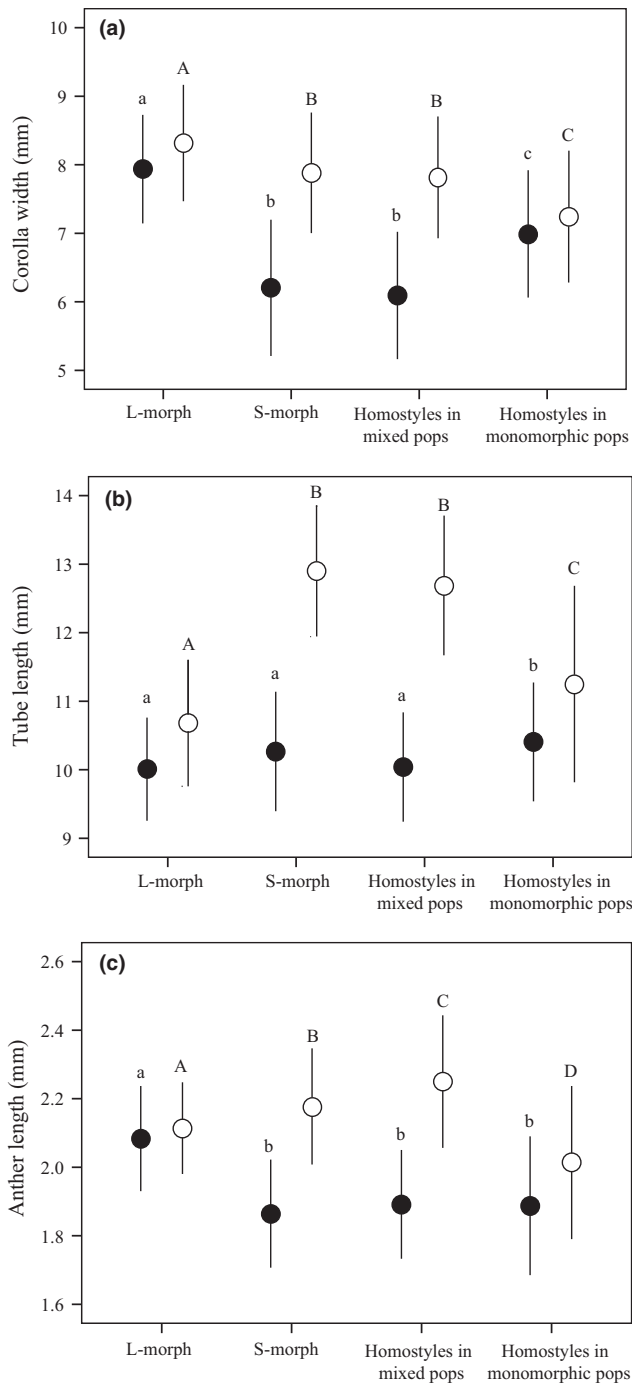
Two features of *P. chungensis* distinguish it from *P. vulgaris*, the only other *Primula* species in which populations containing distylous and homostylous morphs have been studied (Crosby, 1949; Bodmer, 1960; Piper *et al.*, 1984; Curtis & Curtis, 1985). First, in *P. chungensis*, long homostyles are well established across the entire species' range, whereas, in *P. vulgaris*, they are restricted to two small regions in the UK in which they coexist with distylous morphs. In *P. vulgaris*, the vast majority of populations in the UK and elsewhere in Europe are distylous and the breakdown to distyly is a local phenomenon. Second, *P. vulgaris* possesses heteromorphic incompatibility (Darwin, 1877), whereas the distylous morphs in *P. chungensis* are self-compatible. These

differences provide an alternative hypothesis for the origin of homostyly in *P. chungensis* that does not involve genetic changes at the distylous linkage group.

If self-compatibility enabled L-morph colonists of *P. chungensis* to establish and spread, owing to reproductive assurance, this process could account for widespread monomorphism. This scenario assumes that genetic modifiers of stamen position subsequently spread in L-morph populations to produce the range of observed herkogamy variation. Styler monomorphism in this situation would be guaranteed because, in *Primula*, the L-morph breeds true being homozygous recessive at the *S* locus (Lewis & Jones, 1992). By contrast, S-morph colonists would segregate L-morph plants on selfing, making it doubtful that widespread monomorphism arose from this morph. Therefore, this hypothesis predicts that monomorphic populations of *P. chungensis* have originated directly from the L-morph. Elsewhere, this process has been proposed for the evolution of L-morph monomorphism in a small number of populations of distylous *Luculia pinceana* of the Rubiaceae (Zhou *et al.*, 2012). One of the objectives of this study was therefore to attempt to distinguish between these alternative hypotheses for the origin of homostyles in *P. chungensis*.

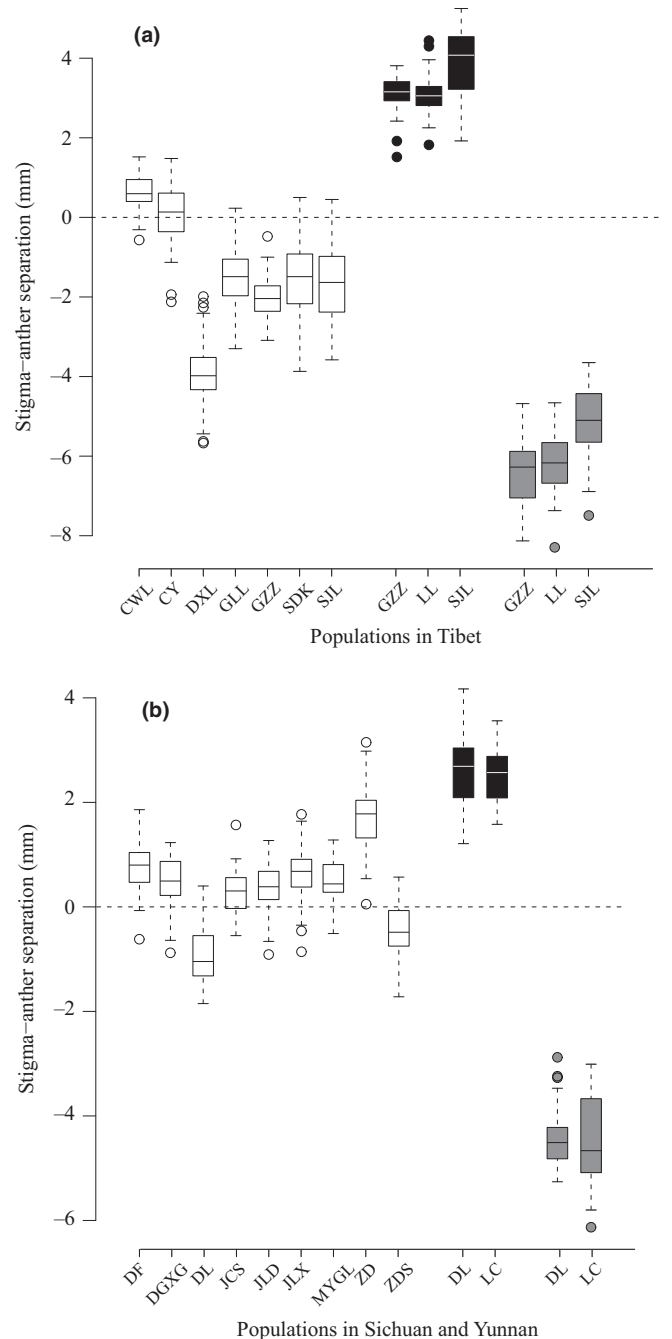
Unfortunately, it was not possible with our data to firmly reject either of the two possible genetic pathways for homostyle origin, although the weight of evidence supports genetic changes at the *S* locus, rather than a process involving only unlinked modifiers. Our investigation of pollen size variation is consistent with the hypothesis that monomorphic populations originated through recombination and/or mutation at the *S* locus. The range of pollen sizes among all monomorphic populations fell within the range of variation exhibited by the S-morph and were consistently larger than pollen of the L-morph (Fig. 6), a pattern





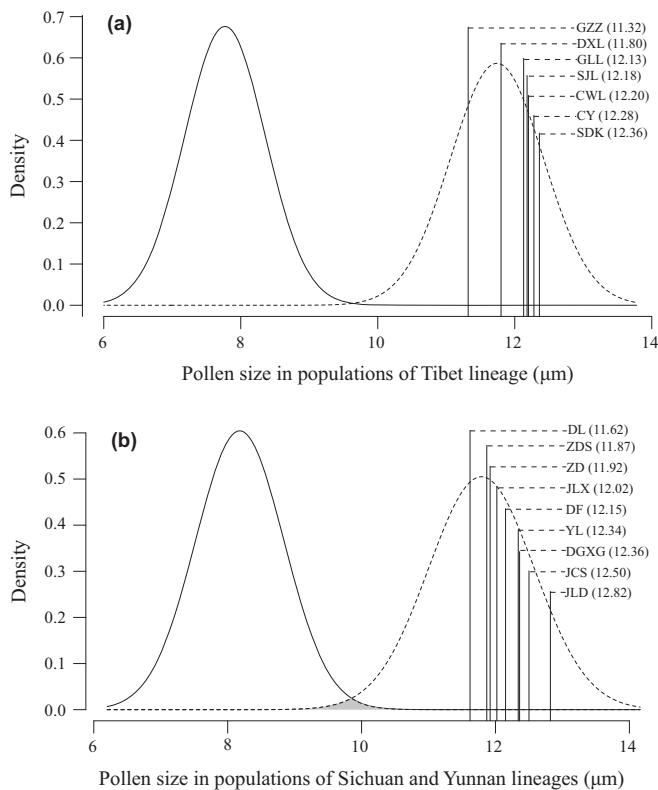
**Fig. 4** Comparison of floral traits (mean  $\pm$  SE) between lineages and floral morphs in *Primula chungensis*. The data from the single population (ZD) of the Yunnan lineage were included with populations of the Sichuan lineage (see the Results section). Homostyles were distinguished depending on whether they were in mixed vs monomorphic populations. (a) Corolla width; (b) floral tube length; and (c) anther length. Open circles, Tibetan lineage; closed circles, Sichuan lineage. The floral traits of morphs sharing the same letter are not significantly different ( $P < 0.05$ ) in the Tibetan (uppercase) and Sichuan (lowercase) lineages.

predicted by the recombination hypothesis. Long homostyles in distylous groups inherit the long-level anthers and pollen size of the S-morph. It is also possible, however, that gradual evolution



**Fig. 5** Stigma-anther separation (herkogamy) of the floral morphs of *Primula chungensis*: (a) Tibet lineage and (b) Sichuan and Yunnan lineages. Each boxplot denotes a population and different colors (black, L-morph; gray, S-morph; white, homostyle) denote the floral morphs. In the boxplot, the horizontal line shows the median; the bottom and top of the box show the first and third quartiles; the circles show outliers. Population codes are identified in Table 1.

of increased pollen size could accompany elongation of short-level stamens in the L-morph, with modifications in stamen height governed by unlinked modifiers. However, among homostylous populations, there was no relation between mean pollen size and stamen height (Pearson's product-moment correlation:  $r = -0.299$ ,  $P = 0.319$ ), an unexpected pattern if pollen size and stamen height evolved in concert. Regardless of which



**Fig. 6** The distribution and means of pollen size in the floral morphs of *Primula chungensis*. (a) Pollen size of the Tibetan lineage; (b) pollen size of the Sichuan and Yunnan lineages. The solid and dotted curves indicate normal fitted density distributions of pollen size in the L-morph and S-morph, respectively. The vertical lines indicate the means of pollen size for each homostylous population. Population codes are identified in Table 1.

genetic mechanism best explains the origins of floral monomorphism, and indeed both might be involved, our data indicate that, in common with most *Primula* species, the derived phenotypes are long homostyles.

Our phylogeographic evidence indicates that at least two independent origins of long homostyly have occurred in *P. chungensis*. Homostylous populations were evident in both the Tibetan and Sichuan lineages. These lineages are separated by the 'Mekong–Salween Divide' which probably acted as a geographical barrier between populations of *P. chungensis*, as has been inferred for several other species occurring in this region of China (e.g. *Taxus* – Gao *et al.*, 2007; *Sinopodophyllum* – Li *et al.*, 2011). Microsatellite variation in populations of *P. chungensis* clustered by geographical location, and there were no nuclear and chloroplast haplotypes shared between the two lineages (Figs 1, 2), indicating a considerable period of isolation and time for homostyles to spread and form monomorphic populations in both regions. To our knowledge, no other *Primula* species exhibits this pattern of morph frequency variation in which homostylous populations are common and distylous populations are rare. This pattern is consistent with the finding that species that have the capacity for autonomous self-pollination consistently have larger geographical ranges than their outcrossing relatives (Grossenbacher *et al.*, 2015).

## Establishment and spread of homostyles

Our phylogeographic analysis identified two primary multi-population lineages with a strong concordance between chloroplast markers, nuclear gene sequences and microsatellite data. The lineages each clustered primarily by geographical location rather than population morph structure. This pattern probably reflects the independent origins of homostylous populations within each region after lineage differentiation. In the Sichuan lineage, the NJ tree indicated that all eight homostylous populations (YL, JLD, JCS, JLX, MYGL, ZDS, DGXG and DF) comprised a subclade (Fig. 3), and the genetic distance among populations was geographically correlated. Significantly, these populations shared the same chloroplast (H6) and nuclear (Hn3) haplotype with some derived low-frequency haplotypes (H7–H10 for chloroplast and Hn4–Hn6 for nuclear haplotype) scattered in a few populations. These results suggest that homostylous populations in this lineage may have originated by sequential colonization northward from the area in which populations YL to DF occur today. The three remaining populations (DL, SW, LC) of the Sichuan lineage contained distylous morphs and formed a sister subclade with the homostylous subclade. Their widely shared haplotypes (H2 and Hn2) were located at the interior of the network, implying ancestral status (Fig. 1b,c). Of these populations, DL contained a substantial frequency of homostyles (20%). However, there was no clear relationship indicating that the homostylous populations were descended from a particular distylous 'parental' population. The several missing haplotypes between subclades implies that homostyles may have dispersed from source population(s) and have been established in the colonized region for a considerable time with limited gene flow between them.

In contrast with the Sichuan lineage, where dispersal appears to have played an important role, some homostylous populations at the western edge of the Tibetan lineage may have evolved by *in situ* fixation, replacing distylous morphs in mixed populations, as modeled by Charlesworth & Charlesworth (1979). Two lines of evidence support this hypothesis. First, there was concordance between NJ and haplotype trees with mixed populations (LL, GZZ and SJL) and their nuclear haplotype (Hn7) forming a distinct terminal branch (Figs 2b, 3). Second, the fixation pathway would be expected to be a more gradual process than founder events allowing the homostylous population to inherit genetic diversity from the 'parental' population, resulting in a low level of genetic differentiation between source (mixed) and derived (homostylous) populations. Our microsatellite data conformed to this pattern; there was no clear relation between population morph structure and genetic distance in the Tibetan lineage, a pattern quite distinct from the Sichuan lineage, where distylous and homostylous populations were strongly differentiated (Fig. 3). Our results are therefore consistent with the hypothesis that, in at least some populations in the Tibetan lineage, homostyles have spread to fixation in distylous populations.

## Floral evolution in distylous and homostylous populations

Our study revealed systematic differences across populations of *P. chungensis* in the size of flowers; in both main lineages, the corolla width of the L-morph was significantly larger than that of the S-morph. This pattern is the reverse of that reported in several other distylous genera in which corollas of the S-morph are larger than those of the L-morph (e.g. *Amsinckia*, *Fagopyrum*, *Lithospermum*, *Rudgea*; see Ganders, 1979). In contrast with corolla size, floral tubes in *P. chungensis* were longer in the S-morph, particularly in the Tibetan lineage. The functional basis of these differences between morphs in heterostylous plants is not well understood. However, homostyles in mixed populations exhibited the corolla size and tube length characteristic of the S-morph, rather than the L-morph. This finding is consistent with the evidence that long homostyles have originated by genetic changes at the *S* locus. With recombination, long homostyles inherit the stamen characteristics of the S-morph, and because, in *Primula*, stamens are inserted on the corolla (epipetal), it is likely that stamen and corolla growth are developmentally associated. Numerous studies have reported close genetic and developmental correlations between petal and stamen growth, including heterostylous species (Richards & Barrett, 1992; Kramer *et al.*, 1998).

One of the most striking findings of our study concerns the wide range of floral variation among homostylous populations. This variation demonstrates that the evolution of homostyly does not always involve the common pathway to the selfing syndrome, but can involve diverse phenotypic responses following homostyle origin. Floral variation among homostylous populations comprised several components, including regional differences in flower size between the two main lineages (Fig. 4), variation in the degree of herkogamy (Fig. 5) and differences in the floral traits of homostyles in monomorphic vs mixed populations (Fig. 4). Five of seven Tibetan populations were largely comprised plants with variable amounts of reverse herkogamy; by contrast, seven of nine populations from Sichuan exhibited a small degree of approach herkogamy (Fig. 5). Variation in herkogamy is likely to have important consequences for mating patterns in populations. For example, long homostylous populations of *Turnera ulmifolia* (= *T. angustifolia*) that have colonized Caribbean islands vary greatly in flower size and degree of herkogamy. This variation is correlated with selfing rates within and between populations (Barrett & Shore, 1987; Belaussoff & Shore, 1995). If at least some of the variation in herkogamy in *P. chungensis* has a heritable basis, as occurs in *T. ulmifolia* (Shore & Barrett, 1990), selective responses to the local pollination environment may occur, with reduced herkogamy favored where pollinator service is unreliable. An unresolved issue is why the two main lineages differ in the types of herkogamy they possess. A variety of selective forces have been invoked to explain the functional significance of approach vs reverse herkogamy (Webb & Lloyd, 1986; Forrest *et al.*, 2011; Kulbaba & Worley, 2012). Studies of pollination and mating in populations would be required to determine whether the observed patterns have an adaptive basis.

Where homostyles occur in distylous populations, it is likely that they will resemble distylous morphs, as mating between the forms in mixed populations would tend to homogenize genetic backgrounds and prevent phenotypic divergence. However, if homostyles establish monomorphic populations by dispersal, we may expect floral differentiation with the trajectories of evolution depending on the reliability of pollen service and the time scale over which reproductive isolation from distylous morphs occurs. Our prediction that homostyles in mixed populations would differ in floral traits from those in monomorphic populations was supported for most contrasts, although patterns in monomorphic populations differed between the Tibetan and Sichuan lineages (Fig. 4). Flower size in homostylous populations from Tibet was significantly reduced with shorter corolla tubes and smaller corolla widths, but this pattern was reversed in the Sichuan lineage.

The domination of stylar monomorphism in both major lineages of *P. chungensis* implies that homostyles possess some advantage over distylous morphs, at least in the colonization of new territory. As all floral forms of *P. chungensis* are self-compatible, this advantage is unlikely to simply reside in the potential for selfing, as implied by Baker's law (Baker, 1955; Pannell, 2015). Rather, it seems more likely that the capacity for autonomous self-pollination combined with the opportunities for outcrossing, promoted by some degree of herkogamy, may provide benefits to homostyles that are not available to their distylous ancestors.

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## Author contributions

W.Z., S.C.H.B., H.W. and D-Z.L. planned and designed the research. W.Z., H-D.L., Z-K.W. and X-J.W. performed the experiments, conducted field work, analysed the data, etc. W.Z., S.C.H.B., H.W. and D-Z.L. wrote the manuscript.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Principal coordinates analysis (PCoA) of the simple sequence repeat (SSR) genotypes from 20 populations of 300 individuals of *Primula chungensis*.

**Fig. S2** The total sample of flowers of *Primula chungensis* measured in this study ranked by style length.

**Fig. S3** The frequency distribution of stigma and anther heights of *Primula chungensis* individuals from 20 populations in south-west China.

**Fig. S4** Scanning electron microscope images of pollen of three morphs of *Primula chungensis*.

**Table S1** Locations of *Primula chungensis* populations investigated in this study

**Table S2** Variation in floral traits of *Primula chungensis* pooled across population morph structures

**Methods S1** Molecular procedures for PCR and genotyping.

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