

FLORAL POLYMORPHISM IN *POLYGONUM BISTORTA* L.

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Abstract

In the present study, new type of floral polymorphism (tristyloous-like flowers) in *Polygonum bistorta* L., i.e., L-, M-, and S-styles (long, medium and short, respectively) is reported. This tristyloous was not only present in different populations but also in a single inflorescence. Although the heights of styles and anthers in the three morphs of flowers differed, their ancillary tepal size, pollen polymorphism, and stigma polymorphism were found similar. The ratio of M-flowers was far lower than those of L- and S-flowers. Unlike the solid nectar of other *Polygonum* species, fluid nectar was present in *Polygonum bistorta*, which reflects its specialized state. Different from most of other heterostylous species with tubular flowers and *Polygonum jucundum* with open distylous flowers, *Polygonum bistorta* contains semi-open flowers. Our findings suggest that *P. bistorta* represents special floral polymorphism in *Polygonum* and is an ideal plant species for studying the evolution of floral breeding system in *Polygonum*.

Introduction

Heterostyly is a floral polymorphism in which the various morphs differ in terms of the sequence of heights at which the anthers and stigmas are presented within its flowers. In these flowers, the sex organs are spatially separated and reciprocally arranged in the morphs; two levels of reproductive organs are present in distylous species, and three levels are present in tristyloous species (Lloyd *et al.*, 1990). Tristyly, which occurs in only seven angiosperm families, is thought to be one of the most complex breeding systems in plants (Barrett, 1993; Naiki, 2012). Traditionally, tristyly was thought to only occur in different individuals or in different populations, and three morphs of flowers (with long, medium and short styles) were never found in the same inflorescence. Tristyly inheritance involves two diallelic loci (*S* and *M*), with the *S*-locus epistatic to the *M*-locus (Nowak *et al.*, 2015).

Heterostyly in Polygonaceae was first documented in *Fagopyrum esculentum* (Hildebrand, 1867). Subsequently, many distylous species have been reported for the genera *Oxygonum* and *Aconogonum* (Hong, 1999). The genus *Polygonum*, which includes over 300 species worldwide (Li and Bao, 2003), is seldom reported to be heterostylous. Reddy *et al.* (1977) reported the first distylous *P. chinense* in *Polygonum*. Recently, some distylous species in *Polygonum* were described (Chen and Zhang, 2010; Chen, 2012), and style-stamen dimorphism in this genus was also reported (Hassan and Khan, 1987), but to date, other new floral polymorphisms have never been reported in the genus.

Polygonum bistorta L. is a perennial herb that reaches up to 90 cm tall. This plant blooms from late spring to autumn, producing tall stems ending in single terminal racemes comprising club-like spikes, 5–7 cm long, which contain rose-pink flowers. *P. bistorta* is distributed in the Americas, western and central Asia, and northern and central Europe, including Britain and the mountains of

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southern Europe. Most studies of this species have focused on its physiology (Kumar *et al.*, 2012), ecology (Egory Starr *et al.*, 2000), and medical applications (Demiray *et al.*, 2009). To date, floral polymorphisms have never been described in detail in this species. Therefore, this species provides unsurpassed opportunities for performing integrated evolutionary studies. The objectives of the current study include: (i) to examine the characteristics of floral polymorphisms and to determine whether *P. bistorta* is tristylous or exhibits anomalous heteromorphisms; (ii) to investigate the relationship between the reproductive system of *P. bistorta* and its insect visitors; and (iii) to explore the systematic and evolutionary significance of floral polymorphism in *Polygonum*.

Material and Methods

Studied species and population sampling

This study was conducted in 2013-2016 in China and in 2014 in Flagstaff, Arizona, USA. Herbarium specimens or living materials from thirteen populations of *P. bistorta* in Anhui (China) and Arizona (USA) were used in this study (Table 1). All vouchers were deposited at the Herbarium of Anhui Normal University, China and Northern Arizona University (NAU), USA. Some voucher specimens for surveying L-, M-, and S-flower ratios of the species (deposited at NAU herbarium) were also collected.

Morphometrics

Floral morphological characters were observed in the field and in the laboratory. To characterize floral polymorphism in *P. bistorta*, tepal height, stigma and anther heights, and the size and number of pollen grains were measured. Since the stamens were present at different positions and angles, their heights were measured in the natural state instead of measuring the length of single dissected stigmas, stamens, and tepals (measure after fully matured so as to rule out the possibility of developmental changes) (Fig. 1). The floral traits recorded included the following: (i) corolla height; (ii) style length (from the basal ovary to the stigmatic surface); (iii) stamen height (from the basal ovary to the midpoint of each anther); (iv) style-nearer anther separation (SNAS); and v) Long-Short anther separation (LSAS) (Fig. 1). The flowers were slit longitudinally and measurements were made from digital photos using the image *Analysis 5.0* software. Forty flowers were randomly sampled from three separate populations (FS1: 14 flowers; FS2: 13 flowers; FS3: 13 flowers) in Flagstaff. All photographs were taken under a Zeiss Discovery V8 stereomicroscope.

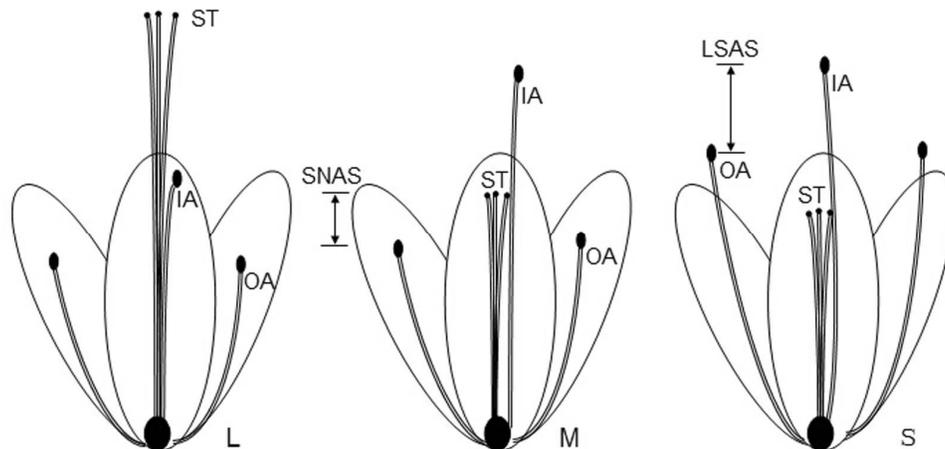


Fig. 1. L-, M-, and S- flowers of *P. bistorta*. ST=Style; IA=Inner anther; OA=Outer anther; SNAS=Style-nearer anther separation; LSAS=Long-Short anther separation.

Size and number of pollen grains

To measure pollen size, samples were collected from fresh flowers. One long (L)-, medium (M)-, and short (S)-level anther was chosen per bud from 25 flowers. Pollen was mounted on each slide in a drop of glycerol and photographed under a Zeiss Axio Scope A1 optical microscope (200X). The polar and equatorial diameters of 75 pollen grains (three moderately sized pollen grains per flower) were measured based on photographs taken with *Analysis 5.0* software. To determine the number of pollen grains produced, anthers from 75 flowers per type (L, M, and S) from the Flagstaff populations were placed on individual microscope slide. Using standard methods (Chen and Zhang, 2010), the number of pollen grains per flower was counted using a light-emitting microscope (LEM), the number of pollen grains in 75 flowers from three populations (25 flowers per population) was counted, and the number of pollen grains from outer and inner anthers was compared. Pollen counts for flowers with different style sizes were compared using Student's *t*-test.

Community ecology

1 m × 1 m sample was selected in 13 study populations, respectively. Plant species compositions, altitude and habitats were recorded for each sample. Some species occurred in populations were recorded as 1, or 0.

SEM observation

To characterize pollen shape, samples from L-, M-, and S- flowers stored in 70% ethanol were prepared according to the acetolysis method (Erdtman, 1960). Pollen grains were mounted in glycerine jelly and sealed with paraffin. The pollen was then observed under a Zeiss Supra 40 VP scanning electron microscope (SEM) operated at 20 kV. The shape and exine ornamentation of pollen grains from each sample were observed. Fresh stigmas and seeds were mounted on double-sided carbon tape and observed under SEM.

Table 1. Populations of *Polygonum bistorta* L. employed in the present study.

Codes	Sites	Habitat	Altitude (m)	Experiments
QLF1	Qingliangfeng, China	Grassy slopes	1650	SR, CE
QLF2	Qingliangfeng, China	Broadleaved deciduous forest	1450	CE
QLF3	Qingliangfeng, China	Broadleaved deciduous forest	1250	CE
QLF4	Qingliangfeng, China	Mixed forest	1050	CE
QLF5	Qingliangfeng, China	Mixed forest	850	CE
BSH1	Bashang 1, China	Uplands	1700	SR, CE
BSH2	Bashang 2, China	Uplands	1500	SR, CE
BSH3	Bashang 3, China	Mixed forest	1300	CE
BSH4	Bashang 4, China	Mixed forest	1100	CE
BSH5	Bashang 5, China	Mixed forest	900	CE
FS1	Flagstaff, Arizona, USA	Slope wastelands	2100	SR, MA, PS, PN, SEM, SSR, CE
FS2	Flagstaff, Arizona, USA	Mountain forest	2100	SR, CE
FS3	Flagstaff, Arizona, USA	Mountain forest	2100	SR, CE

MA: Morphometric analysis; PS: Pollen size; PN: Pollen number; SR: Style morph ratios; SSR: Seed setting rate; CE: Community ecology.

Surveys of style morph ratios and seed set

The relative frequencies of the three morphs of flowers were determined in six populations of *P. bistorta*, as shown in Table 1. At the same time, 24 mature inflorescences were harvested from each individual in the FS1, FS2 and FS3 populations (eight per population) to survey the style morph ratios. Seed set was recorded under natural or bagged conditions in the FS1 and FS2 populations (ten inflorescences were selected in each study population).

Results

Flower morphometrics

The present study reveals that the mature *P. bistorta* flowers are semi-open, and this plant exhibits an unusual type of floral polymorphism, i.e., L-, M-, and S- flowers are present not only in different populations, but also in a single inflorescence. Anthers and stigma are spatially separated in a flower and reciprocally arranged in the morphs, with three levels observed in this species (Fig. 2A, B, C). Normally, eight stamens are positioned at the base of the tepals in two whorls, with five stamens present in the outer whorl and three in the inner whorl. The inner whorl filaments are stronger than the outer whorl filaments, and the basal filaments of the inner whorl are red and inflated compared with those of the outer whorl (Fig. 2D). Fluid nectar was found at the base of the stamen and tepal (Fig. 2E). The stigma and anther heights of the three morphs of flowers are shown in Table 2 and Figure 3. There was no obvious difference in tepal size between the long-, medium-, and short-styled flowers (Table 2; LM: $t_{78} = 1.81$, $P > 0.05$; LS: $t_{78} = 1.66$, $P > 0.05$; MS: $t_{78} = 0.25$, $P > 0.05$) (Note: Here after, flowers with different style-morphs are referred to as L-, M-, and S-flowers and the anther levels are referred to as l, m, and s.) The stigma height of L-flowers was longer than the l anther height of M-flowers ($t_{78} = 14.14$, $P < 0.05$) and the l anther height of S-flowers ($t_{78} = 10.27$, $P < 0.05$). The stigma height of M-flowers was shorter than the m anther height of L-flowers ($t_{78} = 3.44$, $P < 0.05$) and the m anther height of S-flowers ($t_{78} = 13.89$, $P < 0.05$). The stigma height of S-flowers was longer than the s anther height of L-flowers ($t_{78} = 3.70$, $P < 0.05$) and the s anther height of M-flowers ($t_{78} = 2.09$, $P < 0.05$). The LSAS of M-flowers was the largest among the three morphs of flowers (ML: $t_{78} = 71.28$, $P < 0.05$; MS: $t_{78} = 19.51$, $P < 0.05$), while the SNAS of M-flowers was smaller than those of L-flowers ($t_{78} = 29.02$, $P < 0.05$) and S-flower ($t_{78} = 14.27$, $P < 0.05$). There were no obvious differences in pollen number and size among the three morphs of flowers.

The stigma height differed among L-, M-, and S-flowers of *P. bistorta* (Fig. 4A, B, C). The stigmas of the three morphs of flowers were present in a spherical, cerebriform pattern. There were no obvious differences in stigma surface ornamentation among the three morphs of flowers (Fig. 4D, E, F). Anthers of *P. bistorta* are of the adnate type, pink to purple in colour, and elliptical to oblong in shape. The pollen type of *P. bistorta* is 3-zonocolporate, and is elliptical to slightly rectangular in the equatorial view and triangular, angles obtuse, apertures in convex sides in the polar view, and the exine is reticulate. However, many perforations of unequal size and smaller columellae were observed in two polar views and near the colporate pollen. Both the shape and size of pollen were similar in L-flower and S-flower (Fig. 4G–L).

Surveys of style morph ratios and seed set

The style morph ratios of L-, M-, and S-flowers were approximately 237:18:33 among the studied populations (Table 3). No M-flowers in the FS1 population were found. However, in a single inflorescence, some inflorescences composed entirely of long-styled flowers were often found, and some inflorescences which mainly composed of medium-, and short-styled flowers were rarely found (Fig. 5A, B). The most common flowers were L- and S-styled morphs. L-flowers were always situated in the upper part of the inflorescence, while S-flowers were always found in the lower part

of the inflorescence (Fig. 5C). The ratio of L-, M-, and S-type flowers was approximately 103:4:49 among the inflorescences investigated (Table 3). The ratio of M-styled flowers was far lower than those of L-, and S-styled flowers. Under natural conditions, the seed sets were $36.6 \pm 8.45\%$, but under bagged conditions, the seed sets were 0 indicating cross pollination.

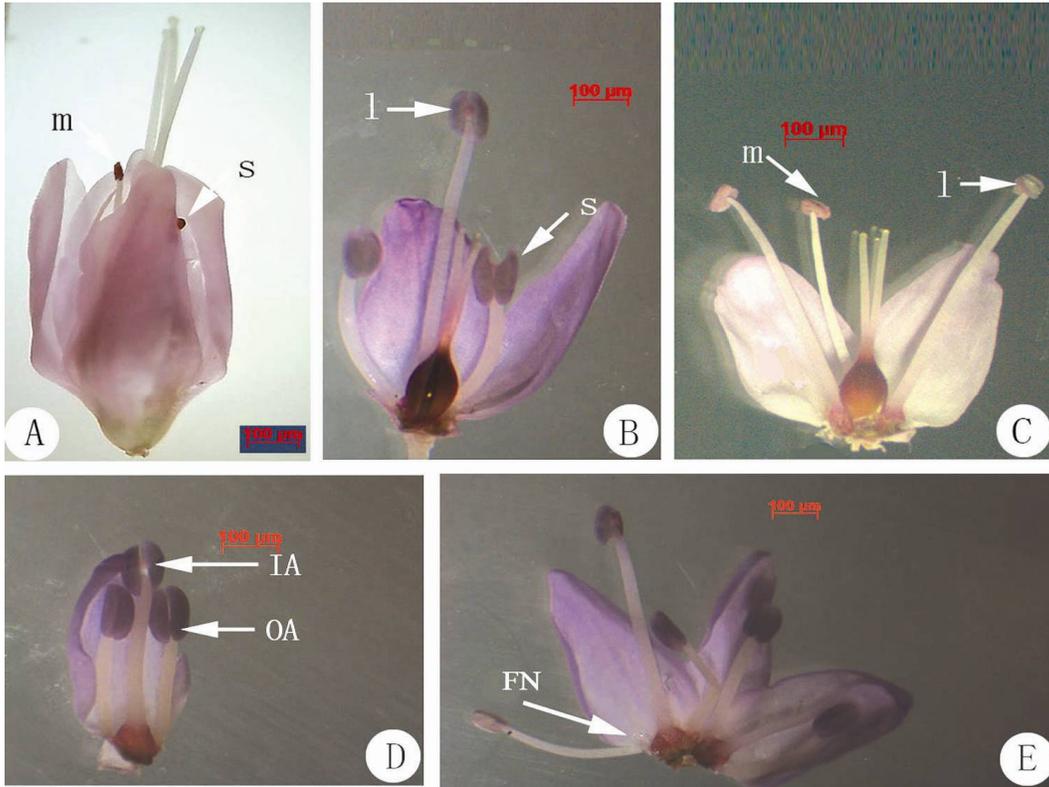


Fig. 2. L-, M-, and S-flowers of *P. bistorta*. A=L-styled flower; B=M-styled flower; C=S-styled flower; D=Inner anther (IA) and outer anther (OA); E=Fluid nectar at the base of the tepal (l: long anther; m: medium anther; s: short anther; FN: fluid nectar).

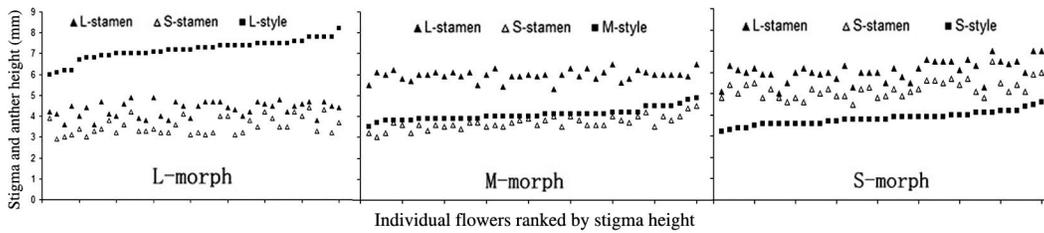


Fig. 3. Characteristics of flowers of *P. bistorta* ranked by style height to illustrate the reciprocal correspondence of stigma and anther positions in the L-, M-, and S-flowers. Positions of stigmas are indicated by solid rectangles (■); Positions of long and short anther are indicated by solid triangles (▲) and open triangles (△), respectively.

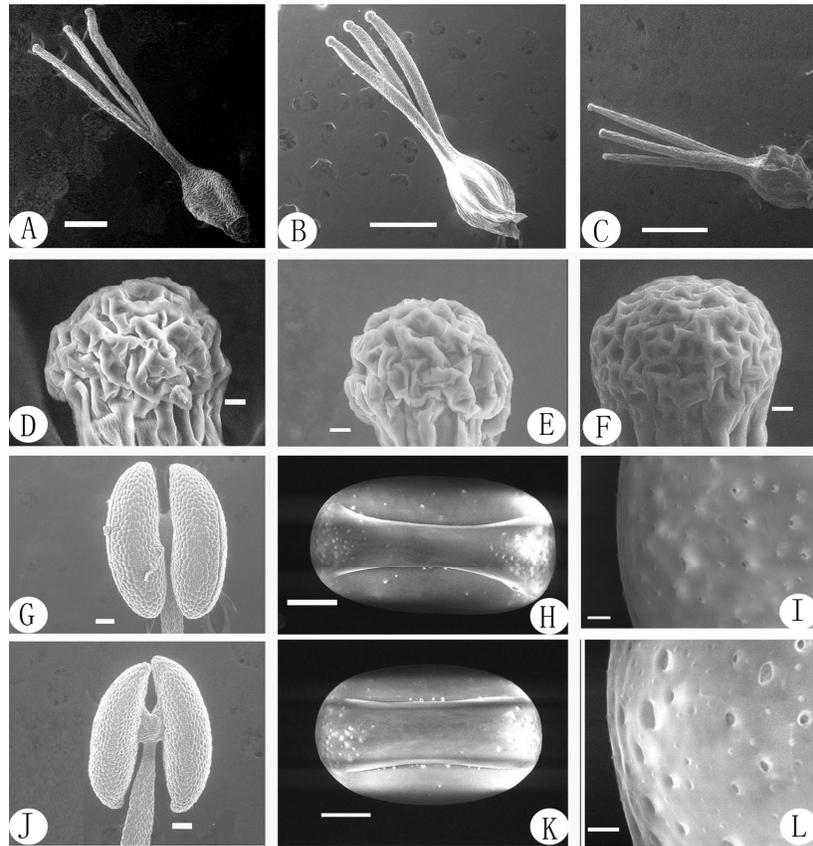


Fig. 4. Micromorphological characteristics of *P. bistorta* (SEM). A=Pistil of L-styled flower; B=Pistil of M-styled flower; C=Pistil of S-styled flower; D=Stigma of L-styled flower; E=Stigma of M-styled flower; F=Stigma of S-styled flower; G=Inner anther; H=Pollen of inner anther; I=Exine ornamentation of pollen of inner anther; J=Outer anther; K=Pollen of outer anther; L=Exine ornamentation of pollen of outer anther. Bars: A,B,C=1 mm; D,E,F,H,K=10 μ m; G,J=100 μ m; I,L=1 μ m.

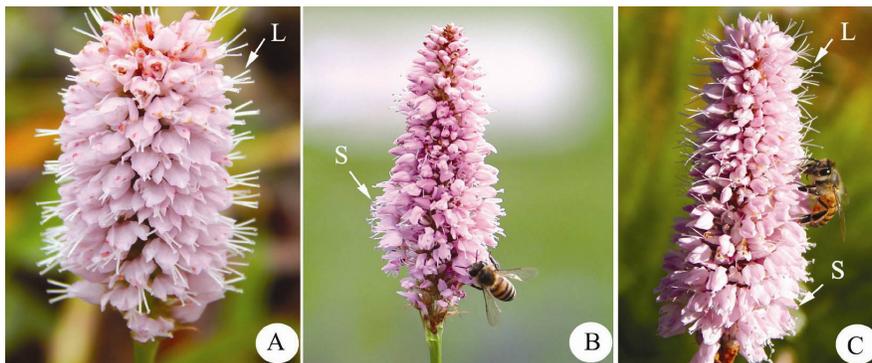


Fig. 5. Inflorescences of *P. bistorta* showing different flowers. A: L-styled; B: S-styled; C: L- and S-styled (Note: L: Long; S: Short).

Table 2. Measurement of floral characters of L-, M-, and S-flowers of *P. bistorta*. Differences between the means were analyzed by Student's *t*-test. [Mean ± standard error (SE)].

Floral characters	L-flower	M-flower	S-flower	N	<i>P</i> <
Height of tepal (mm)	5.02±0.286 ^a	4.91±0.249 ^a	4.93±0.228 ^a	40	-
Height of L-anther (mm)	4.34±0.366 ^a	5.97±0.261 ^b	6.09±0.48 ^c	40	0.01
Height of S-anther (mm)	3.53±0.419 ^a	3.69±0.312 ^b	5.20±0.415 ^c	40	0.01
Height of stigma (mm)	7.19±0.482 ^a	4.09±0.292 ^b	3.84±0.309 ^c	40	0.05
SNAS (mm)	2.85±0.291 ^a	0.395±0.196 ^b	1.37±0.281 ^c	40	0.01
LSAS (mm)	0.81±0.212 ^a	2.27±0.347 ^b	0.88±0.28 ^c	40	0.05
Pollen long-diameter (µm)	49.5±5.01 ^a	48.6±4.94 ^a	50.0 ±4.74 ^a	75	-
P/E Ratio	c. 1.42	c. 1.41	c. 1.43	75	-
Pollen number per flower	402±48 ^a	395±58 ^a	387±49 ^a	90	-

Note: Different letters in the same row indicate significant differences between values. SNAS: Style-nearer anther separation; LSAS: Long-short anther separation.

Community ecology

Thirteen populations of *P. bistorta* were surveyed. Based on data matrix, cluster group was constructed using Canoco for windows 4.5 software. The populations 11, 12 and 13 were clustered together, and the populations 1, 2, 6 and 7 were clustered together, while the other populations were clustered together (Fig. 6). We also found that there was no *P. bistorta* in population 3, 4, 5, 8, 9, 10, whereas *P. bistorta* could be found in the former two cluster groups.

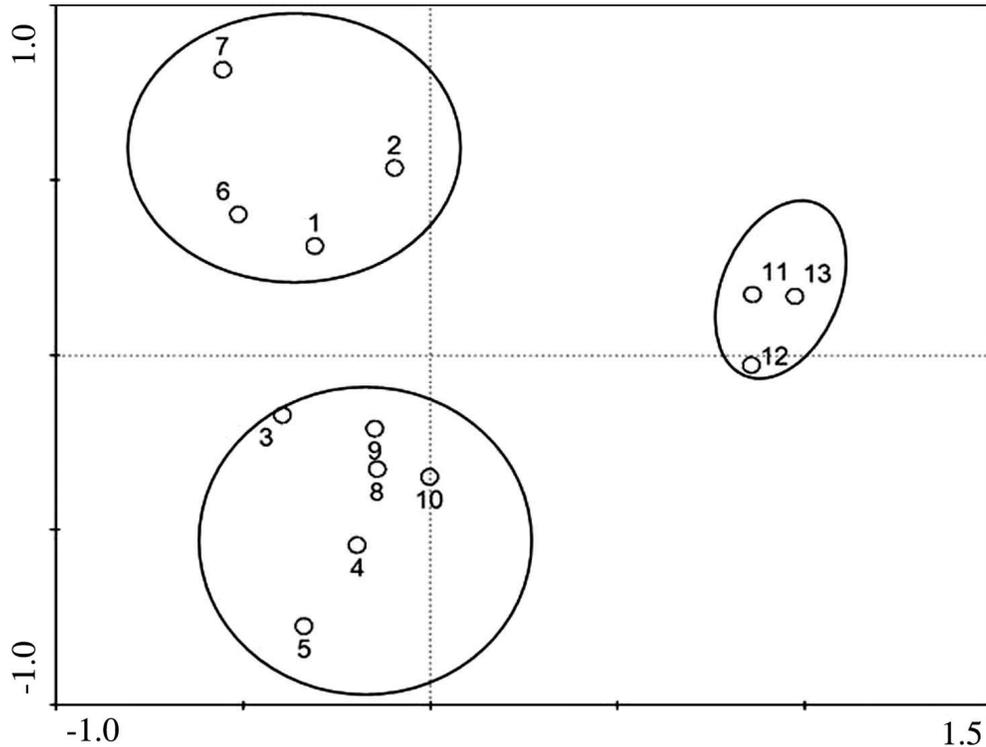


Fig. 6. PCA analysis for species compositions from 13 studied populations of *P. bistorta*.

Table 3. Style morph ratios of *P. bistorta* among populations and among inflorescences.

Scopes	L-styled	M-styled	S-styled	n
Among populations	237.17±47.34	18.17±6.18	33±5.77	6
Among inflorescences	103.38±7.60 ^a	4.21±0.58 ^b	49.21±3.87 ^c	24

Note: Different letters in the same row indicate significant differences between values ($P < 0.05$)

Discussion

Various stochastic and deterministic factors can cause biased morph ratios in tristylous populations. Founder events and genetic drift in small populations are a common cause of anisoplethy and morph loss (Barrett, 1993). Theoretical studies have indicated that the S-morph should be lost most often and the L-morph least often (Costa *et al.*, 2016). However, in the present study, we found that L-, M-, and S-flowers of *P. bistorta* not only could be found in different populations but also in a single inflorescence. This phenomenon is difficult to explain based on the traditional supergene locus control theory (Naiki, 2012). Clearly, *P. bistorta* is an intriguing plant material for studying the floral polymorphism. In trimorphic species, a 1:1:1 style morph ratio (i.e., isoplethy) is expected in populations at equilibrium (Costa *et al.*, 2016), but in field populations, many species present altered morph ratios (Lloyd *et al.*, 1990). For example, no L-morphs are present in tristylous *Oxalis corymbosa* in China (Chen *et al.*, 2007), while in the self-compatible, perennial *Decodon verticillatus* (Lythraceae), populations commonly lack style morphs, particularly M-styled flowers (Eckert and Mavraganis, 1996), which may provide this transitional variation. We found that the L-styled ratio of *P. bistorta* in populations was the largest in a field population, and inflorescences composed primarily of M-styled morphs were seldom found in field populations.

Darwin (1877) hypothesized that the function of the stamen-style polymorphism in heterostylous plants is to increase the likelihood of pollen transfer between sexual organs at the same level. In typical tristylous species, the style height of L-morph is usually approximately the same as that of the l stamen of M-morph and S-morph, and the style height of M-morph is approximately same as that of the m stamens of L-morph and S-morph. The same scenario occurs in *Eichhornia azurea* (Alves Dos Santos and Wittmann, 2000). However, this scenario was not detected in *P. bistorta*. We found that the style height of L-morph was higher than that of the l stamen of M-morph and S-morph. Therefore, the increased reciprocal placement between sexual organs of S- and L-styled floral morphs is expected to have occurred during the evolution of *P. bistorta*. In addition, two whorls of stamens are often the same height in distylous taxa of *Polygonum* (Chen and Zhang, 2010; Chen, 2012), while in *P. bistorta*, the inner whorl stamens are higher than those of the outer whorl, which suggests that the change in stamen height may have occurred in an evolutionary event.

There are no heterostylous taxa in the basal angiosperm groups and basal 'eudicots' (Naiki, 2012), and heterostyly has always been found in medium evolutionary-state tubular flowers (Darwin, 1877; Lloyd and Webb, 1992), with the exception in Polygonaceae and *Turnera* (Barrett, 1992). The Polygonaceae frequently have dish-shaped corolla, which is true for the heterostylous genera, *Fagopyrum* and *Polygonum*. For example, the distylous flowers of *Polygonum jucundum* are broadly open (Chen and Zhang, 2010), but based on the present study, *P. bistorta* flowers are semi-open, which represents a specialized form during petal evolution in *Polygonum*. Along with the generalistic pollination phenomenon of this species, the formation of floral tubes in heterostylous flowers might have coevolved with specialistic pollinators during later evolution.

Typical heterostylous species are always dimorphic or trimorphic for the intrinsic features of style height, anther height, and a series of ancillary characteristics, such as pollen size, exine sculpturing, pollen number and production, and stigma exine ornamentation (Gettys and Wofford,

2008). However, in *P. bistorta*, except for the intrinsic feature of style and anther heights, we observed no difference in these ancillary characteristics. Moreover, eight yellow nectaries are present at the base of the perianth in distylous species such as *P. jucundum* (Chen and Zhang, 2010) and *P. hastato-sagittatum* (Chen, 2012), but fluid nectar rather than solid nectaries are present in *P. bistorta*. Based on the results of community ecology, we found all populations of *P. bistorta* were occurred in high altitude. Both of the findings reflected the specialization in this species and its adaptation to the high altitude, which is consistent with the molecular evidence (Sanchez *et al.*, 2011).

The rate of seed set, which is closely correlated with ovule development, can reflect the degree of successful reproduction in a species (Zhou *et al.*, 1996). However, because we occasionally found two or three morphs in a single inflorescence, it was difficult to survey the seed sets of each morph, and we only obtained two sets of data (0 and 36.5% under bagged and natural conditions, respectively). This result demonstrates that this species can reproduce under natural conditions.

As mentioned above, the results demonstrate that *P. bistorta* is not a typical tristylous species, but it has tristylous-like flowers, which have not previously been found in angiosperms. Therefore, it would be important to investigate the origin and evolution of floral polymorphism in this species. Heterostylous polymorphisms provide opportunities for ecological and genetic investigations into the evolution of plant sexual systems (Barrett, 1992). Although elucidating the evolutionary origins of heterostyly continues to be difficult (Barrett, 1993), significant progress has been made towards understanding these evolutionary pathways (Weller, 1992). At present, several hypotheses about the evolutionary mechanisms favouring heterostyly have been proposed. According to Darwin (1877), heterostyly can be considered as a strategy for reducing the likelihood of self- and intramorph pollination and for promoting legitimate intermorph pollination. Barrett (2002) stated that reciprocal sex-organ positions in style-morphs help increase male fertility by actively promoting more precise pollen dispersal among plants than would occur in populations with uniform sexual organs and herkogamy. However, in the present study, the heights of stigmas and anthers of L-, M-, and S-morphs in *P. bistorta* did not exactly coincide. Lloyd and Webb (1992) proposed a model describing how heterostyly is established, from approach herkogamy to style polymorphism, followed by reciprocal herkogamy (heterostyly). Their model demonstrates that heterostyly could be established from a population that exhibits continuous phenotypic variation, which is not the case in *P. bistorta*. The evolution of distyly from tristily was investigated in populations of *Oxalis alpina* (Weller *et al.*, 2007), and the evolutionary shift from tristily to distyly appears to have been particularly frequent in the Lythraceae (Weller, 1992). Two of the largest genera (*Nesaea* and *Lythrum*) contain both tristylous and distylous taxa, and some of these distylous taxa have retained features that suggest a tristylous ancestry (Ornduff, 1979). Elsewhere in this family, distylous *Pemphis acidula* appears to have evolved distyly through loss of the mid-styled morph from a tristylous ancestor (Lewis, 1975). In the evolutionary model from tristily to distyly in *Pemphis*, the m anthers of the L-morph are closer to the s level, whereas the m anthers of S-morph are closer to the l level (Lewis and Rao, 1971). Similar patterns were also evident in *P. bistorta*, as we found that the m anthers of L-morph were shorter than the tepals of L-morph, while the m anthers of S-morph were longer than the tepals of S-morph. The hypothesis of the evolution of floral polymorphism toward distyly predicts divergence in m anther position between the L- and S-morphs ($mS > mL$) in dimorphic populations, which is also found in *P. jucundum* and *P. hastato-sagittatum* (Chen and zhang, 2010; Chen, 2012). Thus, we argue that heterostyly may arise from floral polymorphism in a single population, followed by heterostyly in different populations, and that *P. bistorta* appears to represent a specialized state from floral polymorphism to heterostyly in *Polygonum*.

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