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The Great Wall of China: a physical barrier to gene flow?

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One population from each of six plant species along both sides of the Juyong-guan Great Wall, together with one population from each of five species along both sides of a path on a mountain top near Juyong-guan, were selected to study the effect of the Great Wall as a barrier on genetic differentiation between two subpopulations using RAPD markers. Significant genetic differentiation was found between the subpopulations on both sides of the Great Wall. A wind-pollinated woody species, *Ulmus pumila*, showed less genetic differentiation than four insect-pollinated species: *Prunus armeniaca*, *Ziziphus jujuba*, *Vitex negundo*, and *Heteropappus hispidus*. *Cleistogenes caespitosa*, a wind-

pollinated perennial herb, displayed more genetic differentiation between subpopulations than the insect-pollinated species because of its propagation strategy. Although AMOVA analysis showed that subpopulations divided by a mountain path had diverged genetically, the variance component between the subpopulations on both sides of the Great Wall was significantly larger than that between the subpopulations at the control site. Therefore, it is reasonable to deduce that the Juyong-guan Great Wall has served as a physical barrier to gene flow between subpopulations separated for more than 600 years.

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Keywords: natural population; genetic differentiation; gene flow; RAPD

Introduction

Geographic isolation is expected to have significant effects on the genetic structure of populations (Smith, 1999). A population may be physically separated when its original habitat becomes divided by a natural barrier (eg, a river, shoreline, mountain range or glacier) or even an artificial barrier (eg, a man-made canal or highway). These barriers can restrict or prevent gene flow and result in the genetic differentiation of isolated subpopulations (Hartl, 1980; Corre *et al.*, 1997; Bauert *et al.*, 1998; Nesbo *et al.*, 1998). Furthermore, the mating system, pollination biology and life history of a species can also have a critical role in the genetic differentiation of its populations (Turner *et al.*, 1982; Wolff *et al.*, 1997; Gauer and Cavalli-Molina, 2000).

The Great Wall was built by the ancient Chinese for the purpose of defence. The earliest record of the Great Wall dates back to 656 BC, and many Emperors in successive dynasties had the Great Wall renovated or extended. The most recent large-scale renovation was in the Ming Dynasty (1368–1644) and was the biggest renovation project in its history. The most famous sites, such as Bada-ling, Jiayu-guan, Juyong-guan, were built during the Ming Dynasty (Luo and Liu, 1992). As an artificial physical barrier, the Great Wall could be an excellent model for studying its effect on the genetic differentiation of plant populations around it.

In this study, six plant species with different habits, pollination modes or reproduction systems along both sides of the Juyong-guan Great Wall at two sites were collected and analyzed. Five of the same or closely related species along both sides of a mountain path without the Great Wall were taken as a control. RAPD markers were used as molecular markers to reveal the genetic variation, since they have been widely used in the studies of plant genetic diversity and population genetic structure (Dawson *et al.*, 1993; Huff *et al.*, 1993; Martin *et al.*, 1997; Buso *et al.*, 1998; Hsiao and Lee, 1999; Zhao *et al.*, 2000).

Materials and methods

Sampling sites and strategy

The Juyong-guan Great Wall (40°13'20"N, 116°04'72"E) was recorded as built in 1368 (Luo and Liu, 1992), and located approximately 60–70 km north of Beijing. Plant materials were collected in September 1999 and April 2000 at two sites (sites A and B) along both sides of the Juyong-guan Great Wall, and one site (site C) along both sides of a path, as control (Figure 1). Sites A and B were about 2 km apart from the control site and separated by an express way. At site A the wall runs from east to west with north and south aspects and at site B the wall runs from south to north with east and west aspects (Figure 1). The Great Wall is 6 m high and 5.8 m wide (on average) at the sampling sites, and the mountain path is approximately 1.5 m wide with north and south aspects. Sampling within populations was carried out randomly in an area approximately 100–150 m long and 7 m wide along both sides of the Wall and the path. Six species

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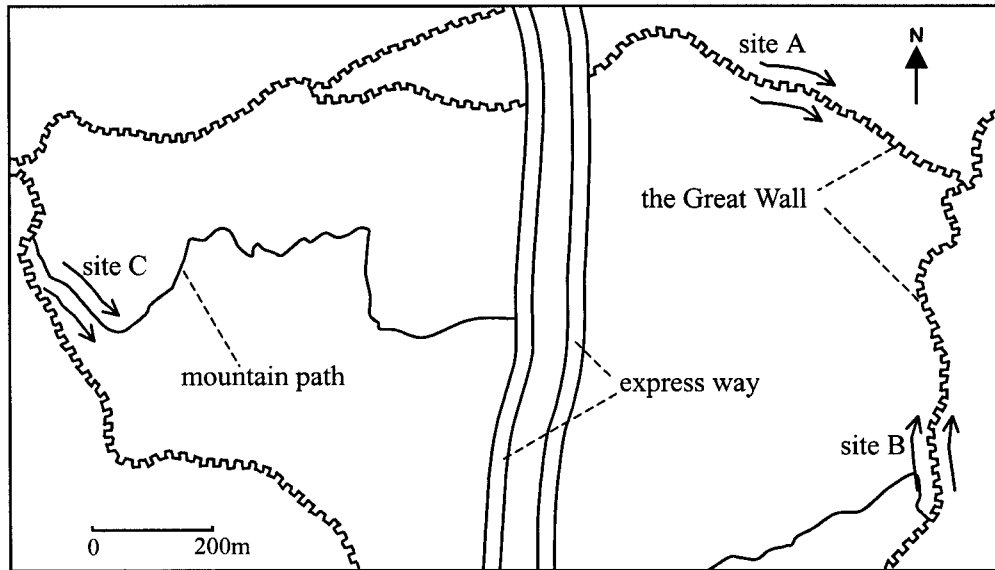


Figure 1 Map of sampling sites.

Table 1 Species and subpopulations studied from the (a) Juyong-guan Great Wall and (b) control site

Species	Population code	Subpopulation code	No. of individual	Habit	Mode of pollination
(a) Juyong-guan Great Wall					
<i>Cleistogenes caespitosa</i> (Poaceae)	CcaJ	CcaJN CcaJS	18 22	Perennial herb	Wind-pollinated
<i>Ulmus pumila</i> (Ulmaceae)	UpuJ	UpuJN UpuJS	21 23	Long-lived tree	Wind-pollinated
<i>Heteropappus hispidus</i> (Asteraceae)	HhiJ	HhiJN HhiJS	14 9	Annual herb	Insect-pollinated
<i>Ziziphus jujuba</i> (Rhamnaceae)	ZjuJ	ZjuJN ZjuJS	13 20	Shrub	Insect-pollinated
<i>Vitex negundo</i> (Verbenaceae)	VneJ	VneJN VneJS	19 21	Shrub	Insect-pollinated
<i>Prunus armeniaca</i> (Rosaceae)	ParJ	ParJE ParJW	20 20	Long-lived tree	Insect-pollinated
(b) Control site					
<i>Cleistogenes caespitosa</i> (Poaceae)	CcaC	CcaCN CcaCS	19 19	Perennial herb	Wind-pollinated
<i>Ulmus macrocarpa</i> (Ulmaceae)	UmaC	UmaCN UmaCS	19 17	Long-lived tree	Wind-pollinated
<i>Ziziphus jujuba</i> (Rhamnaceae)	ZjuC	ZjuCN ZjuCS	20 19	Shrub	Insect-pollinated
<i>Vitex negundo</i> (Verbenaceae)	VneC	VneCN VneCS	23 20	Shrub	Insect-pollinated
<i>Prunus armeniaca</i> (Rosaceae)	ParC	ParCN ParCS	20 20	Long-lived tree	Insect-pollinated

were collected in the Juyong-guan Great Wall area, five of them at site A and one species, *Prunus armeniaca*, at site B (Table 1a). *Heteropappus hispidus*, or its related species, were not found in the control area; therefore, only five species were collected along the path and four of them were of the same species as in the Great Wall area (Table 1b). The fifth one, *Ulmus macrocarpa*, replaced *U. pumila* which was only found in the Great Wall area. For each species, approximately 40 individuals were collected per population, with approximately 20 indi-

viduals on each side of the Great Wall or path, respectively. In total, 11 populations and 416 individual plants were collected (Table 1a, b).

For the convenience of discussion, the two parts of a population divided by the Great Wall or the path were defined as subpopulations. The abbreviation for each subpopulation was designated as follows, the first capitalized letter is the first letter of the genus name, the following two letters are the first two letters of specific epithet; the fourth letter is either J or C

Table 2 Primers, number of total RAPD bands and polymorphic bands detected in subpopulations

Subpopulation code	No. of primers	No. of RAPD bands	No. of polymorphic bands	Mean no. of band/primer	Percent of polymorphic bands
<i>Cca</i> JN	10 ^a	128	64	12.8	50.00
<i>Cca</i> JS	10 ^a	124	71	12.4	57.26
<i>Upu</i> JN	11 ^b	129	83	11.7	64.34
<i>Upu</i> JS	11 ^b	135	88	12.3	65.19
<i>Par</i> JE	10 ^c	137	72	13.7	52.55
<i>Par</i> JW	10 ^c	147	88	14.7	59.86
<i>Zju</i> JN	10 ^d	107	59	10.7	55.14
<i>Zju</i> JS	10 ^d	106	57	10.6	53.77
<i>Vne</i> JN	10 ^e	102	82	10.2	80.39
<i>Vne</i> JS	10 ^e	115	97	11.5	84.35
<i>Hhi</i> JN	11 ^f	151	100	13.7	66.23
<i>Hhi</i> JS	11 ^f	151	93	13.7	61.59
<i>Cca</i> CN	10 ^a	121	75	12.1	61.98
<i>Cca</i> CS	10 ^a	126	67	12.6	53.17
<i>Uma</i> CN	10 ^b	114	50	11.4	43.86
<i>Uma</i> CS	10 ^b	114	57	11.4	50.00
<i>Par</i> CN	10 ^c	141	90	14.1	63.83
<i>Par</i> CS	10 ^c	139	86	13.9	61.87
<i>Zju</i> CN	10 ^d	106	56	10.6	52.83
<i>Zju</i> CS	10 ^d	106	57	10.6	53.77
<i>Vne</i> CN	10 ^e	117	100	11.7	85.47
<i>Vne</i> CS	10 ^e	120	101	12.0	84.17

^aPrimers s52, s201, s207, s418, s485, s489, s494, s2123, s2129, s2130.

^bPrimers s407, s418, s419, s484, s485, s487, s494, s497, s500, s2125, s2138.

^cPrimers s44, s201, s207, s215, s220, s420, s2121, s2123, s2136, s2138.

^dPrimers s42, s43, s45, s48, s201, s206, s207, s212, s214, s2139.

^ePrimers s42, s47, s218, s220, s405, s412, s486, s489, s498, s2121.

^fPrimers s41, s214, s217, s218, s409, s418, s489, s494, s2138, s2139. The sequence information of the primers can be found on the website: <http://www.sangon.com/business/shengong5.htm>.

representing the Juyong-guan or control site, respectively; the last letter represents the sampling position. For example, *Upu*JN means the subpopulation of *U. pumila* collected on the northern side of the Juyong-guan Great Wall.

DNA preparation and RAPD analysis

Young leaves from an individual plant were collected and stored in silica gel at room temperature. Total DNA was extracted from the dried leaves according to the CTAB method of Qu *et al* (2000). A total of 100 primers (S41–60, S201–220, S401–420, S481–500, S2121–2140, Sangon Biotech Company) were used for a two-step primer screening. In the first step, one plant sample per population was randomly selected to identify primers that produced at least three clear bands. In the second step, another three individuals from each population were randomly selected for RAPD analysis with primers screened in the first step. Following the two-step primer screening, 10–11 primers with clear and reproducible bands were chosen for each species (Table 2). Amplification was carried out in 25 µl with 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton-100, 2 mM MgCl₂, 200 µM each of dATP, dGTP, dCTP and dTTP, 30 ng of primer, 2 units *Taq* DNA polymerase and 5–40 ng template DNA. The thermal cycles started with five cycles of 94°C for 1 min, 35°C for 2 min, 72°C for 2 min, then 40 cycles of 94°C for 30 s, 35°C for 2 min, 72°C for 2 min and ended with 72°C for 10 min. The amplification products were separated on 1.4% agarose gel in 1 × TAE buffer with 0.5 µg/ml ethidium bromide at 100–120 V for 2 h, and documented using the AmpGene Gel 100 system.

Statistical analysis

RAPD bands were scored as present (1) or absent (0) for each DNA sample. The genetic diversity within subpopulations was measured by the percentage of polymorphic bands, which was calculated by dividing the number of polymorphic bands by the total number of bands surveyed for the corresponding subpopulations (Table 2). Both Nei and Li's and Euclidean genetic distances within populations were calculated employing RAPDistance version 1.04 (Armstrong *et al*, 1994). Based on Nei and Li's genetic distance matrix, a dendrogram of individuals within a population was constructed using the neighbour-joining method. The variance components between subpopulations and among individuals within subpopulations were calculated with WINAMOVA version 1.55 (Excoffier, 1993) based on Euclidean genetic distances. The significance of each variance component was tested by a random permutation test. The significance of the difference in variance component between the Great Wall subpopulations and the control subpopulations was tested using a paired *t*-test.

Results

Genetic variation and genetic distance among individuals within subpopulation

For each subpopulation, the total number of RAPD bands ranged from 102 to 151, and the number of polymorphic bands ranged from 50 to 101 (Table 2). *Vitex negundo* on the northern side (*Vne*CN) of the path had the highest percentage of polymorphism (85.5%), and *U.*

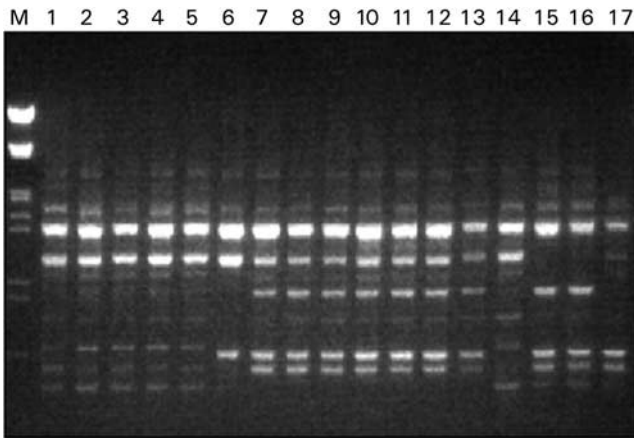


Figure 2 RAPD banding patterns of subpopulation *UmaSC* amplified using primer S419. M: λ DNA/*EcoRI*+*HindIII* markers. 1–17: individuals from subpopulation.

pumila in the same location (*UpuCN*) had the lowest percentage of polymorphism (43.9%).

The results also showed that among 416 individuals studied, 409 had a unique RAPD banding pattern. Two individuals of *Ziziphus jujuba* in subpopulations *ZjuCN* and *ZjuCS* had the same banding pattern as did three individuals of *U. macrocarpa* in the southern subpopulation (*UmaCS*) along the path (Figure 2).

The result of neighbour-joining analyses on the individuals of each population showed that the individuals from the same subpopulation along the Juyong-guan Great Wall tended to cluster together, while the individuals of two subpopulations from one population along the path were always mixed. A typical example of neighbour-joining results for *P. armeniaca* is shown in Figure 3.

Genetic differentiation between the two subpopulations within a population

The results of AMOVA analysis indicated that for the total genetic variation of a population, 71.1–97.1% of the variance was distributed among individuals within subpopulations and 2.9–28.9% of variance was distributed between subpopulations (Table 3). In the Great Wall area, the perennial herb, *Cleistogenes caespitosa*, had the highest variance component within subpopulations (4.7), and *U. pumila* and *P. armeniaca* had the lowest variance component within subpopulations (2.3 and 2.3, respectively). The subpopulations of all species were significantly ($P < 0.001$) genetically differentiated. In the control area, the subpopulations of each species were also significantly differentiated ($P < 0.001$ or < 0.05). However, when the degree of genetic differentiation between subpopulations of the Juyong-guan Great Wall sites and that of the control site was compared by the paired *t*-test, the differentiation between subpopulations of the Juyong-guan Great Wall was significantly greater than that of the control site ($P < 0.05$).

Discussion

The RAPD analysis showed that the degree of polymorphism in six populations along both sides of the

Great Wall and five populations along the path fits into the range of genetic polymorphism (45–100%) of out-crossing plants (Huff *et al*, 1993; Liu, 1994). Except for seven individuals in three subpopulations at the control site, all plant samples studied had unique RAPD banding patterns, giving sufficient polymorphic loci for genetic differentiation analysis.

The variance component analysis indicated that over 71% of genetic variation was attributed to the individual differences within subpopulations (Table 3). Although less genetic variation was detected between corresponding subpopulations, the genetic differentiation between the subpopulations separated by the Great Wall was significant ($P < 0.001$; Table 3). The individual dendrograms, based on neighbour-joining analysis, revealed that all populations in the Great Wall area had two main clusters, and that almost all individuals in a subpopulation were clustered together, except for one to four individuals, indicating that the gene flow between subpopulations separated by the Great Wall was disrupted to a certain extent. *Ulmus pumila*, *Z. jujuba*, *C. caespitosa* and *H. hispidus* have one or two individuals clustered with the individuals from the other subpopulation in both directions, respectively (Figure 4a–d). *Vitex negundo* has only two southern individuals clustered with the northern subpopulation (Figure 4e). Only one western individual of *P. armeniaca* was clustered with the eastern subpopulation (Figure 4f). *Ulmus pumila* is wind-pollinated and has wind-dispersible samara, while *Z. jujuba* and *P. armeniaca* are insect-pollinated and have edible fruits (animal- or human-dispersed). The other three plant species have either insect-pollinated flowers/wind-dispersed fruit or wind-pollinated flowers/insect-dispersed fruit. It is not clear, based on these data, whether the limited gene flow between subpopulations is because of wind, animal, or human activities.

The fact that the subpopulations of the wind-pollinated species, *U. pumila*, along the Juyong-guan Great Wall, has less genetic differentiation between subpopulations than four insect-pollinated species (Tables 1 and 3) follows the general rule that wind-pollinated plants have less genetic differentiation than insect-pollinated plants (Govindaraju, 1988). *Ulmus pumila* flowers in early April and sets wind-dispersed samaras in the same month or early May during the season with strong winds in the Beijing area. In the four insect-pollinated species, the woody species *P. armeniaca* had less genetic variation between subpopulations than the herbaceous *H. hispidus*. The latter had the smallest population size because the individuals were only distributed in an area of $80 \times 1 \text{ m}^2$ along the northern side (14 individuals collected) and of $10 \times 1 \text{ m}^2$ along the southern side (nine individuals collected) of the Great Wall (Figure 4d). In such a small population, genetic drift may play an important role in genetic variation between subpopulations.

Plant population genetic structure is also strongly influenced by life cycle and reproductive strategy (Hamrick *et al*, 1979; Schaal *et al*, 1998; Gauer and Cavalli-Molina, 2000). *Cleistogenes caespitosa*, a perennial herb, is a wind-pollinated species and has a few individuals from both subpopulations clustered together in the Great Wall area. However, it has the greatest genetic differentiation between subpopulations (28.9%)

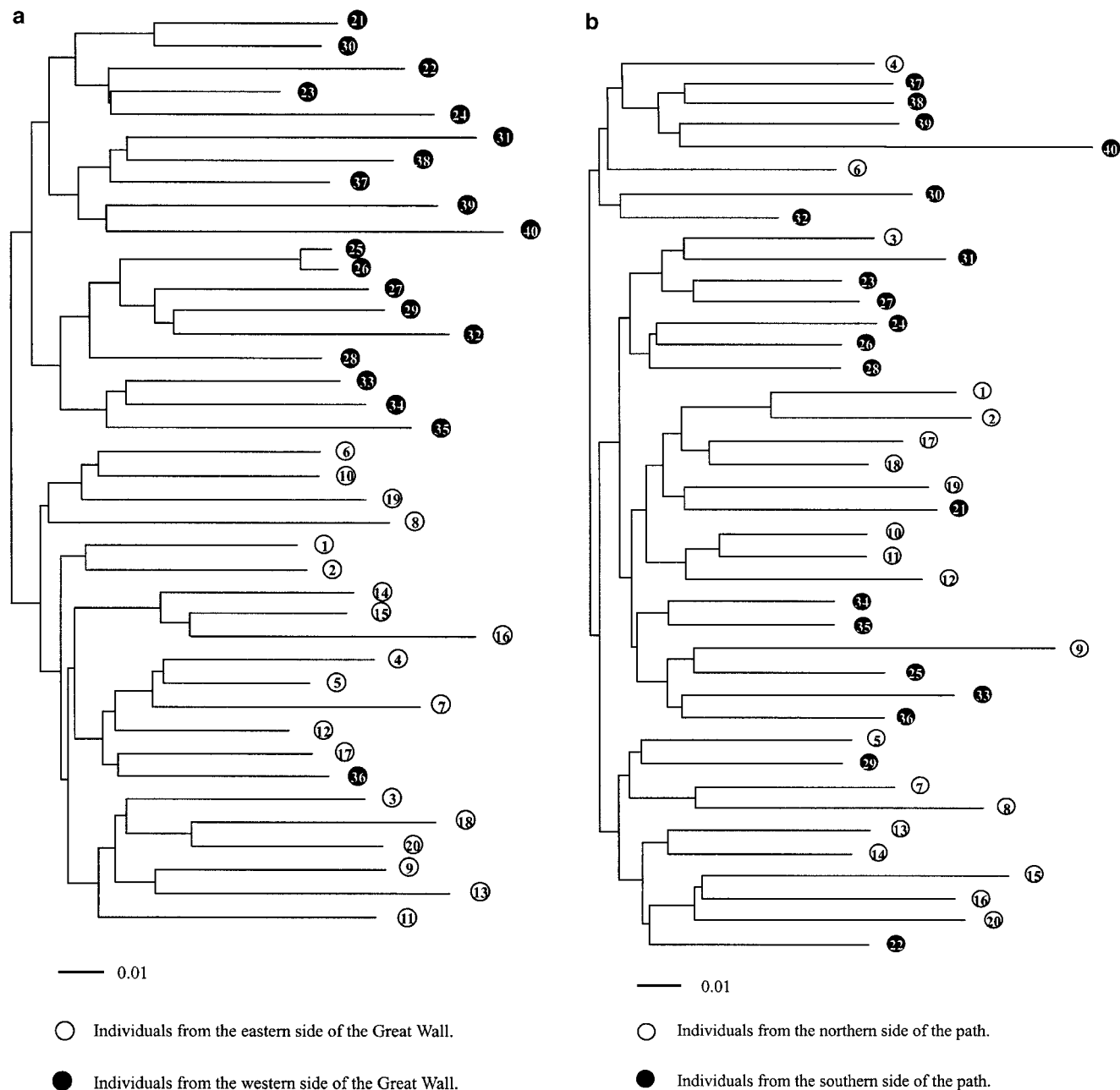


Figure 3 (a) Neighbour-joining dendrogram of 40 *P. armeniaca* individuals from the Great Wall site. (b) Neighbour-joining dendrogram of 40 *P. armeniaca* individuals from the control site.

among the six species. Even at the control site, it has the greatest genetic differentiation between subpopulations among the five species. This phenomenon could have resulted from its reproductive strategy. It is the only species in this study with both sexual and asexual propagation. Therefore, although gene flow between the two sides of the Great Wall or path could be aided by the wind, as indicated by cluster analysis, the individuals at one side may be the progeny of a few clones. Vegetative reproduction will reduce the genetic variation within the subpopulations of *C. caespitosa*, and increase the genetic differentiation between subpopulations.

Most parts of the Great Wall were built on the top of steep mountains, or even on top of cliffs. Naturally, environmental factors, such as light, temperature and moisture, along the two sides could differ slightly, even without the Wall. In order to have an objective evaluation of the effect of the Great Wall on the genetic differentiation of the separated subpopulations, a control site with similar geographic conditions, but without the Wall, on top of a mountain near the Juyong-guan Great Wall was selected. Ideally, the subpopulations at the control site should be separated by the same distance as those at the Wall area, but we failed to find such a

Table 3 Analysis of molecular variation (AMOVA) for 11 subpopulations comparison

Source of variation		df	Variance component	% Total	P-value
<i>Cca</i> JN <i>vs</i> <i>Cca</i> J	Among subpopulations	1	4.72	28.86	<0.001
	Within subpopulations	38	11.65	71.14	
<i>Upu</i> JN <i>vs</i> <i>Upu</i> J	Among subpopulations	1	2.25	13.18	<0.001
	Within subpopulations	42	14.85	86.82	
<i>Par</i> JE <i>vs</i> <i>Par</i> JW	Among subpopulations	1	2.26	14.31	<0.001
	Within subpopulations	38	13.53	85.69	
<i>Zju</i> JN <i>vs</i> <i>Zju</i> J	Among subpopulations	1	2.38	18.91	<0.001
	Within subpopulations	31	10.21	81.09	
<i>Vne</i> JN <i>vs</i> <i>Vne</i> J	Among subpopulations	1	3.38	19.04	<0.001
	Within subpopulations	38	14.38	80.96	
<i>Hhi</i> JN <i>vs</i> <i>Hhi</i> J	Among subpopulations	1	3.50	16.15	<0.001
	Within subpopulations	21	18.16	83.85	
<i>Cca</i> CN <i>vs</i> <i>Cca</i> C	Among subpopulations	1	1.06	11.14	<0.001
	Within subpopulations	36	12.21	88.86	
<i>Uma</i> CN <i>vs</i> <i>Uma</i> C	Among subpopulations	1	0.87	7.60	<0.05
	Within subpopulations	34	10.54	92.40	
<i>Par</i> CN <i>vs</i> <i>Par</i> C	Among subpopulations	1	0.45	2.87	<0.001
	Within subpopulations	38	15.22	97.13	
<i>Zju</i> CN <i>vs</i> <i>Zju</i> C	Among subpopulations	1	1.29	7.96	<0.001
	Within subpopulations	37	10.26	92.04	
<i>Vne</i> CN <i>vs</i> <i>Vne</i> C	Among subpopulations	1	1.46	7.93	<0.001
	Within subpopulations	41	16.90	92.07	

df, degree of freedom. P-values are based on 1000 permutations of the original data set and represent the probability of a more extreme random value (Excoffier, 1993).

site. Therefore, the plant individuals from two subpopulations were collected at the control site in such a way that they were either at least 5.8 m apart, such as *V. negundo* and *C. caespitosa*, or less than 5.8 m apart since no individual could be found at the 'standard' distance. AMOVA analysis showed that the subpopulations of all species at the control site had significant genetic differentiation ($P < 0.001$ or < 0.05 , Table 3). Population genetic differentiation caused by microenvironmental differences has been well documented for plant species, such as *Yushania niitakayamensis* (Hsiao and Lee, 1999), wild emmer wheat (Nevo *et al.*, 1988) and others (Hamrick and Allard, 1972; Schaal, 1975; Nevo, 1988; Nevo *et al.*, 1981, 1994). However, it is unexpected to find that the microgeographic difference between two sides, separated only by a 2-m wide path, could cause significant genetic differentiation between subpopulations. Although AMOVA analysis showed that the

subpopulations within populations at the control site were significantly differentiated genetically, all individuals in two subpopulations were clustered together when neighbour-joining analysis was applied, that is, there was no clear cluster of subpopulations. Nevertheless, the variance component between subpopulations at the control site was about half that between corresponding subpopulations at the Juyong-guan Great Wall, and the variance component at the Great Wall sites was significantly greater than that at the control site. Therefore, based on this study, it is reasonable to deduce that the Juyong-guan Great Wall has served as a physical barrier to gene flow of separated plant subpopulations. It needs to be pointed out that more sampling sites along the Great Wall as well as relevant control areas should be selected in order to evaluate the effect of the Great Wall to the gene flow in general.

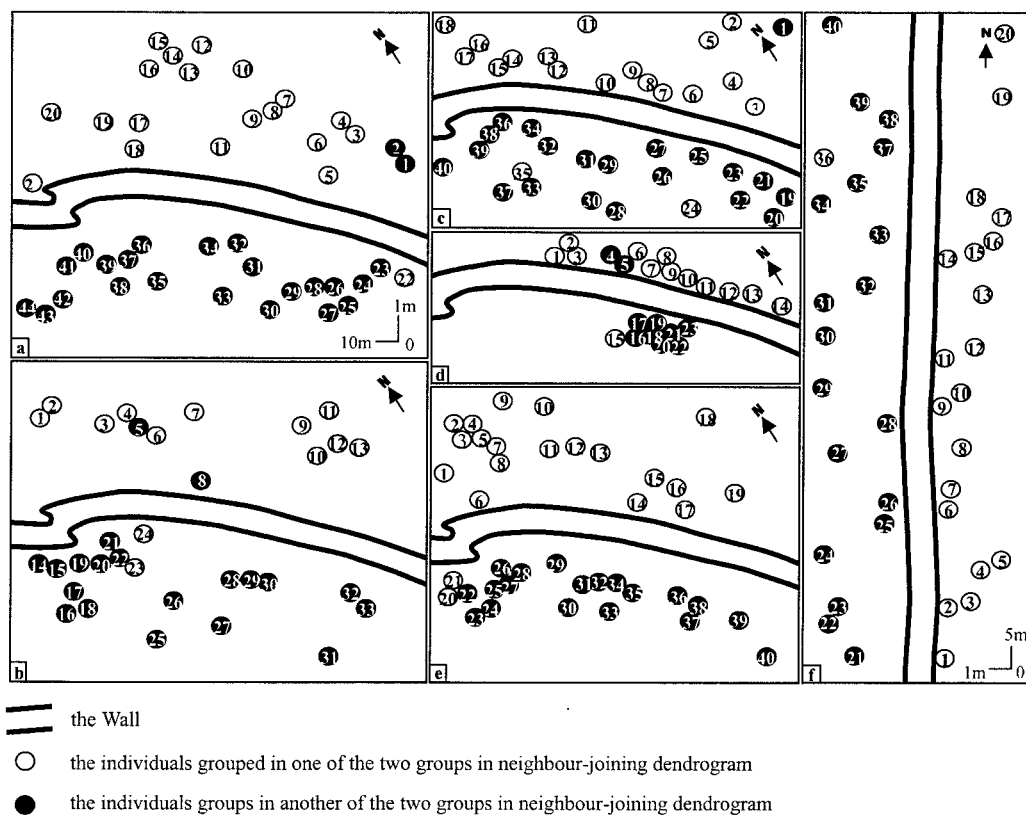


Figure 4 Samples collected along two sides of the Juyong-guan Wall: (a) *U. pumila*, (b) *Z. jujuba*, (c) *C. caespitosa*, (d) *H. hispidus*, (e) *V. negundo*, and (f) *P. armeniaca*. The scales of Figure 4b–e are the same as that in Figure 4a.

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