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Evidence of pre-zygotic reproductive isolation between *Prunus armeniaca* and *Prunus mume*

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ABSTRACT

Background: The trees *Prunus armeniaca* (apricot), *P. mume* (mei or Chinese plum), and *Armeniaca mume* (xingmei) are all members of the *Armeniaca* section of the genus *Prunus*. The three trees are cultivated widely in China.

Hypotheses: Geographical distribution and divergent flowering phenologies constitute pre-zygotic, reproductive isolation barriers that promoted the speciation of *P. armeniaca* and *P. mume*. Xingmei is an artificial hybrid of the other two species.

Methods: We studied the geographic distribution and flowering phenologies of *P. armeniaca* and *P. mume* cultivars from different regions of China. We analysed the copious data of the China Fruit Records (mei and apricot). To examine the origin of xingmei, we analysed the F_2 progenies of *P. armeniaca* × *P. mume* using a species-specific molecular marker. Finally, we researched the genotypes of *P. mume*, *P. armeniaca*, and xingmei to determine whether the three are post-zygotically isolated.

Results: Isolation due to geographical location and flowering phenology are strong pre-zygotic isolation barriers that maintain the separation of *P. armeniaca* and *P. mume*. That separation limits the opportunity for natural hybridization between these two species, and probably prevented the natural appearance of xingmei. Furthermore, no post-zygotic reproductive isolation exists between *P. armeniaca* and *P. mume*.

Conclusions: Pre-zygotic isolation played an important role in the evolution of *P. armeniaca* and *P. mume*. It prevented their natural hybridization. Xingmei is likely to be an artificial hybrid.

Keywords: pre-zygotic isolation, *Prunus armeniaca*, *Prunus mume*, research, speciation.

INTRODUCTION

The evolution of reproductive isolation is central to the speciation process (Kay, 2006). Understanding the genesis and reasons for development of reproductive isolation barriers during speciation of organisms is a formidable challenge of evolutionary biology (Dobzhansky, 1937;

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Mayr, 1942; Stebbins, 1950; Clausen, 1951; Grant, 1981; Coyne and Orr, 2004) due to the role of these barriers in determining gene flow and limiting hybridization between formerly interbreeding populations (Coyne and Orr, 1998). Understanding the types of barriers that contribute to reproductive isolation will help to elucidate the conditions under which speciation is likely to occur and the role of natural selection in speciation, and it can also motivate studies on the genetic basis of speciation (Kay, 2006). Plant evolutionary biologists have recently made major progress in quantifying individual strengths of an array of reproductive isolation barriers in a number of different plant systems (Martin and Willis, 2007; Lowry *et al.*, 2008a, 2008b). Comprehensive studies of some closely related species have led to the identification of components of reproductive barriers over the last decade (Verkleij *et al.*, 1989; Bradshaw and Schemske, 2003; Galliot *et al.*, 2006; Noor and Feder, 2006; Van der Niet *et al.*, 2006; Pascarella, 2007; Marques *et al.*, 2007; Waelti *et al.*, 2008).

Reproductive isolation among pairs of plant species is rarely due to a single isolating factor, but could be a consequence of a large number of intrinsic mechanisms stemming from several pre- and post-zygotic barriers and their potentially complex interactions (Coyne and Orr, 2004; Moyle *et al.*, 2004; Wulff *et al.*., 2004; Bomblies and Weigel, 2007; Bomblies *et al.*, 2007; Hoballah *et al.*, 2007; Moyle, 2007; Rieseberg and Willis, 2007). Pre-zygotic isolation mechanisms include lack of recognition, incomplete or assortative mating as a byproduct of species divergence, or selection against hybrid formation (reinforcement) when hybrids are sterile or less fit. In contrast, post-zygotic mechanisms include hybrid inviability or sterility (Lai *et al.*, 2005; Sweigart *et al.*, 2007; Widmer *et al.*, 2009) among others (Presgraves, 2002; Scopece *et al.*, 2007, 2008). Many studies support the assertion that pre-zygotic isolation barriers, including immigrant inviability, flowering time, and pollinator isolation, make a greater overall contribution to total reproductive isolation than post-zygotic barriers (Chari and Wilson, 2001; Ramsey *et al.*, 2003). This assertion is based on the observation that pre-zygotic barriers are often individually stronger and act earlier over the life history of an organism than do post-zygotic barriers (Coyne and Orr, 2004; Nosil *et al.*, 2005). The degree of reproductive isolation through pre-zygotic mechanisms is also critical for two related sympatric species, as it is an important factor influencing genetic integrity of a species and the probability of the formation of hybrids among related species.

Geographical and floral pre-zygotic ecological barriers are also important to speciation and evolution. Geographical isolation, also known as eco-geographic isolation, is defined as reproductive isolation due to limited contact among related taxa as a result of ecological range limits of those taxa (Schemske, 2000; Ramsey *et al.*, 2003; Angert and Schemske, 2005). Once segments of a species population become geographically isolated, these segments diverge in phenotype and genotype due to natural selection, genetic drift, and adaptability to various regional climatic environments, eventually leading to incompatibility in mating or difficult inter- or intra-species crossing in flowering organisms (Schemske, 2000). Ever since the concept of floral isolation was introduced, it has been embedded in research on species diversification of flowering plants. Differences in floral structures, pollination agents, and phenology could function as reproductive isolation mechanisms for related plant species, especially for sympatric species, and they have the potential to be the most important barrier preventing the formation of viable hybrids in flowering plants (Silvertown *et al.*, 2005; Savolainen *et al.*, 2006; Martin and Willis, 2007; Widmer *et al.*, 2009).

Prunus armeniaca and *P. mume* belong to the sub-family Prunoideae within the Rosaceae and both are native to China, the oldest and largest centre of origin for *Prunus* species. They have both been cultivated for thousands of years and there are very many wild and cultivated varieties in China. Both have played an important role in human diet and health

for millennia. It has been reported that these two species are genetically related and cross-compatible but differ in geographical distribution and floral phenology. The original range of *P. mume* and its races was in the tropical and subtropical regions located between latitude 20°N and 30°N, comprising Taiwan, Guangdong, Fujian, Yunnan, Guizhou, Tibet, and several other southern provinces. The chief centre of origin for *P. armeniaca* includes the northeast, central, west and northeastern Tibet within latitude 34°N–54°N, with only a few races being found in the subtropical regions of China (Fig. 1). The floral phenology of *P. mume* is also different: it flowers 10–90 days earlier than *P. armeniaca* (Zhang and Zhang, 2003). Hybrids of *P. armeniaca* and *P. mume*, collectively named xingmei (alternatively named 'meixing'), exist, but whether these hybrids are natural or artificial is unknown. Although many research findings on inter- or intra-specific relationships between these two species have been discussed (Hormaza, 2002; Fang *et al.*, 2005a, 2005b, 2006a, 2006b; Li *et al.*, 2010), there is little information on the differentiation and isolation barriers to their interbreeding.

In this study, we first conducted research on the factors regulating interspecies mating that could have affected reproductive isolation and speciation between *P. armeniaca* and *P. mume*. We estimated the strengths of their geographical and flowering isolation as two of the possible pre-zygotic barriers, and we verified the artificial identity of the existing hybrids (individual cultivars of xingmei) of *P. armeniaca* and *P. mume* with species-specific markers derived from an AFLP marker. Furthermore, we examined segregation patterns of the species-specific markers and some *P. armeniaca* or *P. mume* species-specific characters in the progenies of self-crosses of 'Takadau Bungo' (a hybrid of *P. armeniaca* and *P. mume*) to confirm the natural reproductive isolation between *P. armeniaca* and *P. mume*.

MATERIALS AND METHODS

Data on geographical distribution and flowering phenology

To determine the latitudinal distributions and flowering phenologies of the two *Prunus* species in nature, we used data on 1423 *P. armeniaca* and 198 *P. mume* cultivars documented in the China Fruit Records – apricot (Zhang and Zhang, 2003) and mei (Chu, 1999). We utilized the flowering phenology data of the two species where the cultivars were separated by latitude and time of floral development. The reference data were the dates of first flowering to the dates of last flower drop. The data recorded in the updated China Fruit Records on both *P. mume* and *P. armeniaca* are scientifically reliable because their reproductive phenologies were studied by taking into account their life-forms. These data are buttressed by historical records on the phenology of the two species. We determined patterns of flowering using more than 25% of the plants of each species in each plot to give the total duration of each phenological phase. High-intensity phenological patterns were established using more than 25% of individuals in each plot to establish times such as day of first flower, peak flowering, and last flower (Chu, 1999; Zhang and Zhang, 2003). Data on flowering schedules of different cultivars of *P. mume*, *P. armeniaca*, and *Armeniaca mume* (xingmei) from different regions were randomly selected, extracted, and tabulated (evolutionary-ecology.com/data/2673appendix.pdf).

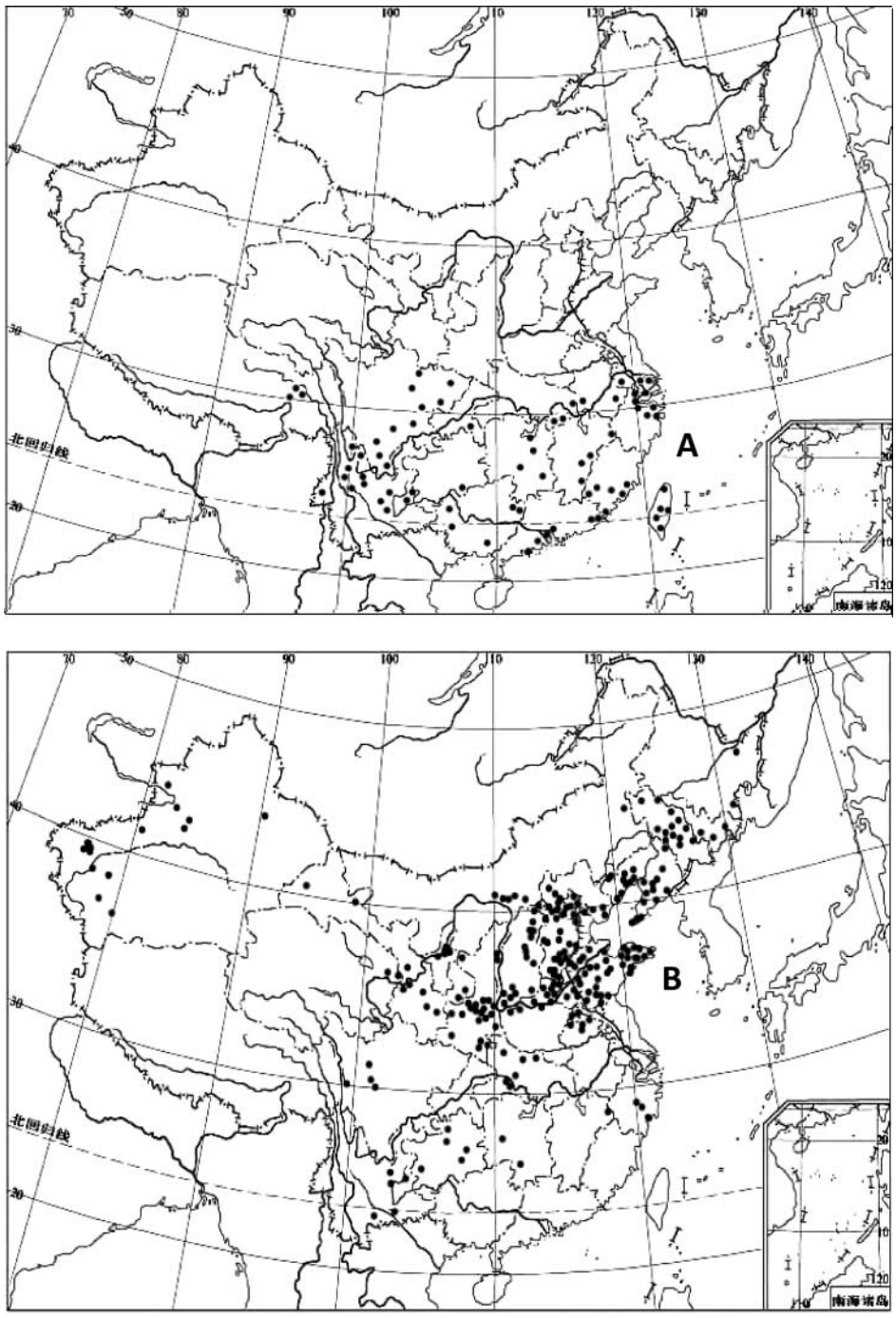

Fig. 1. Geographical distribution of *P. armeniaca* and *P. mume* in China. (A) The original distribution of *P. mume*, which lies between 21°N and 33°N, and includes 11 provinces in China. (B) The original distribution of *P. armeniaca*, which lies between 23°N and 47°N, and includes 21 provinces in China. Most of the distribution of *P. armeniaca* lies above 30°N.

Plant materials

We used 90 *P. mume* and 25 xingmei cultivars collected in the Mei Resources Nursery of Nanjing Agricultural University, China. Their background information had previously been documented by Chen (1996) and Chu (1999). We also used 72 *P. armeniaca* accessions collected from the National Apricot Resources Nursery at the Institute of Pomology and Forestry, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China, the background information having been previously documented by Zhang and Zhang (2003). All the accessions are as listed in Table 1. We made self-crosses of 'Takadau Bungo' in February 2009, collected all the seeds in July, then planted them in separate pots in a greenhouse after stratification for 2–3 months. We also collected the leaves of individual plants of F_2 hybrids of *P. armeniaca* and *P. mume* for DNA isolation.

DNA and RNA isolation

Fresh young leaves of *P. armeniaca* and *P. mume* cultivars were collected, flash frozen in liquid nitrogen, and stored at −40°C until use for DNA extraction. Genomic DNA was isolated from leaves using the DNeasy Plant Mini Kit (Qiagen, City, CA, USA) following the manufacturer's instructions. DNA concentrations were quantified using a Hoefer DyNA Quant 200 (Pharmacia Biotech, Piscataway, NJ, USA) while the quality of DNA was ascertained on 0.7% agarose gels with ethidium bromide. Leaves, flowers, and fruits of *P. armeniaca* ('pingguo') and *P. mume* ('xiyeqing') at different stages were collected for RNA isolation, where total RNA from flowers, leaves, and fruits was extracted using Trizol (Takara Biotechnology, Dalian Co. Ltd.), and total RNA digested by DNase I (RNase Free, Takara Biotechnology, Dalian Co. Ltd.). The quantity and integrity of RNA were assessed by BioPhotometer (Eppendorf, Gene Company Ltd.) and electrophoresis on agarose gels with ethidium bromide.

First strand cDNA synthesis

First strand cDNA was synthesized according to the reverse transcriptase protocol (TAKARA M-MLV RNase H^-, Takara Biotechnology, Dalian Co. Ltd.), where 1 μg RNA sample (organs: flower, leaf, fruit) and 1 μL R11466 adapter (5′-GAGGACTGCAG CTGACTGACTAC$(T)_{30}$VN-3′), together with DEPC-H_2O to a volume of 12 μL, were mixed and incubated at 70°C for 5 min before being placed in ice for 2 min. Next, 4 μL 5 × M-MLV Buffer, 1 μL RNase Inhibitor (20 mM), 2 μL dNTP Mix (10 mM), and 1 μL M-MLV (RNase H^-) were added to a total volume of 20 μL, mixed well, and incubated at 42°C for 1 h, at 70°C for 15 min, then placed on ice for 5 min. The concentration of cDNA from different tissues was adjusted by ubiquinone and then mixed and stored at −20°C until use.

Specific marker and primer

We used the specific marker and primers named 2MMF/2MMR (Table 2 and Fig. 2) previously derived from AFLP that could generate 386 bp and 321 bp PCR products in *P. armeniaca* and *P. mume*, respectively. In addition, these primers can be co-amplified in 'Bungo' and 'Takadau Bungo' (Fang *et al.*, 2006). A 68 bp region was inserted into the target sequence of *P. armeniaca*. This specific marker was tested for its presence in further

Table 1. List of cultivars used for molecular verification

No.	Cultivar name	Type	Origin	No.	Cultivar name	Type	Origin
1	Oushuku	*P. mume*	Japan	33	Hangzhoubaimei	*P. mume*	Zhejiang
2	Sichuanhuangmei	*P. mume*	Sichuan	34	Taihu1	*P. mume*	Jiangsu
3	Nanhong	*P. mume*	Jiangsu	35	Yourou	*P. mume*	Japan
4	Hongding	*P. mume*	Zhejiang	36	Sichuanqingmei	*P. mume*	Sichuan
5	Daqiandi	*P. mume*	Jiangsu	37	Tonglv	*P. mume*	Sichuan
6	QiXingmei	*P. mume*	Jiangsu	38	Nankou	*P. mume*	Japan
7	Momei	*P. mume*	Jiangsu	39	Dalizhong	*P. mume*	Guangdong
8	Yourou2	*P. mume*	Japan	40	Dongqing	*P. mume*	Zhejiang
9	Fujianbaifen	*P. mume*	Fujian	41	Weishangzhong	*P. mume*	Zhejiang
10	Xiyeqing	*P. mume*	Zhejiang	42	Xianmimei	*P. mume*	Hunan
11	Henghe	*P. mume*	Guangdong	43	Xiao'ougongfen	*P. mume*	Jiangsu
12	Hanakami	*P. mume*	Japan	44	Shinoume	*P. mume*	Japan
13	Ruantiaohongmei	*P. mume*	Zhejiang	45	TaoXingmei	*P. mume*	Fujian
14	Koushuu Saisyou	*P. mume*	Japan	46	Gojirou	*P. mume*	Japan
15	Koume	*P. mume*	Japan	47	Taihu3	*P. mume*	Zhejiang
16	Zaohuamei	*P. mume*	Jiangsu	48	Changnong17	*P. mume*	Zhejiang
17	Lve	*P. mume*	Jiangsu	49	Dongshanlimei	*P. mume*	Jiangsu
18	Siyuemei	*P. mume*	Hunan	50	Shuangshuidarou	*P. mume*	Guangdong
19	Yeliqing	*P. mume*	Zhejiang	51	Zaohong	*P. mume*	Guangdong
20	Longyanmei	*P. mume*	Guangdong	52	Gessekai	*P. mume*	Japan
21	Koushuu Koume	*P. mume*	Japan	53	Setsudaume	*P. mume*	Japan
22	Shuangtaomei	*P. mume*	Yunnan	54	Shirokaga	*P. mume*	Japan
23	Sichuanbaimei	*P. mume*	Sichuan	55	Tougorou	*P. mume*	Japan
24	Zhizhimei	*P. mume*	Jiangsu	56	Zonghong	*P. mume*	Jiangsu
25	Yanhua	*P. mume*	Fujian	57	Huangxiaoda	*P. mume*	Zhejiang
26	Lizimei	*P. mume*	Jiangsu	58	Yunnanzhaoshui	*P. mume*	Yunnan
27	Dayezhugan	*P. mume*	Zhejiang	59	Guangdonghaungpi	*P. mume*	Guangdong
28	Gyokuei	*P. mume*	Japan	60	Pinzimei	*P. mume*	Yunnan
29	Wanhong	*P. mume*	Zhejiang	61	Yinvgongfen	*P. mume*	Jiangsu
30	Hongmei	*P. mume*	Jiangsu	62	Fenyungongfen	*P. mume*	Jiangsu
31	Xiaoqingmei	*P. mume*	Jiangsu	63	Huqiugongfen	*P. mume*	Jiangsu
32	Jiuzhongmei	*P. mume*	Jiangsu	64	Xiangfugongfen	*P. mume*	Jiangsu

65	Fenyugongfen	*P. mume*	Jiangsu
66	Xiaogongfen	*P. mume*	Jiangsu
67	Dagongfen	*P. mume*	Jiangsu
68	Fenpigongfen	*P. mume*	Jiangsu
69	Huqiuwanfen	*P. mume*	Jiangsu
70	Yulugongfen	*P. mume*	Jiangsu
71	Renmiantaohua	*P. mume*	Jiangsu
72	Koubandahong	*P. mume*	Jiangsu
73	Sanlunyudie	*P. mume*	Jiangsu
74	Baimudan	*P. mume*	Jiangsu
75	Zaozhusha	*P. mume*	Jiangsu
76	Yinhongzhusha	*P. mume*	Jiangsu
77	Zuoqiaohong	*P. mume*	Jiangsu
78	Nanjingmomei	*P. mume*	Jiangsu
79	Yangzhoumomei	*P. mume*	Jiangsu
80	Nanjinghouding	*P. mume*	Jiangsu
81	Fanhuazhusha	*P. mume*	Jiangsu
82	Nanjingzhaoshuizhusha	*P. mume*	Jiangsu
83	Nanjingtagezhusha	*P. mume*	Jiangsu
84	Yinxutaigezhusha	*P. mume*	Jiangsu
85	Zhoubanyudie	*P. mume*	Jiangsu
86	Xiaoyudie	*P. mume*	Jiangsu
87	Ruxiangyudie	*P. mume*	Jiangsu
88	Fanhuayudie	*P. mume*	Jiangsu
89	Jiuguanlve	*P. mume*	Jiangsu
90	Yiweilve	*P. mume*	Jiangsu
91	Caoxing	*P. armeniaca*	Anhui
92	Caizihuang	*P. armeniaca*	Shandong
93	Xinjiangshaxing	*P. armeniaca*	Xinjiang
94	Zaohetian	*P. armeniaca*	Hebei
95	Shizixing	*P. armeniaca*	Hebei
96	Badou	*P. armeniaca*	Anhui
97	Guanyelian	*P. armeniaca*	Shanxi
98	Fa1	*P. armeniaca*	France
99	Zaoxiangbai	*P.armeniaca*	Beijing
100	Lveshangxing	*P. armeniaca*	Liaoning
101	Xinxiedashi	*P. armeniaca*	Liaoning
102	Honghebao	*P. armeniaca*	Shandong
103	Longjingbaixing	*P. armeniaca*	Shandong
104	Dongning2hao	*P. armeniaca*	Heilong
105	Mituoluo	*P. armeniaca*	Beijing
106	Fa2	*P. armeniaca*	France
107	Longwangmao	*P. armeniaca*	Hebei
108	Hongjinzhen	*P. armeniaca*	Shandong
109	Liguang	*P. armeniaca*	Xinjiang
110	Chuangzhihongxing	*P. armeniaca*	Hebei
111	Xingtaihongjiexing	*P. armeniaca*	Hebei
112	Fangshanhongxing	*P. armeniaca*	Beijing
113	Erhongxing	*P. armeniaca*	Hebei
114	Baiyubian	*P. armeniaca*	Beijing
115	Jidanxing	*P. armeniaca*	Ningxia
116	Dabaixing	*P. armeniaca*	Jilin
117	You1	*P. armeniaca*	Hebei
118	Cangzaotian1	*P. armeniaca*	Hebei
119	Caopixing	*P. armeniaca*	Shanxi
120	Yuhankui	*P. armeniaca*	Hebei
121	Zaohuang	*P. armeniaca*	Heilongjiang
122	Dabada	*P. armeniaca*	Beijing
123	Zihexing	*P. armeniaca*	Hebei
124	Yangshaohuang2	*P. armeniaca*	Henan
125	Beianhe	*P. armeniaca*	Beijing
126	Shanhuangxing	*P. armeniaca*	Beijing
127	Meiwuming	*P. armeniaca*	Beijing
128	Beizhaihong	*P. armeniaca*	Beijing
129	Xinnong25	*P. armeniaca*	Hebei
130	Yiwofeng	*P. armeniaca*	Hebei
131	Hongxing	*P. armeniaca*	Gansu
132	Hongbada	*P. armeniaca*	Beijing

Table 1.—*continued*

No.	Cultivar name	Type	Origin	No.	Cultivar name	Type	Origin
133	Taohexing	*P. armeniaca*	Liaoning	161	Touwojie	*P. armeniaca*	Shaanxi
134	Yingbaixing	*P. armeniaca*	Jilin	162	Dayubada	*P. armeniaca*	Beijng
135	Mixianxing	*P. armeniaca*	Henan	163	Liaomeixing	*Armeniaca mume.*	Liaoning
136	Xiaxianbaixing	*P. armeniaca*	Shanxi	164	Wuzangye	*Armeniaca mume.*	Jiangsu
137	Dafeng	*P. armeniaca*	Hebei	165	Jiangnanwusuo	*Armeniaca mume.*	Unknown
138	Yinbaixing	*P. armeniaca*	Jilin	166	Bungo	*Armeniaca mume.*	Japan
139	Mengguxing	*P. armeniaca*	Heilongjiang	167	Yunanfenghou	*Armeniaca mume.*	Yunan
140	Jiguang	*P. armeniaca*	Beijing	168	Dieyuchong	*Armeniaca mume.*	Jiangsu
141	Maluanling	*P. armeniaca*	Hebei	169	Songchun	*Armeniaca mume.*	Jiangsu
142	Xiaobaoxiangbai	*P. armeniaca*	Beijing	170	Youkihi	*Armeniaca mume.*	Japan
143	Dajiehexing	*P. armeniaca*	Liaoning	171	YunnanXingmei	*Armeniaca mume.*	Yunnan
144	Zaohuang	*P. armeniaca*	Heilongjang	172	Shuhuasongchun	*Armeniaca mume.*	Jiangsu
145	Sanyuancaoxing	*P. armeniaca*	Shannxi	173	Beijingfenghou	*Armeniaca mume.*	Beijing
146	Baiyuxing	*P. armeniaca*	Shandong	174	TakadauBungo	*Armeniaca mume.*	Japan
147	Shajinhong	*P. armeniaca*	Shanxi	175	QijiangXingmei	*Armeniaca mume.*	Sichuan
148	Linbaohongxing	*P. armeniaca*	Henan	176	Yaeageha	*Armeniaca mume.*	Japan
149	Longquanwu	*P. armeniaca*	Beijing	177	Soumeinotuki	*Armeniaca mume.*	Japan
150	Niujiaobangzi	*P. armeniaca*	Shaanxi	178	Mongakushi	*Armeniaca mume.*	Japan
151	Lintonghongxing	*P. armeniaca*	Shaanxi	179	Guxue	*Armeniaca mume.*	Jiangsu
152	Lanzhouhong	*P. armeniaca*	Gansu	180	Nanjingguifei	*Armeniaca mume.*	Jiangsu
153	Shanhuangxing	*P. armeniaca*	Beijing	181	Heitian	*Armeniaca mume.*	Jiangsu
154	Haidongxing	*P. armeniaca*	Shannxi	182	Qiansuiju	*Armeniaca mume.*	Jiangsu
155	Dongning1	*P. armeniaca*	Heilongjang	183	Kaiyunchuizhi	*Armeniaca mume.*	Jiangsu
156	Dahuangxing	*P. armeniaca*	Tianjin	184	Syougahougo	*Armeniaca mume.*	Japan
157	Chibangzi	*P. armeniaca*	Shaanxi	185	Kaiunshidare	*Armeniaca mume.*	Japan
158	Kuhonglian	*P. armeniaca*	Shaanxi	186	Danfenghou	*Armeniaca mume.*	Beijing
159	Fangshanxiangbai	*P. armeniaca*	Beijing	187	Yinafenghou	*Armeniaca mume.*	Jiangsu
160	Zhuyaozi	*P. armeniaca*	Liaoning				

Note: The detailed characteristics of *P. armeniaca* and *P. mume* cultivars are described in Zhang and Zhang (2003) and Chu (1999). The detailed characteristics of *Armeniaca mume* Sieb. (also named Xingmei in this paper) cultivars are described in Chen (1996).

Table 2. Summary of primers used

Primer name	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Expected product (bp)
2MMF	AGGACTCAGAATCAGAATCAAATTAAAA	51	321 and/or 386*
2MMR	TTTTTCCTCACATTTGGAT		
4MF	TATCATCCAAGAAGAAAA	53	68
4MR	TATTCCATGAATATTATA		

* The target.

P. armeniaca, *P. mume*, and xingmei cultivars and could also be used for specific expression analysis between the two species and their cultivars. To identify whether the specific regions existed in *P. mume*, a pair of new primers named 4MF/4MR were designed by Primer 3 (http://www.embnet.sk/cgi-bin/primer3_www.cgi) software in the 68 bp specific region and was amplified from several *P. mume* cultivars (Table 1). The summary of primers is as listed in Table 2.

PCR amplification and PCR product sequencing

The amplification reactions were each performed using an Eppendorf Authorized Thermal Cycler, and the amplification system and PCR conditions followed Li *et al.* (2010), with the annealing temperature set at 51°C. All PCR products were purified with AxyPrep DNA Gel Extraction Kit (Axygen Scientific Inc., Union City, CA, USA) and 5 μL of the products detected on a 1.0% agarose gel. The purified PCR products were ligated into PMD-18 vector (Takara Biotechnology, Dalian Co. Ltd.), and transformed into *Escherichia coli* DH5α for cloning following the instructions of the manufacturer. Fresh clones with target fragments were sent to Invitrogen Company, Shanghai, PR China for sequencing.

Expression of three target fragments in *P. armeniaca* and *P. mume*

We also verified the expression of 321, 386, and 68 bp fragments in the mixed cDNA of *P. armeniaca* and *P. mume*. We performed this verification in a total volume of 30 μL containing 1 μL template cDNA, 3 μL 10 × PCR buffer, 2.4 μL dNTPs (2.5 mM), 1.8 μL $MgCl_2$ (2.5 mM), 1.2 μL (10 pM) of each primer, 2.5 Units Taq DNA polymerase (Takara Biotechnology, Dalian Co. Ltd.), with ddH_2O added to the total volume. The amplification reactions and program, product purification, cloning, and sequencing were the same as described earlier.

RESULTS

Geographic distribution of *P. armeniaca* and *P. mume* in China and their comparative floral phenologies

In China, the centre of origin for *P. armeniaca* includes the northeast, central, west and northeastern Tibet, while *P. mume* was concentrated in the areas northwest of Yunnan, southwest of Sichuan, and east of Tibet (Fig. 1). These regions do not overlap and, based on the distribution of *P. armeniaca* and *P. mume*, geographical isolation may be the crucial

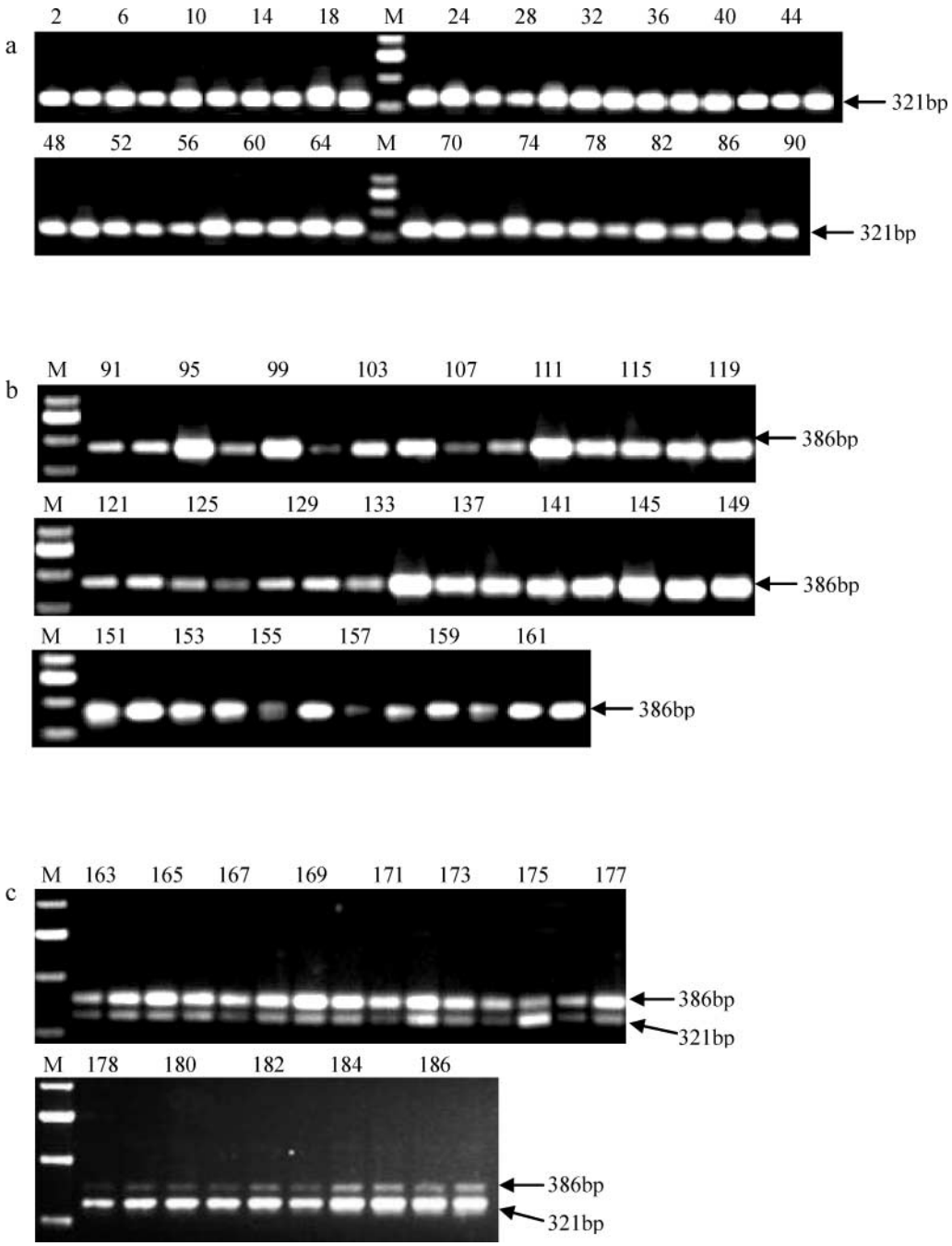

Fig. 2. Amplified results of representative varieties of *P. armeniaca*, *P. mume*, and *Armeniaca mume* Sieb. (xingmei) with 2MMF/2MMR. M: Maker DL2000 plus. Over the top of the strips are some of the accession numbers of *P. mume*, *P. armeniaca*, and xingmei as listed in Table 1. Panel (A) shows that the target fragment amplified from *P. mume* is 321 bp. Panel (B) shows that the target fragment amplified from *P. armeniaca* is 386 bp. Panel (C) shows that two target fragments of sizes 321 bp and 386 bp were amplified from xingmei.

factor needed to maintain species boundaries, thus ensuring that they can evolve independently of each other and evolve radically different phenotypic characteristics. Judging from the distribution of varieties (Table 3), *P. armeniaca* appears to be distributed in more provinces, cities, and autonomous regions, mainly located in central and northern China within latitudes 30°N and 54°N, whereas most *P. mume* cultivars are distributed in southern China below latitude 32°N (Fig. 1). Even though there are also some sympatric areas between 23°N and 32°N where the two species co-occur, these areas are small and the apricots there are largely cultivated cultivars. Because these fruit trees are economically important, people may have introduced them into these areas, in which case their co-occurrence is not a natural phenomenon.

We also investigated the flowering phenology, including day of first flower, peak flowering, and last flowering in *P. armeniaca* and *P. mume*. Table 3 summarizes the results, which show that:

- The main flowering time of *P. mume* ranges from mid-December to mid-February, with cultivars classified as early-, mid- or late-flowering.
- The higher the latitude of *P. mume*, the longer the delay in onset of flowering. (For example, *P. mume* blooms earlier in Guangdong than Henan and Anhui.)
- Most flowering in *P. armeniaca* occurs after February, i.e. between early April and late May, with the same response to latitude as *P. mume*.
- In sympatric areas, fields with both species planted under natural conditions had no overlap in the flowering times of *P. mume and P. armeniaca*: *P. armeniaca* begins flowering 10–30 days after initiation of the last *P. mume* flowers.

These findings suggest that flowering phenology is sufficient to prevent gene flow between *P. mume and P. armeniaca*. Compared with the intra-specific variability and distribution, variations in flowering time also appeared to be an extremely strong and important pre-zygotic barrier between these two species. Due to the difference in geographic distribution and the much earlier flowering phenology of *P. mume* compared with *P. armeniaca* (Table 3), the chance of natural hybridization between these two species appears remote.

Despite this remote possibility, we did notice a very close overlap in flowering times between *P. mume* and *P. armeniaca* in one sympatric area (Yunnan). There, the three *P. mume* cultivars (Yunnanzhaoshui, Chaodoumei, and Shuangtaomei) and one *P. armeniaca* cultivar (Malonghuangxing) all initiate flowering between early January and late February, and their last flowers drop between late February and mid-March. Thus these *P. mume* and *P. armeniaca* cultivars might cross-pollinate in this region (Table 4).

Molecular evidence for reproductive isolation using a species-specific marker in *P. mume, P. armeniaca,* and xingmei

Two specific PCR products have been amplified previously from 'Bungo' and 'Takadau Bungo' cultivars, both of which are hybrids of *P. mume* and *P. armeniaca* (Shimada *et al.*, 1994). Additional analysis confirmed that the longer of the two PCR products was amplified only from *P. armeniaca*, while the shorter product was from *P. mume* only, with the longer PCR product containing a 68 bp insertion fragment lacking in the shorter one. *Prunus mume*, *P. armeniaca*, and xingmei cultivars could easily be separated into three groups of plants. No *P. mume* cultivars had a 386 bp fragment and no *P. armeniaca* cultivars had a 321 bp

Table 3. Characteristics of flowering phenophases of *P. armeniaca* and *P. mume* at different latitudes in China

Species	Distribution range		Floral phenology (time from flower initiation to last flower drop)	
	Province	Latitude		
P. mume	Guandong	20.02–25.14°N	Mid-December to mid-February	
	Taiwan	21.45–25.56°N	Late December to mid-February	
	Guangxi	22.11–25.80°N	Early December to late January	
	Fujian	23.72–27.92°N	Late November to late January	
	Jiangxi	24.76–29.90°N	Late December to late February	
	Tibet	27.98–33.44°N	Mid December to late February	
P. armeniaca and *P. mume*	Yunnan	21.48–28.58°N	Late December to early February*	Late February to early March**
	Hunan	24.97–29.71°N	Early to late February*	Late March to early April**
	Guizhou	25.00–28.57°N	Late December to late February*	Mid-March to early April**
	Sichuan	26.67–33.23°N	Early to late February*	Early March to mid-April**
	Zhejiang	27.08–30.77°N	Mid-February to mid-March*	Early to late April**
	Hubei	29.23–32.65°N	Early to late February*	Mid-March to early April**
	Anhui	29.72–34.42°N	Mid-February to early March*	Late March to mid-April**
	Jiangsu	31.16–34.83°N	Mid-January to late March*	Late March to late April**
	Henan	31.62–36.10°N	Mid-February to early March*	Mid-March to early April**
P. armeniaca	Shandong	31.49–37.74°N	Early April to late April	
	Qinghai	32.23–38.20°N	Early April to mid-April	
	Shaanxi	32.70–39.05°N	Mid-March to mid-April	
	Shanxi	34.12–40.42°N	Late March to late April	
	Gansu	33.33–40.51°N	Late March to late April	
	Ningxia	35.50–39.04°N	Early April to late April	
	Hebei	36.35–41.87°N	Early April to early May	
	Xinjiang	36.86–48.05°N	Late March to late April	
	Neimeng	38.18–50.8°N	Early April to late April	
	Tianjin	39.13–40.05°N	Late March to late April	
	Beijing	39.22–40.47°N	Late March to late April	
	Liaoning	38.92–42.77°N	Early April to early May	
	Jilin	40.88–45.63°N	Late April to mid-May	
	Heilongjiang	44.07–53.48°N	Early May to late May	

* Flowering phenology of *P. mume* in sympatric areas. ** Flowering phenology of *P. armeniaca* in sympatric areas.

Table 4. Analysis of cultivar flowering phenology in a sympatric area (Yunnan Province)

Species	Cultivar	Mid-January	Late January	Early February	Mid-February	Late February*	Early March	Late March
P. mume	Yunnanzhaoshui			×	×	×		
P. mume	Chaodoumei			×	×	×	×	
P. mume	Shuangtaomei	×	×	×	×	×		
P. armeniaca	Malonghuangxing					×	×	×
Armeniaca mume	Yunnan Xingmei	×	×	×	×	×		

Note: × denotes presence of flowers.
*During this period, all the *P. mume, P. armeniaca*, and *Armeniaca mume* cultivars studied in the region have flowers at the same time.

fragment. The two fragments of 386 bp and 321 bp were amplified only from *P. armeniaca* and *P. mume*, respectively. But the xingmei cultivars had both the 386 and 321 bp bands, an aspect which illustrates that reproductive isolation exists between *P. armeniaca* and *P. mume*, and that xingmei is an artificial rather than a natural hybrid, since natural hybridization is difficult under reproductive isolation.

Verification of the origin of xingmei using *P. armeniaca* × *P. mume* F_2 progenies

To further provide evidence of the reproductive isolation and existence of the suggested multiple states of the PCR fragments, we verified the self-fertile progenies of a cultivar named 'Takadau Bungo', which is a hybrid of *P. armeniaca* and *P. mume*. We collected seeds of 'Takadau Bungo' and planted them after a 3 month stratification period. Traits comprising stem, internode, leaf shape and colour, as well as petiole colour of 'Takadau Bungo' plants are phenotypically similar to those of *P. mume*. We performed molecular population genetics studies on 76 individuals using molecular markers to examine their genotypes. All genotypes were consistent with the expected results. Forty individuals had both the 386 and 321 bp bands, 23 had the 321 bp band, and 13 had the 386 bp band (Fig. 3). These quantitative traits show that the separation and recombination proportions of these three situations agreed with the generally accepted ratio of 1:2:1. They also show that there was no inviability or sterility in xingmei, which provides evidence that there was no or negligible post-zygotic reproductive isolation between *P. armeniaca* and *P. mume*, and that pre-zygotic isolation must be the main barrier that has ensured independent evolution of these two species.

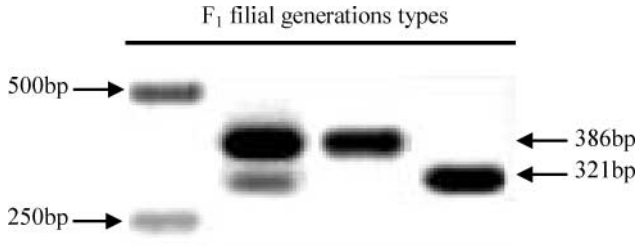

Fig. 3. Representative results of F_1 filial generation types of xingmei amplified by 2MMF/2MMR. Self-fertilized xingmei produces seedlings that amplified three kinds of target fragments (in sequence from left to right): (a) both 386 bp and 321 bp; (b) 386 bp; (c) 321 bp.

Characterization of the two PCR fragments in *P. armeniaca* and *P. mume*

Protein-sequence analysis of 386 bp and 321 bp fragments showed that, in fact, they do not actually code protein sequences. To further characterize these two PCR fragments, we sequenced and aligned several different *P. armeniaca* and *P. mume* cultivars. We found a 68 bp fragment inserted into the target sequence of *P. armeniaca* as shown in Table 5. Verification of the existence of the 68 bp fragment in *P. mume* (using primers 4MF/4MR) indicated that this fragment was amplified in all the *P. mume* cultivars (Fig. 4). The sequencing results showed a 99% similarity compared with the *P. armeniaca* samples, indicating that the 68 bp in *P. mume* was located at different loci of *P. mume* chromosomes. This suggests that the 68 bp fragment has some transposable characters.

We also carried out RT-PCR analysis of these three (386 bp, 321 bp, and 68 bp) fragments between these two species. Results showed that the two species-specific fragments of 386 and 321 bp were expressed in all *P. armeniaca* and *P. mume* cultivars, respectively (Fig. 5). Both of these fragments may be retro-transposable elements. RT-PCR verification results for the 68 bp insert region showed that it was expressed in both *P. armeniaca* and *P. mume* at the cDNA level (Fig. 6), indicating that this small fragment could also be a retro-transposable element but whose activation may affect transformation in the genome sequence of *P. armeniaca* or *P. mume*. The transpositional behaviours of this retro-element not only significantly affect the plant genome size, but are also closely related to plant evolution and may become evidence of evolution and differentiation between plant species (Grandbastien, 1992).

DISCUSSION

For a good understanding of speciation processes, it is important to study reproductive isolation across the full range of isolating mechanisms and across a range of divergence, from differentiated populations to distinct species. Since not all divergence among populations will lead to speciation, deeply diverged species will have continued to accumulate ecological and morphological differences, potentially obscuring the relative contributions of various isolating mechanisms during species formation. Studying incipient or very closely related species can minimize these problems and may provide the most relevant data on the conditions necessary for speciation. Although much is known about the genetic basis of reproductive isolation between species, little is understood about its underlying

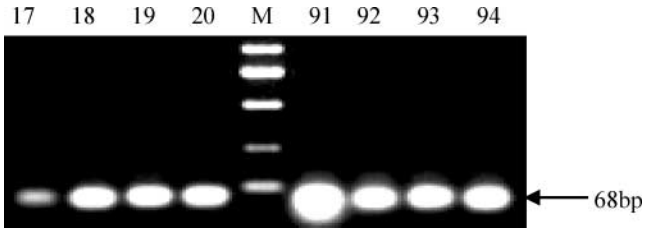

Fig. 4. Amplified results of the 68 bp insert between *P. armeniaca* and *P. mume* by primer 4MF/4MR. M: AXYGEN 100 bp Ladder DNA marker. The target fragments on the left of the marker are amplified results of *P. mume*, the ones on the right are *P. armeniaca*. 17–20: Accession numbers of *P. mume*; 91–94: Accession numbers of *P. armeniaca*. Cultivar names are shown in Table 1.

Table 5. The 68 bp inserted region detected in the target sequence of *P. armeniaca*

Species	Cultivar no.	Start site	Sequence (5′ → 3′)	Terminal site
P. armeniaca	94	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAAGTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	95	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAAGTCCCAATAATAAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	96	283	CGTAATTATCATCCAAGAAGAAAATCGTAACCAATCAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	97	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	98	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	99	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	100	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	101	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAATTTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	102	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAAACAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. armeniaca	103	283	CGAAATTATCATCCAAGAAGAAAATCGTAACCAATCAAATTCCCAATAATTAAATTTATAATATTCATGGAATA TATCTT	362
P. mume	19	286	CGAAAT . . . . . TATCTT	297
P. mume	20	286	CGAAAT . . . . . TATCTT	297
P. mume	21	286	CGAAAT . . . . . TATCTT	297
P. mume	22	286	CGAAAT . . . . . TATCTT	297
P. mume	23	286	CGAAAT . . . . . TATCTT	297
P. mume	24	286	CGTAAT . . . . . TATCTT	297
P. mume	25	286	CGAAAT . . . . . TATCTT	297
P. mume	26	286	CGAAAT . . . . . TATCTT	297
P. mume	27	286	CGAAAT . . . . . TATCTT	297
P. mume	28	286	CGAAAT . . . . . TATCTT	297

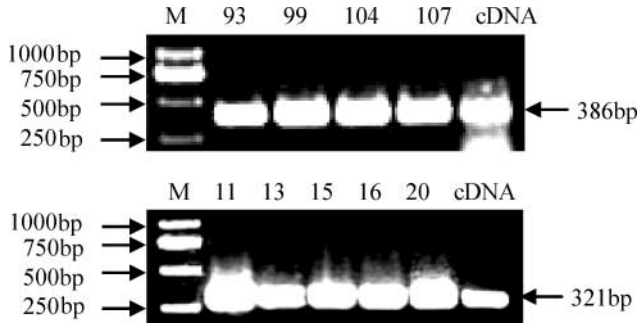

Fig. 5. Verification and expression results of the two target fragments in *P. armeniaca* and *P. mume* amplified by 2MMF/2MMR. M: DL2000 marker. The upper panel shows the amplified results from mix cDNA of *P. armeniaca*. The lower panel shows *P. mume*, whose target fragments were 386 bp and 321 bp. 11, 13, 15, 16, 20 and 93, 99, 104, 107: Accession numbers of *P. mume* and *P. armeniaca*, respectively. Cultivar names are listed in Table 1.

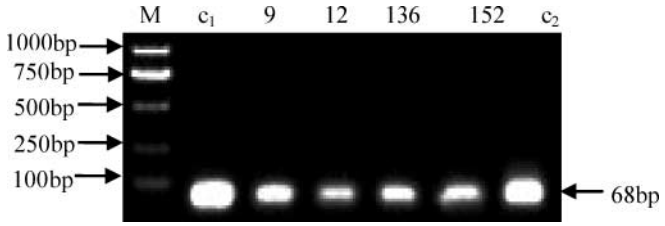

Fig. 6. Verification and expression of the inserted 68 bp target fragment. M: DL2000 marker. Accession numbers of *P. mume* and *P. armeniaca*: 9, 12, 136, 152; the cultivar names are listed in Table 1. c_1 and c_2: cDNA from *P. mume* and *P. armeniaca*, respectively. This picture shows that the 68 bp inserted fragment was successfully amplified from *P. mume* and *P. armeniaca*. Four amplified results on DNA level served as the control.

evolutionary causes. The study of two very closely related, but reproductively isolated, plant species has provided some valuable insights (Charlesworth and Charlesworth, 2000; Widmer *et al.*, 2009).

Geographical isolation

Studies have suggested that geographical isolation is a form of reproductive isolation in which members of a population become separated from another population by geographical barriers that prevent the interchange of genes between the separated populations and lead to different distributions or other aspects conducive to the speciation and evolution. *Prunus armeniaca* and *P. mume* are sister species of stone-fruit trees in China, and are grown in climates with well-differentiated seasons. Studies of these two species have demonstrated that they have different centres of origin and have spread in different ways based on different geographical distribution, with areas with specific climates being able to maintain species boundaries and prevent the interchange of genes between the separated populations. This has ensured that these species can evolve independently with different phenotypes and genotypes as a consequence of natural selection, genetic drift, and adaptability to various regional climatic environments, hence avoiding inter- or intra-species crossing.

Isolation by floral phenology

Floral isolation has been considered to be an important reproductive mechanism governing species diversification in many genera (Tang *et al.*, 2007). In this study, we were concerned with a specific case in which the parental species have differences in flowering phenology. It has been argued from first principles that plants will mate assortatively at flowering time (Breese, 1956; Wagner, 1976; Jain, 1979; Hartl and Clark, 1989; Lyons and Mulley, 1992; Lynch and Walsh, 1998; Hedrick, 2000; Kirkpatrick, 2000; Fox, 2003). Early bloomers will mate disproportionately with other early flowering plants, while late flowering individuals will mate disproportionately with late bloomers. There is no doubt that phenotypic variation is the rule in natural populations (Augspurger, 1981; Rathke and Lacey, 1985) and many studies have confirmed a genetic cause of this variation (Pors and Werner, 1989; Fox, 1990; Dorn and Mitchell-Olds, 1991; Kelly, 1992; Conner and Via, 1993; O'Neil, 1997; Quinn and Weitherington, 2002).

As with many other closely related taxa that have the potential to hybridize, a number of reproductive isolation mechanisms interact to allow F_1 hybrid formation (Arnold, 1997, 2006). Divergent flowering phenologies between *P. armeniaca* and *P. mume* have been shown to be an important pre-zygotic barrier to hybridization, with *P. mume* initiating and completing flowering earlier in the season than *P. armeniaca*. This divergence of flowering time has the potential to be the most important barrier to gene flow between sympatric populations, since the two species must clearly overlap in their reproductive times before later-acting barriers may come into play. Our results agree with this assertion. In this study, we investigated various aspects of the flowering phenology, in particular the day of first flower and last flower drop as well as the time of peak flowering. Our results show that the main flowering time of *P. mume* ranges from mid-December to mid-February, and that the higher the latitude, the later the flowering time. In contrast, the floral phenology of *P. armeniaca* is distributed between early April and late May, with the same change in trend with respect to latitude as in *P. mume*. The floral phenology of their hybrid, xingmei (*Armeniaca mume*), is distributed between mid-February and early April and is also affected by latitude (evolutionaryecology.com/data/2673appendix.pdf). Even in sympatric sites (with one exception – see next paragraph), there was no evident overlapping in flowering times: *P. mume* produced its last flowers 10–30 days before the first *P. armeniaca* flowers. Evidently, flowering time is an extremely important pre-zygotic barrier that prevents natural hybridization between the two species so that the formation of F_1 hybrid populations would be likely to result only by artificial means.

The closest to an overlap in flowering times between *P. mume* and *P. armeniaca* occurs in a sympatric area in Yunnan where the three *P. mume* cultivars (Yunnanzhaoshui, Chaodoumei, and Shuangtaomei) and one *P. armeniaca* cultivar (Malonghuangxing) all initiate flowering between early January and late February, and their last flowers drop between late February and mid-March. In late February, depending on weather and cultivation conditions, all four cultivars may flower simultaneously, thus increasing the possibility of cross-pollination between *P. mume* and *P. armeniaca* cultivars in this region.

It is interesting to note that in Yunnan, one cultivar of xingmei, aptly named 'Yunnan Xingmei', flowers at the same time as the four *P. mume* and *P. armeniaca* cultivars. Perhaps 'Yunnan Xingmei' is a hybrid of *P. armeniaca* 'Malonghuangxing' and one of the three *P. mume* cultivars. From closer scrutiny of the flowering times of 'Yunnan Xingmei' versus the three *P. mume* cultivars in Yunnan we learn, in fact, that *P. mume* 'Shuangtaomei' and *P. armeniaca* 'Malonghuangxing' are the likely parents of this hybrid because 'Yunnan

Xingmei' and 'Shuangtaomei' have similar flowering phenology (Table 4). So one must admit the possibility that xingmei originated from Yunnan as a natural hybrid. This observation calls for further investigations encompassing more cultivars and wild types of both *P. mume* and *P. armeniaca* in Yunnan.

As *P. mume* and *P. armeniaca* are both *Prunus* species, they can be cross-compatible (Chu, 1999). Some hybrids (e.g. the xingmei group) have phenotypes intermediate between *P. mume* and *P. armeniaca*. However, it is not yet clear whether these hybrids were produced by natural or artificial hybridization.

Franks and Weis (2009) point out that climate change may cause changes in reproductive isolation within and among populations. It is clear that speciation is unlikely to be a consequence of a single isolating barrier. Instead, floral isolation may be a complex variable that represents an ecological shift in direct response to climate change. Such a shift could evolve relatively quickly (Franks *et al.*, 2007). Combining results from the current study with our previous analyses, we expect to discover a significant association between shifts in flowering time and latitude ranges in sympatric areas that induce independent evolution and adaptability between *P. armeniaca* and *P. mume*.

Molecular evidence for reproductive isolation

Recent advances in molecular techniques enable researchers to identify the particular genomic regions and genes responsible for reproductive isolation barriers in plants (Josefsson *et al.*, 2006; Sweigart *et al.*, 2006; Bomblies *et al.*, 2007; Rieseberg and Willis, 2007; Case and Willis, 2008; Lexer and Widmer, 2008). Uncovering the genetic mechanisms of reproductive isolation promises to provide an insight into how these isolation barriers evolve. Importantly, the genetic architecture of reproductive isolation can control the genome-wide pattern of introgression among hybridizing species, since genomic regions that are tightly linked to reproductive isolation genes will not introgress across species boundaries at the same rate as other regions of the genome (Wu, 2001; Wu and Ting, 2004; Turner *et al.*, 2005).

We used two specific fragments to identify the genetic characteristics of the sequences of *P. armeniaca*, *P. mume* and their hybrid, xingmei. Our results show that there must be reproductive isolation between *P. armeniaca* and *P. mume*, and that xingmei was probably produced artificially. By studying the offspring of xingmei, we observed that pre-zygotic isolation is the strongest influence between these two species, much more than are viability and fertility of xingmei. The characterization and transposition of the 68 bp insert fragment on chromosomes may be an important factor for speciation, the activation of which could affect transformation of the genome sequence, leading to differences of phenotypic traits, adaptation, or could even provide an important impetus for evolution. Our findings are the first molecular evidence to qualify patterns of speciation by genomic regions for reproductive isolation barriers between *P. armeniaca* and *P. mume*. This information could provide insight into the spatial structuring of genetic variation in both species, which will be useful in the formulation of conservation strategies and may identify novel genotypes for use in the improvement and development of cultivars.

CONCLUSIONS

Our comparative analyses demonstrate that pre-zygotic reproductive isolation through geographical distribution and through differences in floral phenology plays an important

role in evolution between *P. armeniaca* and *P. mume*, and that xingmei is their fertile hybrid probably generated artificially. The empirical and molecular evidence in this paper can be informative and helpful in further studies on evolution and speciation of *P. armeniaca* and *P. mume* and even for all other related species.

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