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Taxonomic status of *Pinus henryi* using multiplexed microsatellite markers

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ABSTRACT. The taxonomic status of *Pinus henryi*, a rare species endemic to China, is still ambiguous. In this study, the genetic relationships among *P. henryi* and its congeners (*P. tabulaeformis*, *P. tabulaeformis* var. *mukdensis*, and *P. massoniana*) were revealed using multiplexed microsatellite markers, including chloroplast microsatellites, nuclear microsatellites, and expressed sequence tag microsatellites. The results refute the hypothesis that *P. henryi* is a subspecies of *P. tabulaeformis* or *P. massoniana* and support the suggestion that it may be a distinct species closely related to *P. tabulaeformis*.

Key words: *Pinus henryi*; SSR markers; Narrow endemic; Taxonomic status

INTRODUCTION

Pinus henryi, first described in 1902 by Masters, is a rare and endemic pine in China. There is controversy regarding the taxonomic position of *P. henryi*, and it has been a matter of debate from the 19th century to the present. *P. henryi* was initially synonymized with *P. tabulaeformis* by Cheng (1930), then treated as a variety of *P. massoniana* (Wu, 1956; Kuan, 1983), and finally it has been treated as a variety (Guan, 1982) or subspecies (Businsky, 1999) of *P. tabulaeformis*. However, the taxon has previously been treated as a distinct species (Zheng and Fu, 1978) and is still accepted as such in some studies (Niu, 1990; Zhang et al., 1995; Zhang, 1996; Zhao and Liu, 2010).

To settle the taxonomic status of *P. henryi*, extensive research has been carried out over the last 20 years using traditional morphological characters (Li and Xu, 1989; An and Zhao, 1992; Zhao and Liu, 2010). Based on these studies, *P. henryi* was treated as a distinct species. However, identification of closely related species based on morphological analysis may be distorted by a high level of homoplasy (Nyffeler et al., 2005). Recently, developed molecular methods offer significant opportunities to explore species delimitation (Bodo Slota and Porter, 2006; Liu et al., 2011; Yan et al., 2014).

Microsatellite markers (simple sequence repeats; SSR) are highly polymorphic, informative, co-dominant, technically simple, and reproducible (Gupta et al., 1996). Microsatellites have been successfully used for cultivar identification (Cardoso et al., 2014), hybrid species discrimination (Dong et al., 2011), and genetic map construction (Wang et al., 2014), as well as intra- and interspecies genetic diversity and relationship determination in closely related taxa (Guo and Luo, 2011; Qin et al., 2013).

In this study, we applied multiplexed microsatellite markers (including chloroplast microsatellites, nuclear microsatellites, and expressed sequence tag microsatellites) to elucidate the genetic relationships among *P. henryi* and its congeners: *P. tabulaeformis*, *P. tabulaeformis* var. *mukdensis*, and *P. massoniana*. All 4 pines are geographically and phylogenetically closer to each other than to any other Chinese pines (Wang et al., 1999). Our aims were to verify the reliability of previous systematic hypotheses, in particular whether *P. henryi* should be classified as a subspecies of *P. tabulaeformis* or *P. massoniana*.

MATERIAL AND METHODS

Plant material

Four taxa of *Pinus* were analyzed in this study: *P. henryi*, *P. tabulaeformis*, *P. tabulaeformis* var. *mukdensis* (the variety of *P. tabulaeformis*), and *P. massoniana*. The geographic location of taxa and the number of accessions sampled are shown in Table 1. Fresh needles were sampled from individual adult trees from each population. Distances between sampled trees ranged from 50-100 m depending on the population size, to ensure that the sample trees were representative of their populations. To avoid degradation of plant tissues, all samples were labeled and stored in sealed bags with silica gel as described by Sytsma et al. (1993) until DNA extraction.

Table 1. Locations of the sampled *Pinus* taxa and sample number (N).

Taxa	Code	Locality	N	Latitude (°N)/Longitude (°E)	Elevation (m)
P. henryi	B	Nanzheng, Shaaxi	30	32.857/106.586	1254
P. tabulaeformis	Y	Huanglong, Shaanxi	30	35.632/109.772	1127
P. tabulaeformis var. *mukdensis*	H	Anshan, Liaoning	30	40.960/123.147	294
P. massoniana	M	Yangxian, Shaanxi	30	33.326/107.624	722

DNA extraction and SSR-polymerase chain reactions (PCR) amplification

Total genomic DNA was extracted from silica gel-dried needles of each plant using the DNAsecure Plant kit (TIANGEN Biotech Co., Ltd., Beijing, China). Quality of the total DNA was verified by gel electrophoresis (1% agarose gel) and quantified using a BioPhotometer plus (Eppendorf, Hamburg, Germany). DNA samples were stored at -20°C until SSR amplification.

Six CpSSR primers (Pt48210, Pt110048, Pt41093, Pt30204, Pt1254, and Pt26081), 4 NSSR primers (B4D05, PtTX3107, NZPR114, and NZPR554), and 3 EST-SSR primers (Ptctg5167, Ptctg64, and RPtest5) were selected based on previous reports (Vendramin et al., 1996; Liewlaksaneeyanawin et al., 2004; Marum et al., 2009) (Table 2). PCRs were performed using 50 ng DNA in a volume of 20 μL containing 1X PCR buffer, 0.2 mM dNTPs, 0.2 mM Mg^{2+}, 0.2 mM of each primer pair, and 1 U *Taq* DNA polymerase. The cycle program included an initial 5 min denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, with a 10-min final extension at 72°C.

The amplification products were separated by 6% polyacrylamide gel electrophoresis and visualized using a simplified silver-staining method previously described by Xu et al. (2002).

Data analysis

Only clear, well-defined, and reproducible bands were recorded, and SSR loci were scored individually. For the statistical analysis, the patterns at all SSR loci were scored 1 for the presence of a band and 0 for the absence of a band in a binary data matrix for further analysis. Percentage of polymorphic bands (PPB) were calculated according to Smith et al. (1997). The binary matrix was transformed into a Nei and Li genetic distance matrix using FreeTree (Hampl et al., 2001). The phenograms were constructed using the neighbor-joining (NJ) method in the MEGA 4.0 software (Tamura et al., 2007). A principal coordinate analysis (PCoA) was performed based on the variance covariance matrix calculated from marker data using the program GenAlEx v6.5 (Peakall and Smouse, 2012). Bayesian analysis of SSR population structure was performed on the entire data set using the program STRUCTURE 2.3.2 (Pritchard et al., 2000) to detect population structure and estimate the number of populations (*K*) in a sample as well as to assign individuals to 1 or more of these populations (*K*). The number of genetically distinct clusters (*K*) was set to vary from 1-4. The model was run for 10 independent simulations for each *K*, using a burn-in length of 50,000 and a run length of 100,000 iterations. The most likely number of clusters was estimated according to the model value (ΔK) based on the second-order rate of change, with respect to *K*, of the likelihood function, following the procedure described by Evanno et al. (2005).

RESULTS

Amplification was successful for all 13 SSR markers. The total number of bands scored

per primer combination ranged from 3 to 15, with an average of 6.4 bands per primer set. The PPB for the 18 primer combinations were all 100%, confirming their utility to show differences between the samples analyzed in this study (Table 2).

Table 2. Primer sequences and characteristics of microsatellite loci in the *Pinus* taxa.

Primer	Primer sequence (5'-3')	Allele size (bp)	Number of alleles	PPB
Pt48210	F-CGAGATTGATCCGATACCAG R-GAGAGAACTCTCGAATTTTTCG	89-121	6	100
Pt110048	F-TAAGGGGACTAGAGCAGGCTA R-TTCGATATTGAACCTTGGACA	67-138	6	100
Pt41093	F-TCCCGAAAATACTAAAAAAGCA R-CTCATTGTTGAACTCATCGAGA	78-154	3	100
Pt30204	F-TCATAGCGGAAGATCCTCTTT R-CGGATTGATCCTAACCATACC	112-145	6	100
Pt1254	F-CAATTGGAATGAGAACAGATAGG R-TGCGTTGCACTTCGTTATAG	72-174	15	100
Pt26081	F-CCCGTATCCAGATATACTTCCA R-TGGTTTGATTCATTCGTTCAT	89-112	4	100
B4D05	F-TTTCGGCATCACAACAGC R-GTTTGGAAGCTGGAAGTTGG	218-234	3	100
PtTX3107	F-AAACAAGCCCACATCGTCAATC R-TCCCCTGGATCTGAGGA	150-164	8	100
NZPR114	F-AAGATGACCCACATGAAGTTTGG R-GGAGCTTTATAACATATCTCGATGC	179-187	4	100
NZPR554	F-GCGATGTGCAACCCTTGATA R-TGCTATTCCGTCAAAAACCC	246-252	9	100
Ptctg5167	F-TGCAGAGAGATTCGATGGG R-ATTTTGGTTTGTTTGCTGGC	293-358	10	100
Ptctg64	F-GGAAGCTGTTACAAGTGCGG R-ATCGAGAAAGAGAGGAAGGG	236-284	4	100
RPtest5	F-ACAACAATAATAACGGGGGC R-ACGCTTTAGATCCTCCTGCA	197-226	6	100

PPB = percentage of polymorphic bands.

To assess the taxonomic status of *P. henryi*, NJ cluster analysis was performed for all individuals of the *Pinus* taxa (Figure 1). Samples of *P*. *tabulaeformis* (Y) and *P*. *tabulaeformis* var. *mukdensis* (H) clustered together, while samples of *P. henryi* and *P. massoniana* clustered within 2 well-differentiated groups in the NJ dendrogram (Figure 1). This confirmed the grouping of individuals within their own taxonomic range. *P. massoniana* (M) appeared to be the most genetically differentiated. The genetic relationship between *P. henryi* and *P. tabulaeformis* was closer than that between *P. henryi* and *P. massoniana.*

PCoA was performed to provide spatial representation of the relative genetic distances among individuals and to determine the consistency of differentiation among taxa defined by the cluster analysis. The first 2 principal components explained 44.70 and 17.20% of the total variation, respectively, while 74.43% was explained by the first 3 components (Figure 2). The first principal coordinate separated most individuals of *P. henryi* (B) from the individuals of the other 3 taxa. The second principal coordinate separated most individuals of *P. massoniana* (M) from the individuals of the other 3 taxa. The results of the PCoA indicated that *P. henryi* might be a distinct species closely related to *P. tabulaeformis*.

In the SSR admixture analysis using STRUCTURE (Figure 3), the highest likelihood of the data was obtained when samples were clustered into 3 groups ($K = 3$). For the 4 *Pinus* taxa dataset, the 3 clusters corresponded to *P*. *henryi* (B, green cluster), the combination of *P*. *tabulaeformis* (Y), and *P. tabulaeformis* var. *mukdensis* (H, red cluster), and to *P*. *massoniana* (M, blue cluster), further indicating that these results agreed with the PCoA and the NJ cluster results.

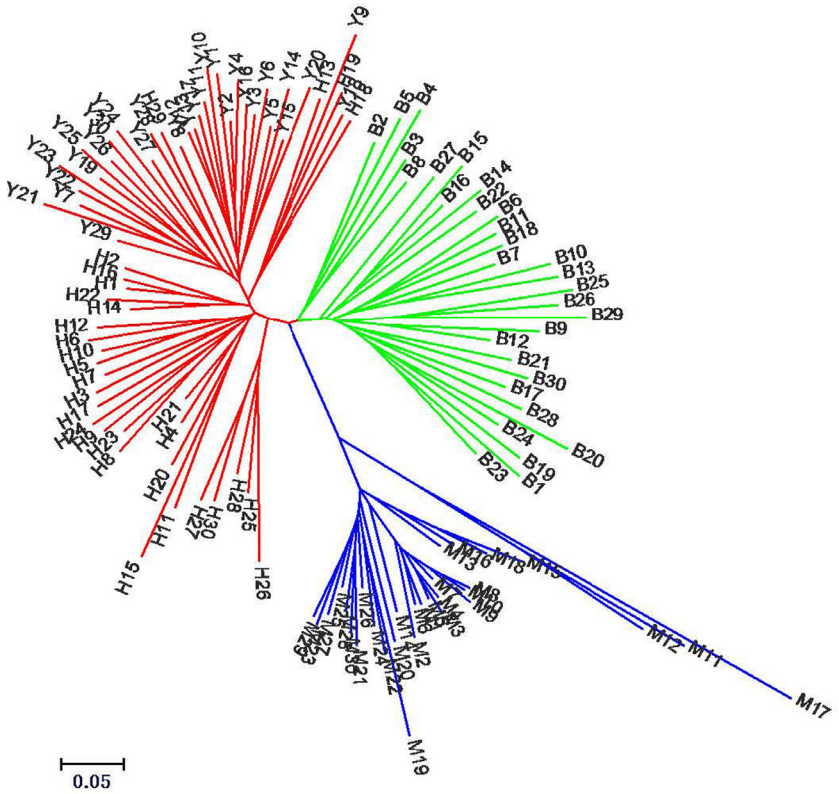

Figure 1. Dendrogram for all individuals of the *Pinus* taxa using the neighbor-joining method.

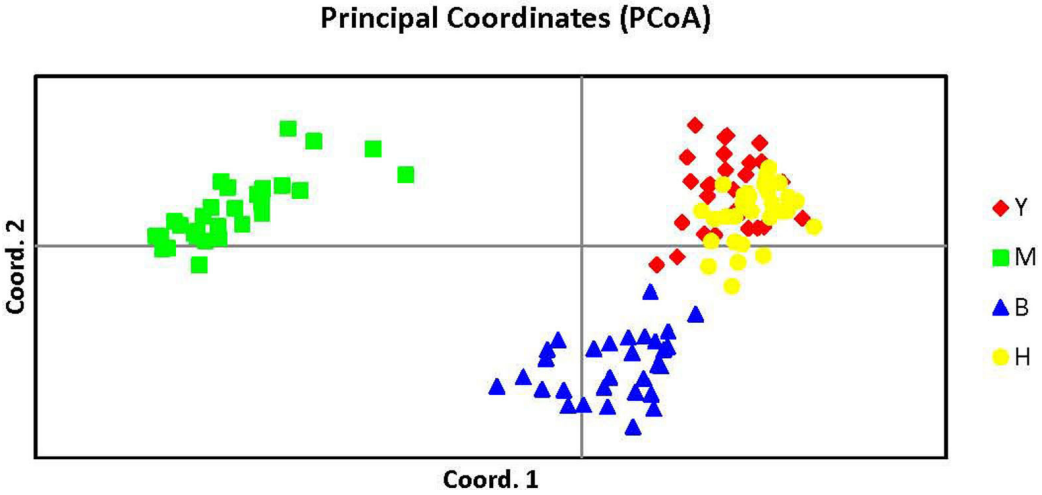

Figure 2. Two-dimensional plot of the principal coordinate analysis of SSR data showing the clustering of populations of *Pinus* taxa. The first and second principal coordinates account for 44.70 and 17.20% of the total variation, respectively.

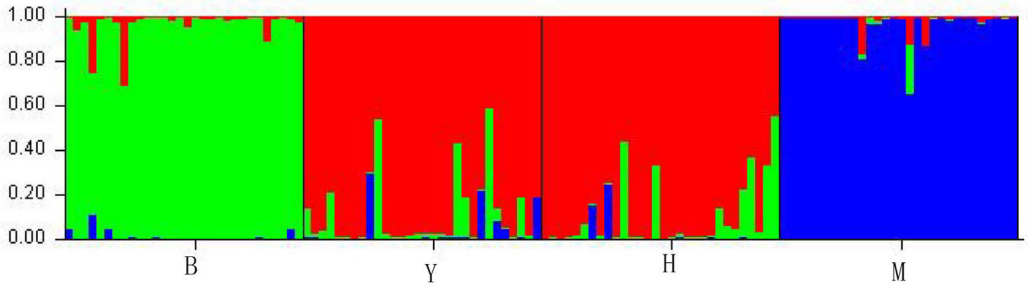

Figure 3. Genetic relationships among the *Pinus* taxa estimated using STRUCTURE based on SSR data. The model with $K = 3$ showed the highest K value.

DISCUSSION

The controversy over the taxonomic status of the 4 pines in this study mainly lay in the relationship between *P. henryi* and *P. tabulaeformis*. These two species have a similar morphology and adjacent distributions (Li and Xu, 1989). However, the NJ dendrogram (Figure 1), the PCoA diagram (Figure 2), and the STRUCTURE plot (Figure 3) exhibited similar patterns, revealing that *P. henryi*, *P. tabulaeformis* and *P. tabulaeformis* var. *mukdensis*, and *P. massoniana* were distinctly differentiated, suggesting they have different genetic backgrounds. These results indicate that *P. henryi* should be a distinct species rather than a subspecies of *P. tabulaeformis* or *P. massoniana*.

In addition, our results are strongly supported by morphological and biochemical studies. Compared to *P. tabulaeformis*, *P. henryi* has a wider stem taper, smaller seeds (6.37 *vs* 7.02 mm) and cones (4.44 *vs* 5.58 cm), and shorter (10.31 *vs* 11.81 cm), narrower (1.21 *vs* 1.24 cm), and thinner (0.70 *vs* 0.79 mm) needles (Mao and Liu, 1989; Li and Xu, 1989). Furthermore, *P. henryi* exhibits a diagnostic karyotype with diacritic satellite positions on its chromosomes (Zhang, 1990). Wood anatomical characteristics (e.g., number of secretory cells in the resin duct, thickness of the inner wall of ray tracheids, the number of uniseriate wing cells in fusiform ray; An and Zhao, 1992), and physio-biochemical traits (chlorophyll and nuclear acid content, peroxidase isozymes; Zhang et al., 1995) have congruously demonstrated the differences between *P. henryi* and *P. tabulaeformis*. Furthermore, these species have allopatric distributions with their morphologic-geographic division running in the Daba Mountains (Li and Xu, 1989). *P. tabulaeformis* is distributed on the northern slope of the Daba Mountains, while *P. henryi* is found on the southern slope.

Therefore, we conclude that all evidence from geographical, morphological, biochemical, karyotype, and genetic data refute the subspecies hypothesis and indicate that *Pinus henryi* may be a distinct species closely related to *P. tabulaeformis*.

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