

Genetic structure and trait variation within a maple hybrid zone in North China provide evolutionary and ecological insights for ancient lineage evolution

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Article

Keywords: Acer, ecological niche divergence, flowering phenology, morphology variation, reproductive barriers, secondary contact zone

Posted Date: March 30th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1487543/v1>

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Abstract

Secondary contact zones can provide valuable evolutionary and ecological insights for elucidating patterns of divergent lineage evolution. The tertiary relict flora in East Asia can be divided into northern (“NEA”) and southern (“SEA”) regions. To date however, secondary contact zones of lineages from the NEA and SEA regions have rarely been studied. To test whether introgression occur between the ancient lineages and their evolutionary and ecological consequences, we conducted detailed genetic and ecological work within a maple hybrid zone in North China. Genetic structures of both adults and seeds populations were characterized. Spatial genetic pattern was checked along an altitude gradient. Flowering phenology was analyzed to test the existence of assortative mating. Leaf and fruit morphologies were compared between lineages. Our study confirm introgression occur between NEA and SEA lineages. Post-pollinator barriers predominantly contribute to a reduction in interlineage gene flow. Ecological niche differs between lineages along an altitudinal gradient. We clarify a long-standing classification debate with respect to *Acer pictum* subsp. *mono* and *A. truncatum*, though there are extensive morphological variation and overlaps between the species pair. Our study represents the first few detailed evolution and ecological investigation focusing on ancient NEA-SEA lineages hybrid zone.

Introduction

Globally, East Asia harbors the most diverse temperate flora, among which there are numerous Tertiary relict species^{8–11}. Milne and Abbott¹² have proposed that there are two independent relict species regions within East Asia, with one being centered in South/Southeast China, with extensions to the Himalayas (hereafter “SEA”) and the other in a region encompassing Northeast China, the Korean Peninsula, and the Japanese Archipelago (hereafter “NEA”) (Fig. 1a). It is speculated that a climatic barrier, demarcated by an arid belt around the North China Plain, may mediate the divergence of SEA and NEA flora. Taxonomically, numerous SEA–NEA divergence patterns have been reported at the genus level or above^{12,13}. Moreover, some species (or species pair) have been reported to show a clear SEA–NEA divergence^{14–19}, which may provide evidence to indicate that long-standing barriers continue to play important roles in recent evolutionary processes. From the studied cases, it can be speculated that Mt. Taihang and Mt. Yanshan constitute major meeting corridors of NEA and SEA lineages¹⁹. However, although hybridization events have been reported to occur within these putative corridors^{16,20}, fewer studies have been conducted to investigate the evolutionary and ecological consequences of these secondary contact zones. Accordingly, it would be of particular interest to determine whether NEA and SEA lineages actually undergo mergence, introgression, or reinforcement within these contact zones, and also whether they have ecological niche and trait divergence. Such insights would undoubtedly enhance our current understanding of the evolution of the two relict lineages, and clarify the overlooked importance of North China in biodiversity origin⁶.

Characterizing genetic structures within hybrid zones is a major approach that can be used to determine the evolutionary and ecological consequences of secondary lineage contact²¹. Under circumstances in

which there are larger populations of parental lineages than hybrids (bimodal pattern), the two lineages can be considered largely reproductively isolated e.g.^{22,23–26}, whereas when most individuals are hybrids (unimodal pattern), there may be an absence of reproductive barriers between the two lineages^{20,27}. Moreover, when most hybrids are F_1 individuals, the gene flow between lineages is weak, whereas when most hybrids are backcrosses, introgression may occur between lineages^{26,28}. A further important character is the width of hybrid zones, with narrower hybrid zones indicating stronger reproductive isolation between lineages²⁹. Moreover, differing strengths of pre-zygotic and/or post-zygotic barriers may lead to varied genetic structures within hybrid zones³⁰. Hence, characterizing the genetic structure and width of hybrid zones would be predicted to yield valuable insights regarding the evolutionary and ecological consequences of hybridization.

In North China, two sister species, *Acer pictum* subsp. *mono* and *A. truncatum*, are reported to be sympatrically distributed and constitute the predominant components of the deciduous forests^{31–33}. However, there is wide debate regarding the classification of these two species. Although many scientists treat *A. truncatum* as a separate species, some still consider it to be a subspecies of *A. pictum*. In this regard, the two species are generally distinguished based on a comparison of the respective leaf and fruits morphologies. However, given the wide variability in traits, their validity in differentiating these species is questionable. According to Flora of China, the leaf base of *A. truncatum* is typically truncate, whereas that of *A. pictum* subsp. *mono* is generally heart-shaped. Furthermore, in *A. truncatum*, the fruit wing is generally as long as the seed, whereas it is considerably longer in *A. pictum* subsp. *mono*. However, we have observed marked variations in leaf and fruit morphologies, even within a single tree (personal observations). Consequently, given the dubious merits of the established morphological diagnoses, it remains unclear whether these are two reproductively compatible species or a single species characterized by a large morphological variation.

By treating these trees as a species complex (hereafter “*Acer*”), a previous phylogeographical study identified a clear NEA–SEA nuclear genetic division, with the two lineages co-existing in North China¹⁴. However, compared with other species studies in this region^{16,20}, only two sampled *Acer* populations have been found to show clear sympatric distribution at a local scale^{14,18} (Fig. 1b). This thus raises the questions as to why there are so few populations comprising sympatrically distributed NEA-SEA lineages within secondary contact zones and the factors govern the spatial distribution of the two lineages. Moreover, it would be of interest to determine whether the two lineages can hybridize and, if so, the extent of hybridization, and also to establish whether the two genetic lineages correspond to the *A. pictum* subsp. *mono* and *A. truncatum* species division. By answering these questions, we can gain valuable insights into the evolutionary and ecological processes occurring subsequent to NEA–SEA lineage secondary contact, as well as going some way to clarifying the long-standing uncertainty regarding the taxonomic status of *A. pictum* subsp. *mono* and *A. truncatum*.

The Daheishan National Nature Reserve, located to the east of Mt. Yanshan, is one of the two previously sampled populations with sympatrically distributed NEA–SEA lineages (Fig. 1b). Among the 22 sampled

individuals in the reserve, six and eight were NEA and SEA lineages, respectively, with few hybrids, which are characterized by a typical bimodal pattern ¹⁴. However, given the limited number of sampled trees and the lack of precise locations, further analyses are required to establish the genetic structure and width of the hybrid zone.

In this study, based on the precise location of each individual, an extensive genetic survey of both adult and offspring populations, the monitoring of flowering phenology, and measurement of leaf and fruit morphologies, we sought to determine the evolutionary and ecological consequences of secondary contact between the NEA and SEA. Specifically, we strived to provide answers to the following questions: (1) Do the two lineages show ecological niche differentiation, and are they mixed or isolated at a local spatial scale? (2) Are the two lineages reproductively isolated and do they differ in terms of flowering phenology? (3) Do the two lineages differ with respect to leaf and fruit morphology and, if so, do these differences correspond to those described for *A. pictum* subsp. *mono* and *A. truncatum* in the standard of Floras?

Results

Genetic structure of the parental population

On the basis of the values obtained for both $\ln PD$ and ΔK , we identified two distinct genetic groups within the DHS *Acer* population (Figure S1). Among the 70 individual trees, 72.9% were assigned a q -value smaller than 0.1 or larger than 0.9, thereby signifying a typical bimodal distribution (Fig. 2b). Individuals with q values greater than 0.9 and with consistent genetic origin from the NEA region were defined as the NEA lineage (hereafter “NEA-DHS”), whereas those with values less than 0.1 and with consistent genetic origin from the SEA region were defined as the SEA lineage (hereafter “SEA-DHS”). Individuals with intermediate q values between 0.1 and 0.9 were defined as hybrid genetic types (hereafter “Hybrid-DHS”). We accordingly identified totals of 27 SEA-DHS (38.6%), 24 NEA-DHS (34.3%), and 19 Hybrid-DHS (27.1%) (Fig. 2b).

Both PCoA and NewHybrids analyses revealed patterns similar to those obtained based on STRUCTURE analysis. The PCoA results indicated that the SEA-DHS and NEA-DHS based on STRUCTURE assignment were divergent and clustered along the first axis of the ordination plot, whereas the Hybrid-DHS plotted intermediately between the SEA-DHS and NEA-DHS (Fig. 2c). Newhybrids analysis indicated that 65.7% of individuals were parental types (30% SEA-DHS and 35.7% NEA-DHS) and 4.3% were F_2 hybrids. Among the remaining unclassified 21 individuals, 12 can be classified as F_2 individuals, and two appear to be NEA-DHS backcross types with a posterior probability criterion of 0.55.

Interestingly, we detected a tendency of the different genetic types to cluster spatially along the study transect (Fig. 2a). q values were found to be positively correlated with altitude (Pearson's $r = 0.83$, $p = 0.000$) and there were significant differences in the altitudinal distributions of the three genotypes, with NEA-DHS being primarily concentrated at the hilltop end of the transect (altitude > 670 m) and SEA-DHS

being mainly clustered at the foot of the hill (< 600 m) with just a few individuals scattered at higher altitudes. Hybrid-DHS were found to be mainly distributed in a region extending from the midpoint of the transect to the hilltop (570–730 m).

A comparison of the assignment results obtained based exclusively on the DHS dataset (DHS-only: 70 individuals and 11 loci) and that of the entire assessed range (whole-range: 1278 individuals and six loci) revealed broadly similar patterns. On the basis of the whole-range dataset, 27% and 31% of individuals are SEA-DHS and NEA-DHS, respectively, and were accordingly respectively clustered in PCoA plots (Figures S2 and S3). However, although, we still detected a significant correlation between q values and altitude the Pearson's r was somewhat lower (0.63, Figure S2). In this regard, given that for STRUCTURE assignment analysis, a larger number of assessed loci has been reported to be more effective than increasing number of sampled individuals^{34,35}, we have only reported the results obtained based on the DHS-only dataset.

Genetic analysis of the offspring population

Among the 410 analyzed seeds, 198, 170, and 42 were collected from the SEA-DHS, Hybrid-DHS, and NEA-DHS maternal trees, respectively (Table S2). Similar to the parental population, a typical bimodal pattern was observed for the offspring population (Fig. 2b, Table S2). The NEA-DHS maternal trees produced 97.6% pure NEA-DHS seeds, and the remaining seeds (2.4%) were of a backcross type with a high genetic proportion of NEA ($q > 0.85$). The SEA-DHS maternal trees produced 72.2% pure SEA-DHS seeds, 10.1% pure NEA-DHS seeds, and 17.7% Hybrid seeds. Almost all seeds produced by the SEA-DHS with q values > 0.5 were obtained from a single tree, which was identified as SEA-DHS based on the DHS-only dataset, although was indicated to be Hybrid-DHS based on the whole-range dataset.

The Hybrid-DHS maternal trees produced 17.6% pure SEA-DHS seeds, 57.6% pure NEA-DHS seeds, and 24.7% Hybrid seeds.

Flowering phenology

During the flowering season, we undertook daily monitoring of a total of 10074 flowers produced by 29 trees, among which, one tree (SEA-DHS) was protandrous, four trees (3 Hybrid-DHS and 1 NEA-DHS) were protogynous, and the remaining 24 trees were duodichogamous. We observed that the blooming phenology of SEA-DHS and NEA-DHS differed significantly with respect to the majority of the assessed phenological indices, with a single exception being a marginally significant difference in the peak blooming time of Male I (Table 1). Compared with NEA-DHS, SEA-DHS were characterized by a significantly later flowering phenology, with the commencement and cessation of blooming being on average 2 and 3 days later, respectively. Similarly, the commencement, peak, and cessation of Female were later by averages of 4, 4, and 5 days, respectively, whereas those of Male II were later by 5, 4, and 5 days, respectively. Furthermore, the duration of blooming was significantly longer in the SEA-DHS than in NEA-DHS by 3 days. In the case of Hybrid-DHS, the values obtained for all assessed phenological indices were found to be intermediate between those of the two parental types. Among these, the values of six

indices differed significantly from one or the other parental type, with the majority (5/6) differing from those of the SEA-DHS. Thus, phenologically, the Hybrid-DHS appear to be closer to the NEA-DHS.

Table 1

Flowering phenology of SEA-DHS, Hybrid-DHS, and NEA-DHS. Observations commenced on April 20th. Significant differences between pairs determined at the $p < 0.05$ level based a one-way ANOVA are indicated by different superscript letters. a*: $p = 0.06$

Statistical Index	Genetic Type SEA-DHS (N = 9)	Hybrid-DHS (N = 14)	NEA-DHS (N = 6)
Male I commencement	7.44 ± 0.58 ^a	6.45 ± 0.49 ^{ab}	5.20 ± 0.86 ^b
Male I peak	11.44 ± 1.06 ^{a*}	10.18 ± 0.63 ^a	8.80 ± 1.10 ^{a*}
Male I cessation	13.33 ± 1.08 ^a	11.09 ± 0.74 ^{ab}	9.8 ± 0.37 ^b
Female commencement	14.33 ± 1.08 ^a	11.14 ± 0.80 ^b	10.00 ± 0.86 ^b
Female peak	16.11 ± 1.09 ^a	13.29 ± 0.71 ^b	11.67 ± 0.67 ^b
Female cessation	17.89 ± 1.16 ^a	14.57 ± 0.72 ^b	12.67 ± 0.84 ^b
Male II commencement	18.22 ± 0.66 ^a	15.57 ± 0.72 ^b	13.67 ± 0.84 ^b
Male II peak	19.67 ± 0.62 ^a	17.07 ± 0.74 ^b	15.50 ± 0.43 ^b
Male II cessation	24.22 ± 0.68 ^a	22.36 ± 0.77 ^a	19.17 ± 0.40 ^b
Total duration	18.00 ± 0.33 ^a	16.64 ± 0.65 ^{ab}	14.83 ± 0.75 ^b
SEA-DHS: southern East Asian lineage of <i>Acer</i> in the Daheishan National Nature Reserve (DHS); NEA-DHS: northern East Asian lineage of <i>Acer</i> in the DHS; Hybrid-DHS: hybrids between the SEA-DHS and NEA-DHS lineages.			

However, despite the differing phenology of the SEA-DHS and NEA-DHS, we did, nevertheless, observe instances of overlaps in the blooming periods of male or female flowers in one genetic type with those of flowers of opposite sex in another genetic type. For example, the peak of Female among NEA-DHS (11.67 ± 0.67) was found to coincide with the peak of Male I (11.44 ± 1.06; $p = 0.879$) in SEA-DHS. Similarly, Female blooming in SEA-DHS peaked (16.11 ± 1.09) just 1 day after the peak of Male II (15.50 ± 0.43) in NEA-DHS ($p = 0.667$), which at this time still retained an abundance of male flowers in bloom. In contrast, however, we detected no overlapping phenology with respect to the blooming of Male I of NEA-DHS or Male II of SEA-DHS with the Female in another genetic type.

Morphological variation of leaves and fruit

Leaves The first two axes of the PCoA plot were found to explain 71.6% of the variation in leaf morphology (Fig. 3a), with InflectionLength, InflectionWidth and CentralLength contributing most to the

first axis (PC1, 42.1%), whereas Lobes#, TotalArea and CentralWidth contributed most to the second axis (PC2, 29.5%) (Table S3). Although we detected some overlap in the data points, the leaves of SEA-DHS and NEA-DHS largely clustered in separate groups (Fig. 3a). Generally, the leaves of NEA-DHS were found to have seven lobes, whereas those of SEA-DHS are typically five lobed (Lobes#), thereby contributing to significantly larger leaves in NEA-DHS than in SEA-DHS (TotalArea). Furthermore, NEA-DHS leaves have shorter and wider central lobes (CentralLength and CentralWidth), as well as an earlier and narrower inflection of the central lobes (InflectionLength and InflectionWidth), compared with those of SEA-DHS (Table 2).

Table 2

Morphological variation in the leaves and fruits of *Acer* trees in the Daheishan National Nature Reserve. Definitions of morphological indices are presented in Figure S5. Significant differences between pairs determined at the $p < 0.05$ level based a one-way ANOVA are indicated by different superscript letters.

Definition	Morphological Indices	SEA-DHS	Hybrid-DHS	NEA-DHS
Leaf		N = 269	N = 188	N = 233
Number of leaf lobes	Lobes#	5.27 ± 0.04^c	5.97 ± 0.07^b	6.59 ± 0.05^a
Leaf area	TotalArea	32.66 ± 0.66^c	38.22 ± 1.04^b	46.94 ± 0.85^a
Length of inflection point	InflectionLength	1.75 ± 0.04^a	1.65 ± 0.06^a	1.36 ± 0.06^b
Width of inflection point	InflectionWidth	1.04 ± 0.03^a	1.07 ± 0.05^a	0.83 ± 0.03^b
Length of central lobe	CentralLength	4.68 ± 0.05^a	4.41 ± 0.07^b	4.24 ± 0.05^b
Width of central lobe	CentralWidth	2.46 ± 0.04^c	2.66 ± 0.04^b	3.03 ± 0.04^a
Fruit		N = 170	N = 256	N = 189
Fruit opening angle	FruitAngle	96.94 ± 1.45^c	103.12 ± 1.12^b	111.55 ± 1.53^a
Fruit junction width	JunctionWidth	0.70 ± 0.01^a	0.60 ± 0.01^b	0.51 ± 0.00^c
Length of seed	SeedLength	1.22 ± 0.01^a	1.16 ± 0.01^b	0.89 ± 0.01^c
Width of seed	SeedWidth	0.79 ± 0.01^a	0.73 ± 0.01^b	0.61 ± 0.00^c
Fruit wing length	WingLength	1.25 ± 0.01^b	1.39 ± 0.02^a	1.09 ± 0.01^c
Fruit wing width	WingWidth	0.76 ± 0.15^a	0.79 ± 0.15^a	0.65 ± 0.09^b
Fruit length	FruitLength	3.85 ± 0.06^b	4.17 ± 0.04^a	3.53 ± 0.04^c
Fruit width	FruitWidth	1.87 ± 0.02^a	1.80 ± 0.03^a	1.29 ± 0.02^b
SEA-DHS: southern East Asian lineage of <i>Acer</i> in the Daheishan National Nature Reserve (DHS); NEA-DHS: northern East Asian lineage of <i>Acer</i> in the DHS; Hybrid-DHS: hybrids between the SEA-DHS and NEA-DHS lineages.				

With regards to Hybrid-DHS, the leaves were found to be morphologically intermediate between those of the two parental types (Fig. 3a), as were the values of the assessed morphological trait indices (Table 2).

Fruits The first two axes of the PCoA plot were found to explain 76.8% of the variation in fruit morphology (Fig. 3b), with SeedLength and SeedWidth making the largest contribution to the first axis (PC1, 61.7%),

and FruitAngle and FruitLength contributing most to the second axis (PC2, 15.1%) (Table S3). Similar to leaf morphology, although there was a degree of overlap in the data points, the fruits of SEA-DHS and NEA-DHS were clustered in discrete groups (Fig. 3b), with fruits showing significant divergence with respect to all eight assessed morphological indices. NEA-DHS tend to be characterized by smaller fruits (FruitLength and FruitWidth), seeds (SeedLength, SeedWidth and JunctionWidth), and fruit wings (WingLength and WingWidth). Moreover, the seed wings of NEA-DHS fruits are typically oriented at an obtuse angle, whereas those of SEA-DHS fruits tend to be aligned at a right angle (FruitAngle) (Table 2).

Similar to leaf morphology, we found that the morphology of Hybrid-DHS fruits was generally intermediate between that of the two parental types (Fig. 3b), as reflected in the values of the different morphological traits. The exceptions in this regard were FruitLength and WingLength, with hybrid trees typically producing longer fruit with longer fruit wings (Table 2).

Discussion

Our findings in this study indicate that NEA and SEA lineages of *Acer* co-exist in the DHS region of North China, and that both adult and offspring populations show a typical bimodal distribution pattern. The two lineages are characterized by a largely well-differentiated spatial distribution along an altitudinal gradient, and tend to differ with respect to flowering phenology and the morphologies of leaves and fruits. Most hybrids, which are probably later generation of F_1 individuals, tend to be distributed at intermediate altitudes between those of the two parental lineages. Collectively, these results would tend to provide evidence of a reproductive barrier between the two lineages, whereas interfertile hybrids occur incidentally.

Ecological niche divergence has evolved between the NEA and SEA lineages

NEA and SEA lineages in the DHS were found to show a clear pattern of spatial isolation, with NEA-DHS tending to be predominantly distributed at altitudes greater than 670 m, whereas SEA-DHS were found to be scattered at lower altitudes, with rare overlap between the two lineages. Nevertheless, despite the limited size of our study population, we believe our findings to be reasonably representative of the regional population, given that we sampled all accessible individuals along a 5-km-long, 20-m-wide transect, with only four or five inaccessible individuals being excluded from sampling. It is unconvincing whether inclusion of these latter unsampled individuals would change the established spatial genetic pattern. Moreover, consistent with the findings of the present study, 100 trees sampled during a preliminary survey undertaken in an area near the transect at an altitude of less than 650 m all proved to be SEA-DHS individuals (unpublished data). In addition, the trees of both lineages, as well their hybrids, appear to have a similar age structure, as indicated by DBH measurements, which for most individuals ranged between 26 and 45 cm (Figure S5), thereby tending to indicate a long persistence of the observed spatial pattern.

We speculate that spatial isolation along altitudinal gradients may be common feature of *Acer* populations in North China. According to the findings of a previous phylogeographical study, the NEA and SEA lineages meet at an area to the North of Mt. Taihang and in the Jiaodong Peninsula (Fig. 1b) ¹⁴. However, most *Acer* populations in North China comprise exclusively NEA or SEA lineages, with only two populations near the DHS showing clear evidence of more than one lineage. We believe that the apparent absence of any substantial overlap at the local scale can be ascribed to two overriding factors. Firstly, on the basis of our characterization of the DHS population, the hybrid zone width appears to be relatively narrow (approx. 150 m in the DHS), thereby indicating little overlap of the NEA and SEA lineages at a local scale. Hence, though 20 more scattered individuals are generally collected and analyzed in each population, it is possible that one lineage is excluded owing to sampling within a limited altitudinal range. By examining the GPS locations of trees sampled by Guo et al. (2014), we found that pure NEA populations found to the North of Mts. Taihang and Yanshan are generally distributed at altitudes higher than 700 m, whereas pure SEA populations in this region are typically found at altitudes lower than 500 m (Fig. 1b), which is consistent with the distribution pattern identified in the DHS. Secondly, as a consequence of human population density in North China, particularly in the region to the North of Mts. Taihang and Yanshan, large areas of natural forest have disappeared at low altitudes ^{36,37}, which may have led to the local extinction of SEA lineages in many *Acer* populations in this region. Consequently, we speculate that the secondary contact zone between the NEA and SEA lineages of *Acer* should be more extensive than it now appears. Confirmation of this supposition will, nevertheless, necessitate further extensive sampling along altitudinal gradients in North China.

The natural long-persisting pattern of the isolation of NEA and SEA lineages along an altitudinal gradient in the DHS (and potentially for other populations in North China) may reflect the local adaptation of *Acer* prior to secondary contact. Generally, the SEA lineage is found at low latitudes, where it is mainly distributed at high altitudes in warm temperate forests, whereas the NEA lineage occurs at higher latitudes, and is distributed primarily in cool temperate forest (Fig. 1b). The bioclimatic conditions characterizing NEA and SEA habitats are relatively distinct ³⁸. Bioclimatic divergence as well as allopatric distribution can be considered prerequisites for the evolution of local adaptation, which can lead to niche divergence when different lineages subsequently meet ^{39–41}. In this context, species of *Acer* are not the only one for which the local adaptation of NEA and SEA lineages has been reported (eg. *Juglans mandshurica* species complex ¹⁶, *Lindera obtusiloba* ³⁸). However, species of *Acer* are the first for which niche divergence along an altitudinal gradient in North China has been described. However, establishing whether this pattern is typical of NEA–SEA divergent species (or pairs), will necessitate further analyses.

Post-pollination barriers contribute predominantly to the reproductive isolation of NEA-DHS and SEA-DHS lineages

Our results reveal that both adult and offspring populations of the NEA and SEA lineages of *Acer* are characterized by a typical bimodal genetic pattern, with most hybrids being later generation F_1 individuals, which thus tends to indicate a reduction in inter-lineage gene flow, although introgression

between lineages does indeed occur. Nevertheless, even though the two lineages are isolated in terms of altitudinal distribution, they are still comparatively spatially adjacent (over a range of approx. 6–1800 m). In this regard, the findings of a study examining the mating patterns and population genetic structure of an *Acer* population in Beijing⁴² have revealed that long-distance pollen dispersal can occur with a high probability. Moreover, it has been reported that the mean pollen dispersal distance of protogynous morphs of *Acer opalus* subsp. *granatense*, is 2.8 km⁴³. Hence, we speculate that spatial isolation is unlikely to be a major factor limiting inter-lineage gene flow. Given the evolution of local adaptation and ecological niche divergence in the NEA and SEA lineages, as discussed above, there are opportunities for reproductive barriers to develop. Therefore, we infer that a reproductive barrier rather than spatial isolation between lineages, is a major factor contributing to a limited inter-lineage gene flow in the DHS.

The very limited number of identified hybrids and narrow hybrid zone in the DHS are indicative of a reproductive barrier between the two lineages, which is predicted to be strong^{29,44}. On the basis of our observations, we infer that the reproductive barrier between the NEA-DHS and SEA-DHS lineages probably operates prior to seed development and maturation, which tends to be supported by the consistent genetic structure between seed and adult populations (Fig. 2b). Generally, prezygotic barriers, such as assortative mating, evolve more rapidly than postzygotic barriers³⁰, and can lead to a pronounced reduction in inter-lineage gene flow and the development of narrow hybrid zones^{29,45,46}. Flowering phenology and/or pollinator community divergence between lineages are common pre-pollination barriers that can contribute to assortative mating^{27,47,48}. In the present study, we found that the NEA-DHS and SEA-DHS lineages differ with respect to almost all the flowering phenology indices we assessed (Table 1), thereby indicating trait divergence. However, we established that a majority of the individuals surveyed in the DHS are duodichogamous (82.76%), characterized by two separate functional male stages. Consequently, the prolonged male function can give rise to an overlap of the blooming periods of the female and male flowers on trees of the opposite lineage (Table 1), even though blooming rhythms differs. Furthermore, given that we found no evidence of any appreciable differences between lineages with respect to flower shape, and that inter-lineage spatial isolation is relatively limited in the DHS, we would tend to assume that there is no divergence between the two lineages regarding the insect pollinator community. Accordingly, although further verification is necessary, we speculate that post-pollination rather than pre-pollination barriers, are the major factor contributing to the reproductive isolation of lineages in the DHS. Such post-pollination barriers including pre-mating barriers associated with the stigma, as well as prezygotic or postzygotic barriers downstream of the stigma^{30,49}.

Morphological divergence of NEA-DHS and SEA-DHS lineages corresponds to *A. pictum* subsp. *mono* and *A. truncatum*

Notably, NEA-DHS and SEA-DHS lineage trees can be differentiated with respect to the morphologies of both leaves and fruits (Fig. 3a and b, Table 2). NEA-DHS typically produce larger seven-lobed leaves, characterized by a heart-shaped leaf base, whereas the generally smaller leaves of SEA-DHS typically have five lobes and a truncate-shaped leaf base. In terms of fruit, NEA-DHS have smaller seeds and seed

wings spreading at larger angles compared with those produced by SEA-DHS. Furthermore, whereas the wings of SEA-DHS seeds are as long as the seeds (1.06 ± 0.02), those of NEA-DHS seeds tend to be longer than the seeds (1.24 ± 0.01). Although the criteria used to differentiate *A. pictum* subsp. *mono* and *A. truncatum* are not identical among the different Floras, our morphological characterization in this study would tend to be consistent with the identity of NEA-DHS individuals as *A. pictum* subsp. *mono* and SEA-DHS individuals as *A. truncatum*. Given that there are no other closely related *Acer* species in North China, we are thus reasonably confident in assigning NEA and SEA lineages in the DHS as *A. pictum* subsp. *mono* and *A. truncatum*, respectively. Moreover, the clear indication of a reproductive barrier between the NEA-DHS and SEA-DHS lineages provides compelling evidence regarding the current classification status of these two controversial species.

However, despite this convincing support for the separate species status of *A. pictum* subsp. *mono* and *A. truncatum*, we suggest that leaf and fruit morphologies would not constitute solid identification criteria. Both species in the DHS tend to be characterized by large-range morphological variation, with a certain degree of overlap between species (Fig. 3a and b). Moreover, the presence of hybrids may exacerbate the difficulty in identifying these species based solely on morphology. If indeed niche divergence of the two species along an altitudinal gradient in the DHS is confirmed to be common phenomenon throughout the secondary contact zone, a combination of morphological and habitat-related criteria may provide relatively reliable and readily assessed clues that could be used to identify *A. pictum* subsp. *mono* and *A. truncatum* in North China. For conclusive verification, however, the most precise approach to identification is one based on the analyses of molecular markers, such as nSSRs or nuclear single-copy genes^{18,50,51}.

Conclusion

Collectively, the findings of our detailed genetic structure and trait analyses of *Acer* population in the DHS clarify the identities of *A. pictum* subsp. *mono* and *A. truncatum* as two separate species, which correspond to the NEA and SEA lineages, respectively, identified in a previous phylogeographical study. Within the study region, the two species are characterized by ecological niche divergence and different habitats along an altitudinal gradient. Although we detected introgression between the two species, interspecific gene flow appears to be uncommon and the established hybrid zone is narrow. Our observations tend to indicate that post-pollination rather than pre-pollination mechanisms constitute a major reproductive barrier between the two species. Although the species differ significantly with respect to both leaf and fruit morphologies, we detected a large variance in these traits and a degree of overlap between the species, thereby indicating that neither trait would be a reliable criterion on which to base the differentiation of this cryptic species pair.

This study is the first that has focused on the evolutionary and ecological consequence of the convergence of Tertiary relict NEA and SEA lineages. Although secondary contact between *Juglans mandshurica* (NEA lineage) and *J. cathayensis* (SEA lineage) has previously been examined in the Mt. Taihang and Mt. Yanshan regions, which probably generates a hybrid lineage in the latter region.¹⁶, the

ecological consequences for the *Juglans* species pair in the secondary contact zone have yet to be studied. Similarly, the sympatrically distributed *Quercus mongolica* and *Q. liaotungensis* have also been found to meet in the region to the north of Mt. Taihang and Yanshan, although to date no clear niche divergence, morphological divergence, or reproductive barriers have been identified in this secondary contact zone^{20,52}. Moreover, the *Quercus* species pair appears to be not typical NEA–SEA distribution pattern, and it remains to be determined whether the differences in distribution contribute to different ecological consequences for *Acer* and *Quercus*. Given the paucity of evolutionary and ecological studies focusing on secondary contact zones in North China, it has yet to be established whether the pattern identified for the *Acer* species pair is a more widespread common feature of these zones. However, as North China is considered a key secondary contact region for Tertiary relict NEA and SEA lineages, we believe that further evolutionary and ecological studies should be conducted to gain a better understanding of the origins and maintenance of biodiversity in this high-latitude region.

Materials And Methods

Study area

The Daheishan National Nature Reserve (hereafter “DHS”) is located to the east of Mt. Yanshan, with altitudes ranging from 590 to 1074 m. The regional climate is temperate monsoon with an annual average temperature of 4.9–7.5°C and annual precipitation of 400–450 mm. The vegetation in the reserve is typical temperate deciduous forest.

Genetic structure of the parental population

On the basis of previous study¹⁴, we selected a 5.5-km-long, 20-m-wide hillside transect in DHS, where the two genetic types of *Acer* might coexist. Along this transect, from the foot of the hill to the summit, we located all accessible *Acer* adults using a mobile phone GPStoolbox APP, with a location error within 4 m. In total, we located 70 individuals, with an average diameter at breast height (DBH) of 37.07 ± 1.66 cm (Figure S4). Leaves were collected from all identified individuals and desiccated using silica gel at room temperature until used for DNA extraction.

Total genomic DNA was extracted using a plant genomic kit (Tiangen, Beijing). All individuals were genotyped by 11 previously used microsatellite loci (Table S1)^{42,53}. The 20-μL PCR mixtures contained 10–20 ng DNA, 0.4 μL of each forward and reverse primer (10 μM), 7.7 μL of ddH₂O, and 10 μL of 2x TSINGKE Master Mix (Tsingke Biotechnology Co., Ltd.). Reactions were performed in a Veriti™ PCR thermal cycler (Applied Biosystems) using the following amplification program: an initial denaturation of 94°C for 5 min; six cycles of 94°C for 50 s, locus-specific annealing temperatures[™] for 50 s, and 72°C for 30 s; a following 24 cycles of 94°C for 30 s, locus-specific annealing temperatures for 50 s, and 72°C for 50 s; and a final extension at 72°C for 10 min.

Allele sizes were determined using GeneMarkerv.2.2.0⁵⁴, with all alleles being assessed independently by two persons to reduce scoring errors. To infer the genetic structure of the DHS population, we

implemented both a Bayesian clustering approach and principal co-ordinates analysis (PCoA), the latter of which was conducted using GenAlex6.5⁵⁵.

Bayesian clustering was conducted using STRUCTUREv.2.3.4⁵⁶ with K values from 1 to 10 and an admixture model and correlated allele frequencies being applied. For each K, we ran 10 independent simulations with a burn-in of 1,000,000 iterations followed by 2,000,000 rounds MCMC sampling. The optimal K was determined using Structure Harvester^{56,57} based on both the maximum likelihood value (lnPD) and change rate of lnPD (ΔK)⁵⁸. To verify the Bayesian clustering results obtained for the 70 trees sampled in the DHS, we conducted an additional STRUCTURE run for K = 2 for a separate dataset comprising the 70 individuals examined in the present study and a further 1208 individuals analyzed by Guo et al. 2014 from sites other than the DHS. Given that Guo et al. assessed only six SSR loci, we used a sub-dataset of the 70 individuals screened with the same six loci during the additional calculations, the procedure of which was the same as that described above.

Using NewHybridsv.1.1⁵⁹, we quantitatively determined the posterior probability that each individual fell into one of the following six categories: the two pure parents, first-generation hybrids (F_1), second-generation hybrids (F_2), and backcrosses to the two pure parents. MCMC sampling was set as 100,000, preceded by a burn-in of 100,000 iterations with “Jeffreys-type” priors. Assignment of categories was based on the highest probability greater than 85%.

Genetic analysis of offspring populations

To determine whether random mating occurs between the different genetic types, we collected seeds during September in both 2020 and 2021. However, in these two years, we collected sufficient amounts of viable seed for analysis from only 20 and 17 individuals, respectively (Fig. 2a). Most of the remaining focal trees either failed to produce seeds or had produced only a few seeds that were inaccessible at the canopy. DNA was extracted from a total of 410 seeds, which were genotyped using the aforementioned 11 microsatellite loci. Assignment analysis for K = 2, applied using STRUCTUREv.2.3.4, was conducted for the 410 seeds, as well as the 70 parental trees. The experimental procedure and parameter settings were the same as those described above.

Flowering phenology

The sexual system of *Acer* has four phenotypes: duodichogamous, protogynous, protandrous, and male⁴². Hence, there are three functional sex types: (1) “Male I” flowers open earlier than “Female” flowers, with mature stamens, no style and ovary; (2) “Female” flowers have mature pistils, short filaments and indehiscence anthers; (3) “Male II” flowers open later than “Female”, with mature stamens, ovary, and separated stigmas. Duodichogamy is characterized by “Male I,” “Female,” and “Male II” types; protandry by “Male I” and “Female” types; and protogyny by “Female” and “Male II” types⁴².

To investigate the divergence of flowering phenology among the different genetic types, we recorded flowering phenology during the period from April 20 to May 18, 2020. Daily observations of flowering

were performed for 29 individuals with readily accessed branches, among which there were 9 SEA lineage, 14 hybrid, and 6 NEA lineage trees based on STRUCTURE analysis of the parental population (Fig. 2a). Initially, we selected four branches on each tree, evenly distributed in the four cardinal directions, which were marked for subsequent daily investigation. The total numbers of focal flowers produced by each tree were counted at the alabastrum stage, and during the monitoring period, the sex of each blooming flower was recorded. The data obtained for the numbers of flowers of each sex blooming (hereafter Blooming#-“sex”) are presented as the numbers recorded daily. No further flowers bloomed on focal branches after May 16.

On the basis of the daily Blooming#-“sex” data for each tree, we calculated indices of flowering phenology for each individual, including the commencement, peak, and cessation of each sex blooming. Using these data, we examined the potential phenological divergence among genetic types by performing a one-way ANOVA.

Analysis of leaf and fruit morphology

During August 2020, we collected a total of 690 healthy leaves from the 70 focal trees, which were scanned using an HP LaserJet 1100A scanner, and used DIGIMIZERv4.5.2 to measure six indices reflecting leaf size and shape (Figure S5a and Table 2).

Similarly, we collected and scanned a total of 615 fruits from 22 trees, among which, 170 were from SEA-DHS maternal sources, 256 from Hybrid-DHS, and 189 from NEA-DHS, as identified based on STRUCTURE analysis of parental population (Fig. 2a). Using DIGIMIZERv4.5.2, we obtained values for eight indices reflecting fruit size and shape (Figure S5b and Table 2).

To visualize potential morphological divergence among the different genetic types of leaf and fruit traits, we performed principal component analysis using R 4.1.2 software.

Statement of ethical approval

All the plant materials were sampled from natural populations in North China and no damages were caused to the studied trees. This study was conducted in accordance with local legislation and permission was issued to collect such samples. Each individual was marked with a tag which was possible to check in future. Voucher specimens collected were deposited at Beijing Normal University Herbarium.

Declarations

Acknowledgements

This work was supported by the National Natural Science Foundation of China [32071494, 32171654] and the National Science and Technology Basic Resources Survey Program of China [2019FY101700].

Author Contributions

Wang HF, Bao L and Ge JP designed the work. Yang R, Liu Y and Zhao J conducted all field work. Yang R and Deng YW performed molecular trials and analyzed data; Yang R, Deng YW and Wang HF drafted the work. Deng YW and Wang HF substantively revised it. All authors interpreted results and contributed to the final manuscript.

Data Accessibility Statement

Additional details are available in Figures S1-S5 and Tables S1–S3. All sample, SSR, flowering and morphological data are available on Dryad repository (<https://doi.org/10.5061/dryad.08kpr54n>).

Competing Interests Statement

A research collaboration was developed with local government during all field work, including locating focal trees, collecting genetic samples, recording flowering phenology and scanning leaves and fruits. All collaborators are included as co-authors. The research addresses a priority concern, in this case the identify and conservation of Tertiary relict species hybrid zone in North China. The results of research have been shared with all collaborators and local government. All data have been shared with the broader public via appropriate biological databases.

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Figures

Figure 1

Geographical range of NEA-SEA lineages area and the sampling population locations near the divide belt from GUO et al. 2014.

(a) Geographical range of two Tertiary relict regions [southern East Asian (SEA) and: northern East Asian (NEA)] for temperate flora in East Asia, according to Milne and Abbott (2002) (b) An enlarged representation of the area demarcated by the red border in (a) showing the geography of North China, which is a key secondary contact zone for Tertiary relict NEA and SEA lineages. The colored circles indicate the location of *Acer* populations sampled by Guo, et al. (2014b). The pie charts of sampled populations are STRUCTURE results based on a whole-range data set (see text), with the red and green sectors representing SEA and NEA lineages, respectively. The numbers adjacent to circles denote the altitudinal range in meters of all sampled individuals in populations. The focal population in the Daheishan National Nature Reserve examined in this study is indicated by a yellow star.

Figure 2

Genetic structure of the parental and offspring population.

(a) Ancestral proportion (q value) of the 70 *Acer* trees sampled in the Daheishan National Nature Reserve (DHS) based on STRUCTURE analysis of a DHS-only dataset. The q value is positively correlated with

altitude (Pearson $r = 0.83$, $p = 0.000$). Different colors or filled/empty of circles represent individuals used for different analyses in the study. The legend abbreviates adult/offspring genetic structure as adult/offspring, flowering phenology as phenology, leaf/fruit morphology as leaf/fruit. (b) Frequency distributions of q values in adult (gray) and offspring (colored) populations. (c) Principal coordinates analysis results obtained for the adult population.

SEA-DHS: southern East Asian lineage of the *Acer* species complex in the Daheishan National Nature Reserve (DHS); NEA-DHS: northern East Asian lineage of the *Acer* species complex in the DHS; Hybrid-DHS: hybrids between the SEA-DHS and NEA-DHS lineages.

Figure 3

Morphological variation in the leaves (a) and fruits (b) of southern and northern East Asian lineages (SEA-DHS and NEA-DHS, respectively) of the *Acer* species complex in the Daheishan National Nature Reserve based on principal component analysis.

Supplementary Files

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