Maintenance of strong morphological differentiation despite ongoing natural hybridization between sympatric species of *Lomatia* (Proteaceae)

Emma J. McIntosh¹,²,*, Maurizio Rossetto¹, Peter H. Weston¹ and Glenda M. Wardle²

¹The Royal Botanic Gardens and Domain Trust, Sydney, New South Wales, Australia and ²School of Biological Sciences, the University of Sydney, Sydney, New South Wales, Australia

* For correspondence. E-mail emci3309@uni.sydney.edu.au

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**Background and Aims** When species cohesion is maintained despite ongoing natural hybridization, many questions are raised about the evolutionary processes operating in the species complex. This study examined the extensive natural hybridization between the Australian native shrubs *Lomatia myricoides* and *L. silaifolia* (Proteaceae). These species exhibit striking differences in morphology and ecological preferences, exceeding those found in most studies of hybridization to date.

**Methods** Nuclear microsatellite markers (nSSRs), genotyping methods and morphometric analyses were used to uncover patterns of hybridization and the role of gene flow in morphological differentiation between sympatric species.

**Key Results** The complexity of hybridization patterns differed markedly between sites, however, signals of introgression were present at all sites. One site provided evidence of a large hybrid swarm and the likely presence of multiple hybrid generations and backcrosses, another site a handful of early generational hybrids and a third site only traces of admixture from a past hybridization event. The presence of cryptic hybrids and a pattern of morphological bimodality amongst hybrids often disguised the extent of underlying genetic admixture.

**Conclusions** Distinct parental habitats and phenotypes are expected to form barriers that contribute to the rapid reversion of hybrid populations to their parental character state, due to limited opportunities for hybrid/intermediate advantage. Furthermore, strong genomic filters may facilitate continued gene flow between species without the danger of assimilation. Stochastic fire events facilitate temporal phenological isolation between species and may partly explain the bi-directional and site-specific patterns of hybridization observed. Furthermore, the findings suggest that *F₁* hybrids are rare, and backcrosses may occur rapidly following these initial hybridization events.

**Key words:** Hybridization, *Lomatia myricoides*, *Lomatia silaifolia*, Proteaceae, microsatellite, morphometrics, admixture, hybrid zone, introgression, species cohesion, Australia, fire.

**INTRODUCTION**

The maintenance of species cohesion despite ongoing gene exchange via natural hybridization in plants is a phenomenon attracting increasing research attention (e.g. Moccia et al., 2007; Sambatti et al., 2012). Natural hybridization can create bridges for gene flow, offering a platform for adaptive evolution by introducing variation and novel traits into populations, potentially resulting in introgression and admixture of genotypes (Rieseberg and Wendel, 1993; Ellstrand et al., 1996; Arnold, 1997; Broyles, 2002).

With advances in genetic technologies, researchers have been able to uncover greater complexity within hybrid populations (Lai et al., 2012; Twyford and Ennos, 2012) and we can now delve deeper into interspecific gene exchange can be ongoing despite the presence of strong reproductive barriers (Sambatti et al., 2012), and pinpoint evidence of adaptive introgression (Heliconius Genome Consortium, 2012). There is substantial evidence that evolution can occur at the level of loci (Wu, 2001; Nosil and Schluter, 2011) and that the hybrid form is often a transitional phase in a much larger dynamic exchange of genetic material between parental lines, via backcrossing and introgression (Salvini et al., 2008; Gompert et al., 2010).

Evidence of extensive hybridization has been detected in the genus *Lomatia* (Weston and Crisp, 1994; Milner et al., 2012), making it an ideal system for exploring the role of natural hybridization in speciation and adaptation across the genus. This study focused on hybridization patterns between *Lomatia myricoides* (C.F. Gaertn.) Domin and *L. silaifolia* 21 (Sm.) R.Br. (Proteaceae), two Australian native shrub species that are highly differentiated in morphology and habitat preferences (Pellow et al., 2009). Although closely related, they are not sister species, and a geographical pattern of shared chlorotypes provides further evidence for hybridization (Milner et al., 2012).

*Lomatia myricoides* (river lomatia) is a tall, woody shrub (to 7 m) with simple leaves up to 20 cm long (dentate in seedlings and young adults, becoming entire in large adults) and lateral inflorescences (Weston and Crisp, 1994; Wilson et al., 1995). In contrast, *Lomatia silaifolia* (crinkle bush) is a shrub to 2 m in height, with highly dissected leaves [mostly pinnately divided twice or three times (Fig. 1)] and terminal inflorescences (Wilson et al., 1995; Pellow et al., 2009). The species have wide distributions, broadly overlapping at the boundaries of their ranges in the Sydney region (Wilson et al., 1995), where their flowering times overlap in summer (Beadle et al., 1972).
Lomatia myricoides and L. silaifolia are both capable of resprouting from subterranean lignotubers after fire, but their susceptibility to fire and their post-fire growth are distinctively different (Benson and McDougall, 2000). Lomatia myricoides is found growing on the banks of watercourses in fire-resistant moist forest or fire-prone eucalypt forest or at the interface of these communities. In contrast, L. silaifolia occurs in fire-prone open eucalypt woodlands and forests (Benson and McDougall, 2000; Pellow et al., 2009), raising questions about the role of selection for divergent habitats in the maintenance of species cohesion.

Fire plays an important role in the reproductive biology of L. silaifolia, as prolific flowering occurs in the first and second seasons after a fire and rarely thereafter (Denham and Whelan, 2000). Flowering of L. myricoides, on the other hand, is not fire-dependent and plants are more susceptible to fire damage. Consequently, these species are likely to flower concurrently at a site only if some L. myricoides plants remain unaffected in the years immediately following a fire. Under such circumstances, flowers of L. myricoides are likely to outnumber those of L. silaifolia. This suggests the potential for asymmetrical gene flow from L. myricoides into L. silaifolia and their hybrids where the two species co-occur, and for a large number of backcrossed hybrids towards L. myricoides.

Intriguingly, the frequency of morphological intermediates [inferred as putative hybrids (Weston and Crisp, 1994)] varies between sympatric populations of the parental species, raising questions about the degree of intrapopulation gene flow at different sites. The contrasting phenologies, habitat preferences and susceptibilities to fire of the parental species are likely to form strong selective pressures against the persistence of the intermediate hybrid form. Furthermore, the evolutionary potential of a population restricted to F₁ (first-generation hybrid) individuals (such as in Milne et al., 2003) may be significantly less than a population with a high frequency of gene flow and weak differentiation between parental species and their hybrids (Minder et al., 2007; Oberprieler et al., 2010).

Prior to detailed genomic studies, natural hybridization events must be confirmed, using affordable, rapid screening methods such as morphological analyses and genotyping techniques (Gompert et al., 2010; Marques et al., 2010). In this study we employed morphometric and genetic methods to investigate the magnitude and the potential causes and consequences of natural hybridization events between L. myricoides and L. silaifolia. In particular we sought to: (1) determine the extent of genetic admixture present in hybridizing populations and whether it was accurately reflected by morphological variation; (2) determine whether the patterns of hybridization were uniform between geographically separated hybrid populations seemingly presenting different degrees of in situ sympathy; and (3) analyse the directionality of interspecific gene flow in view of the fire dependency of the reproductive biology of Lomatia.

**MATERIALS AND METHODS**

**Study species**

Lomatia myricoides occurs along the south-east coast of Australia, south-west of the Great Dividing Range to Melbourne (Fig. 1). Lomatia silaifolia also occurs along the east coast and Dividing Range, but extends northward to the Tropic of Capricorn. The species are broadly sympatric in the Sydney Basin and the Blue Mountains region.

Conflorescences in Lomatia are racemes or panicles of lateral flower pairs, and flowers appear to be protandrous (Weston, 2007). Following fertilization, fruits are dehiscent follicles containing numerous, flattened, winged seeds that are wind-dispersed (Pellow et al., 2009). Lomatia myricoides flowers between December and January, whilst L. silaifolia flowers...
between November and February (Beadle et al., 1972), and the species share insect pollen visitors (E. J. McIntosh, pers. observ.). Preliminary seed germination trials using genetically determined hybrids and pure species revealed that hybrids were fertile, because flowering produced seeds with germination rates comparable to those of the parental species (results not presented).

**Study sites**

This study focused on populations within the broader sympatric zone between *L. myricoides* and *L. silaifolia*. Herbarium records (www.chah.gov.au/avh) Last accessed April 18, 2011 and field surveys were used to select three sites representing different degrees of *in situ* sympathy and hybridization between these species. The upland site at Newnes State Forest (NSF) appeared to be a hybrid swarm of multiple interbreeding and backcrossing hybrid generations, *L. silaifolia* and *L. myricoides* growing alongside many plants of intermediate morphology. Both species occurred in close proximity along a creek at the southern coastal site in the Royal National Park (RNP), with a small number of morphological intermediates. At the northern coastal site, Bobbin Head (BH), the two species were separated by a steep altitudinal gradient along the edge of a gully and morphological intermediates were not detected despite such a herbarium specimen having been collected in the area in 1969 (accession number NSW130756).

**Sampling strategy**

Transects were constructed to sample representative individuals of *L. myricoides*, *L. silaifolia* and putative hybrids at 10 m intervals (Cornelissen et al., 2003). For morphological analysis, 72 *Lomatia* plants (26 at NSF, 25 at BH and 21 at RNP) were sampled from across the study sites in October 2010. From each plant, five mature leaves from the most recent growth season were sampled consecutively from the base of the shoot, excluding those with evidence of damage or disease. Herbarium specimens are lodged at the National Herbarium of New South Wales.

For genetic analysis, an additional two transects were sampled in the NSF population (total *n* = 101) as the large number of morphological intermediates suggested the potential for a hybrid swarm to exist, which was worthy of closer examination. Fresh leaves were silica-dried and stored at −20 °C prior to extraction. Total DNA was extracted from 50 mg of leaf tissue using the DNeasy® 96 Protocol (Qiagen®, Hilden, Germany) and quantified by agarose gel electrophoresis.

**Molecular analyses**

Nuclear microsatellites (nSSRs) were used to investigate intrapopulation level gene flow and diversity between *L. myricoides*, *L. silaifolia* and their hybrids, due to their high heterozygosity, specificity at low taxonomic levels and highly polymorphic and co-dominant traits (Goldstein and Pollock, 1997). A total of 147 plants were genotyped at 12 polymorphic nSSR loci using primers developed for *L. silaifolia* (LS013, LS046, LS050, LS059, LS064a, LS122, LS129, LS136, LS163, LS167, LS178 and LS189a) (Milner et al., 2013). nSSR loci were amplified in 10 μL reactions containing 0.5 ng of genomic DNA following the PCR protocol in Milner et al. (2013). PCRs were repeated for 20% of samples across all primers and PCR products were visualized by agarose gel electrophoresis before genotyping on an ABI 3730 Genetic Analyzer (Applied Biosystems, Macrogen, Korea) in a diluted 20 μL multiplex sample containing 1 μL from each primer pair. Genotyping was also repeated for 20% of samples to ensure consistent and reliable allele calling. A GeneScan™ 500 LIZ® size standard was applied and nSSR profiles were examined using GeneMapper v4.0 (Applied Biosystems, 2005). *Lomatia* species and their hybrids are diploid and allele calls consistently yielded no more than two peaks, hence no evidence of polyploidy was encountered in *Lomatia* hybrids. The presence of null alleles was tested using Micro-Checker v2.2.3 (van Oosterhout et al., 2004).

**Assignment methods**

In order to understand the genotypic structure of each population and the evolutionary consequences of interspecific gene flow in *Lomatia*, model-based Bayesian clustering analyses were conducted in NewHybrids v1-1 (Anderson and Thomson, 2002). This software allocates individuals to one of six genotypic classes (pure *L. myricoides*, pure *L. silaifolia*, F1, F2, backcross to *L. myricoides* or backcross to *L. silaifolia*). No a priori knowledge of an individual’s genetic background is required using this method, meaning that admixture can be detected without the need for diagnostic alleles (Anderson and Thomson, 2002). Preliminary tests were conducted using random combinations of priors, to ensure consistent partitioning of genotypic classes. Bayesian estimates were stable and did not vary greatly depending on the length of burn-in period, run length or the application of priors, hence no priors were applied in final NewHybrids analyses. Analyses were conducted by study site. Mean posterior probabilities were computed from five runs each of a burn-in period of 30 000 runs (to allow the Markov chain Monte Carlo simulations to converge) and a run time of 300 000 sweeps, using the uniform prior.

The original intention was to assign each specimen to one of the six genotypic classes when its posterior probability of belonging to that genotypic class equaled or exceeded *P* = 0.90 (cf. Viscosi et al., 2009; Pinheiro et al., 2010). No specimen met the condition of *P* ≥ 0.90 for being allocated to the *F1*, *F2* or backcross genotypes as 12 nuclear microsatellite markers provided insufficient genetic resolution (cf. Vaha and Primmer, 2006; Field et al., 2009). As a result, individuals with *P* < 0.90 of belonging to a pure *L. myricoides* or *L. silaifolia* genotypic class were considered admixed, of hybrid origin, and were lumped into a single class. Three genotypic classes were used for further analysis: pure *L. myricoides*, pure *L. silaifolia* and the hybrid pool. It appeared that a substantial genotyping effort, involving more than 48 loci, would be required to separate backcrosses from pure parental individuals in future studies (Vaha and Primmer, 2006). Hereafter, the term ‘hybrid’ refers to individuals genetically assigned as admixed, and species assignments are based on genotypic analysis.

**Genetic diversity**

The mean number of alleles per polymorphic locus (AP), allelic richness (*R*<sub>a</sub>) (Mousadik and Petit, 1996), and observed
and expected heterozygosity ($H_e$ and $H_s$, respectively) were calculated by pure species and hybrid cohorts, within sites. Pairwise genetic distances between species and hybrids within sites, and between pure species across sites, were estimated using Weir and Cockerham’s $F_{ST}$ [Weir and Cockerham, 1984; calculated in FSTAT version 2.9.3 (Goudet, 2001) following 10,000 permutations]. Jost’s $D$ (Jost, 2008) was also calculated to account for the dependency of $F_{ST}$ measures on within-population diversity (Meirmans and Hendrick, 2011).

**Genetic differentiation between parental species and hybrids**

Principal coordinate analyses (PCoAs) were used to produce comparative graphical representations of genotypic similarity based on a matrix of Codom—genotypic distances, in GenAlEx (Peakall and Smouse, 2006).

**Morphometric analysis**

Variation in leaf morphology and total plant height between *L. myricoides*, *L. silaifolia* and their putative hybrids was examined in an attempt to detect phenetic groupings of individuals and to quantify variation present within and between these groups. Reproductive material was not included due to limited availability during the study season. Leaf images were scanned in Adobe Photoshop version 7.0 with 400 dpi resolution and saved as jpeg files.

Seven single-parameter shape descriptors were selected for morphometric analysis: degree of leaf division; number of leaf lobes; leaf dissection index (LDI); petiole:lamina length ratio; leaf length:width ratio; lamina circularity; and plant height. The selected variables are important taxonomic traits separating these species (Pellow et al., 2009), and were screened to avoid a high degree of collinearity with other predictor variables (Sokal and Rohlf, 1995). The variables were also required to display a difference in median values between *L. myricoides* and *L. silaifolia* as inferred from box and whisker plots.

Manual counts of leaf lobes and leaf divisions were conducted on colour image files and morphometric measures were computed using ImageJ software (version 1.43u, W. S. Rasband, National Institutes of Health, Bethesda, MD, USA). The degree of leaf division and number of leaf lobes were defined according to Hickey (1973) and Huff et al. (2003). Many *L. silaifolia* leaves had overlapping leaflets, hence measures of leaf area and perimeter and calculations of LDI (McLellan and Endler, 1998) are acknowledged as under-estimations; however, they remain valuable measures for separating the species. The petiole:lamina length ratio was selected as a means of standardizing petiole length, and leaf length:width ratios and lamina circularity (Huff et al., 2003) were included as alternative measures of leaf shape. Plant height was measured according to Cornelissen et al. (2003).

In order to examine morphological relationships between species and hybrids within sites, principal component analyses (PCAs) based on covariance matrices were used to produce ordinations from morphometric data using Primer v6 software, and methods detailed in Clarke and Gorley (2006) were followed. Specimen data were normalized by trait, and leaf length:width ratio, petiole:lamina length ratio and LDI were log$_{10}$-transformed to reduce skew. The degree of leaf division was excluded from the PCA due to the non-normal distribution of the variable and the correlation with number of leaf lobes.

**Correlating genetics and morphology**

We sought to determine whether genotypic and morphologic variations in hybridizing populations were correlated. *Lomatia* individuals that had been both successfully genotyped and included in morphometric analyses from three study sites (24 from BH, 20 from RNP and 24 from NSF) were included in Mantel tests (Mantel, 1967), conducted in GenAlEx following 999 permutations. Mantel tests check for a statistical relationship between the elements of two distance matrices with corresponding entries. Genetic and morphometric matrices had previously been calculated (Codom—genotypic distance in GenAlEx and Euclidean distance in Primer v6 respectively).

**RESULTS**

**Assignment results**

NewHybrids results revealed the presence of pure parental species and admixed individuals with varying degrees of introgression at all three sites. The majority of genotyped plants in the large NSF population were of hybrid origin (78 %, $n = 79$); few were pure parental species (*L. myricoides* $n = 15$, *L. silaifolia* $n = 7$) (Figs 2 and 3). Hybrids ranged from highly to partially admixed at this site (Fig. 2B), a pattern also demonstrated by the gradation or ‘V’ shape of admixture in Fig. 3. Hybrids were detected at lower numbers in RNP ($n = 5$), alongside pure *L. myricoides* ($n = 11$) and *L. silaifolia* ($n = 5$) (Table 1, Fig. 3).

At RNP the composition of hybrid genotypic classes (Supplementary Data Fig. S1) suggested that several hybrid individuals were early generational backcrosses towards *L. silaifolia*. For example, RS1 had a posterior probability of $P = 0.242$ of belonging to the backcross to *L. silaifolia* genotypic class. Three individuals had a high, although not significant, posterior probability of belonging to the $F_1$ generation, including RH13 ($P = 0.743$). RH13 was also one of the most morphologically intermediate plants, with numbers of leaf divisions and lobes intermediate between, but outside, the ranges of either parental species (Fig. 2C).

Only one hybrid was uncovered at BH, with a moderately high posterior probability of being of pure *L. silaifolia* background ($P = 0.859$) and morphologically grouping amongst individuals of *L. silaifolia*.

Comparisons between initial in-field morphological assignment notes and genotyping assignment results revealed that large numbers of plants had initially been misidentified, particularly at NSF. At this site, 51 % ($n = 52$) of genotyped plants were initially misidentified: 31 plants suspected to be *L. silaifolia* were in fact hybrids, eight of which had a low posterior probability ($P < 0.50$) of being pure *L. silaifolia*. A further 17 plants suspected to be *L. myricoides* were also hybrids, three of which had $P < 0.50$ of being pure *L. myricoides*. A further four plants were misidentified as hybrids, despite the genetics revealing that these were pure *L. myricoides* ($P > 0.90$) and one was pure *L. silaifolia* ($P > 0.90$).

**Genetic diversity**

All 12 nSSR loci were found to be polymorphic and highly variable across both species at all three sites, amplifying a total of 229 alleles across 147 *Lomatia* plants. The most variable
marker across all sites was LS178, with a mean of 30 alleles, whereas LS136 and LS064A were the least variable, with seven and eight alleles, respectively.

Hybrids at NSF had the highest allelic diversity of all samples across all sites, with a mean of 16.333 alleles per locus (AP), and allelic richness of 15.957 (RS) (Table 1). Differentiation between
The distinction between parental species was also low at RNP (pairwise $F_{ST} = 0.146 \pm 0.043$, $P < 0.001$, Table 2; $D = 0.533$) and observed heterozygosity in both parental species exceeded that expected (Table 1). This, combined with high overall heterozygosity measures ($H_e = 0.752 \pm 0.032$, $H_s = 0.740 \pm 0.032$) added to evidence of a hybrid swarm at this site.

The distinction between parental species was also low at RNP (pairwise $F_{ST} = 0.214 \pm 0.048$, $P < 0.01$, Table 2; $D = 0.679$), although greater than between parental species at NSF. Gene flow at RNP between L. silaifolia and hybrids was slightly higher than that between L. myricoides and hybrids, as inferred from $F_{ST}$ values (Table 2) and the overall heterozygosity was high ($H_e = 0.662 \pm 0.029$, $H_s = 0.752 \pm 0.040$).

Gene flow between the parental species at BH (pairwise $F_{ST} = 0.215 \pm 0.053$, $P < 0.001$, Table 2) did not differ greatly from that at RNP, which was interesting given the lack of recent hybrids found at this site (Fig. 2E, F). However, the two species were most differentiated at BH according to Jost’s $D$ ($D = 0.741$).

Intriguingly, gene flow between L. myricoides from each site (Table 3) was lower than, or comparable to, interspecific gene flow between L. myricoides and L. silaifolia at any one site (Table 2). L. silaifolia plants in comparison displayed a high level of gene flow between sites, particularly between RNP and BH ($F_{ST} = 0.009 \pm 0.013$) (Table 3).

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**Table 1. Population diversity indices for Lomatia at three sites where the two parental species occur in sympatry**

<table>
<thead>
<tr>
<th>Site</th>
<th>Species*</th>
<th>$n$</th>
<th>AP</th>
<th>$R_s$</th>
<th>$H_o$</th>
<th>$H_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSF</td>
<td>L. myricoides</td>
<td>15</td>
<td>7.750 ± 0.914</td>
<td>7.592 ± 0.884</td>
<td>0.731 ± 0.066</td>
<td>0.719 ± 0.064</td>
</tr>
<tr>
<td></td>
<td>L. silaifolia</td>
<td>7</td>
<td>6.167 ± 0.683</td>
<td>5.842 ± 0.633</td>
<td>0.748 ± 0.063</td>
<td>0.717 ± 0.054</td>
</tr>
<tr>
<td>RNP</td>
<td>L. myricoides</td>
<td>11</td>
<td>4.917 ± 0.583</td>
<td>4.703 ± 0.545</td>
<td>0.698 ± 0.069</td>
<td>0.633 ± 0.051</td>
</tr>
<tr>
<td></td>
<td>L. silaifolia</td>
<td>5</td>
<td>5.250 ± 0.552</td>
<td>4.700 ± 0.444</td>
<td>0.763 ± 0.064</td>
<td>0.707 ± 0.047</td>
</tr>
<tr>
<td></td>
<td>Hybrids</td>
<td>5</td>
<td>4.333 ± 0.414</td>
<td>3.372 ± 0.334</td>
<td>0.796 ± 0.078</td>
<td>0.647 ± 0.053</td>
</tr>
<tr>
<td>BH</td>
<td>L. myricoides</td>
<td>12</td>
<td>6.083 ± 0.839</td>
<td>5.638 ± 0.764</td>
<td>0.612 ± 0.071</td>
<td>0.649 ± 0.074</td>
</tr>
<tr>
<td></td>
<td>L. silaifolia</td>
<td>12</td>
<td>8.667 ± 0.907</td>
<td>8.667 ± 0.907</td>
<td>0.729 ± 0.057</td>
<td>0.744 ± 0.048</td>
</tr>
<tr>
<td></td>
<td>Hybrids</td>
<td>1</td>
<td>1.833 ± 0.112</td>
<td>1.833 ± 0.112</td>
<td>0.833 ± 0.112</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Abbreviations: $n$, number of samples; AP, mean number of alleles per polymorphic locus; $R_s$, allelic richness; $H_o$, observed heterozygosity; $H_e$, expected heterozygosity ($\text{mean} \pm \text{s.e.}$); n/a, not applicable (there was only one hybrid at BH).

*Species assignments are based on genotypic analysis at $P \geq 0.90$.
†Values of $H_e$ for hybrids should be treated with caution as they were calculated in violation of the assumptions of Hardy–Weinberg equilibrium.

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**Table 2. Pairwise $F_{ST}$ between parental species and between parents and the hybrid cohort within sites (mean across 12 nSSR loci ± s.e.)**

<table>
<thead>
<tr>
<th>Species*</th>
<th>NSF</th>
<th>RNP</th>
<th>BH</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. myricoides</td>
<td>0.146 ± 0.043***</td>
<td>0.214 ± 0.048**</td>
<td>0.215 ± 0.053***</td>
</tr>
<tr>
<td>and L. silaifolia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. myricoides and hybrids</td>
<td>0.035 ± 0.011***</td>
<td>0.126 ± 0.022**</td>
<td>0.162 ± 0.039</td>
</tr>
<tr>
<td>L. silaifolia and hybrids</td>
<td>0.032 ± 0.014**</td>
<td>0.113 ± 0.036*</td>
<td>0.009 ± 0.037</td>
</tr>
</tbody>
</table>

There was only one hybrid at BH.

*Species assignments are based on genotypic analysis at $P \geq 0.90$.
**$P < 0.05$; ***$P < 0.01$; +++$P < 0.001$.

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**Genetic differentiation between parental species and hybrids**

The PCoAs using 12 nSSRs proved effective means of visualizing the degree of genetic differentiation and population structures at each site. At NSF, a PCoA showed a cloud of genetic variation spanning the parental extremes and indicated the presence of a hybrid swarm and extensive interspecies gene flow (Fig. 2B). In the RNP genetic PCoA three clusters were
revealed, with hybrids intermediate between the parental species clusters, as expected for early generational hybrids (Fig. 2D). A PCoA of genetic distance at BH supported genotypic assignment methods by confirming that the two morphologically distinct species clusters were also genetically distinct (Figs 2F and 3).

**Morphometry**

Pure L. myricoides and L. silaifolia could be clearly distinguished using morphometric methods. The species did not overlap even at the extremes of variation in each character state (degree of leaf division, number of leaf lobes, LDI, petiole:lamina length ratio, leaf length:width ratio and lamina circularity) (Fig. 4), except for plant height, which is highly variable.

Lomatia silaifolia was characterized as a low shrub with a complex leaf outline; a mode of three leaf divisions, many lobes and a high LDI (Figs 2A, C, E and 4). Low leaf length:width ratios and lamina circularity indices confirmed the comparable length and width of L. silaifolia laminae, unlike the narrow, linear laminae of L. myricoides. The latter was characterized by its tall stature, simple leaf outlines, no leaf divisions and a low LDI (Figs 2A, C, E and 4). The low petiole:lamina ratio of L. myricoides indicated a short petiole, in contrast to the long petioles of L. silaifolia (Fig. 4).

In a PCA of morphometric data across all three sites (not shown), L. silaifolia clustered tightly when the first and second principal components were visualized, but spread in the third due to variation in the number of leaf lobes, LDI and the petiole:lamina length ratio. Lomatia myricoides was tightly clustered when the first and third principal components were visualized due to low variation in lamina circularity and the lamina length:width ratio, but was spread in the second component due to wide variation in plant height.

Hybrid individuals were characterized by extreme morphological variability, spanning the parental extremes (Fig. 4). The pooled hybrid cohort was similar to L. silaifolia in height, LDI and lamina circularity but closer to L. myricoides in number of leaf lobes (Fig. 4).

The PCAs of morphometric data by site showed distinct parental species clusters, but hybrids were not evenly distributed across the continuum between the two and most fell towards the margins of a particular parental species cluster (Fig. 2A, C, E). At each site, more than 95% of the variance was explained by the first three principal components and between 79 and 91% by the first alone. At NSF the PCA was slightly bimodal, with few intermediate hybrids and a large number of hybrids morphologically similar to one or other parental species (Fig. 2A).

At RNP, several hybrids separated towards the centre of the ordination space, phenotypically more similar to L. silaifolia than L. myricoides (Fig. 2C). This was primarily due to the effects of leaf outline complexity (LDI and number of lobes) and petiole length (vectors in Fig. 2C). One hybrid shared the ordination space of L. silaifolia and was misidentified as L. silaifolia in the field, but genotypic assignment methods suggested it was a late-generation backcross. Another hybrid was exceptionally tall, apparently due to its old age, separating from all other individuals in the ordination space.

Phenetic clustering was strongest at BH, where a bimodal PCA of morphometric variation separated parental species due to marked differences in leaf shape and plant height (Fig. 2E), and the single late-generation backcross individual was located within the L. silaifolia cluster.

Five individuals at NSF were morphologically cryptic, clustering tightly with L. silaifolia in the morphometric ordination despite being genetically more similar to pure L. myricoides. Two clustered particularly tightly amongst L. silaifolia individuals and both were within the range of variation expected for that species for six out of the seven character traits analysed, despite genetic assignment methods placing them closer to L. myricoides. Another plant at NSF, misidentified as L. silaifolia on the basis of morphology, had a posterior probability of P = 0.501 of being from a pure L. myricoides background and only P < 0.002 of being from a pure L. silaifolia background.

**Correlating genetics and morphology**

Two-way Mantel tests applied to Codon—genotypic distance and Euclidean distance matrices across all sites revealed a significant, low correlation between genetic and morphometric datasets (r = 0.442, P < 0.001). When Mantel tests were performed site by site, a significant, high correlation was detected between morphometric and genetic distances amongst Lomatia plants at BH (r = 0.807, P < 0.001); a lesser, but still significant correlation was found at RNP (r = 0.616, P < 0.001) and a significant, albeit low, correlation was found at NSF (r = 0.241, P = 0.001). A Mantel test for hybrids only at NSF revealed a non-significant, low correlation (r = 0.073, P = 0.183).

**DISCUSSION**

**Extent of genetic admixture in hybridizing Lomatia populations**

Despite their distinct ecological and morphometric character traits, molecular evidence of admixture was detected at all

**Table 3. Pairwise Fst within species between sites (mean across 12 nSSR loci ± s.e.)**

<table>
<thead>
<tr>
<th></th>
<th>NSF</th>
<th>RNP</th>
<th>BH</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSF</td>
<td>–</td>
<td>0.200 ± 0.045***</td>
<td>0.041 ± 0.013**</td>
</tr>
<tr>
<td>RNP</td>
<td>0.200 ± 0.045***</td>
<td>–</td>
<td>0.020 ± 0.012**</td>
</tr>
<tr>
<td>BH</td>
<td>0.167 ± 0.035***</td>
<td>0.144 ± 0.034***</td>
<td>–</td>
</tr>
</tbody>
</table>

Values for L. silaifolia are at the top right, values for L. myricoides are at the bottom left.

*P < 0.01; **P < 0.001.*
study sites where *L. myricoides* and *L. silaifolia* occurred in sympatry. However, the patterns of hybridization differed markedly between sites. Suggestions of multiple hybrid generations and extensive introgression characterized the hybrid swarm at NSF, whereas hybrids were uncommon and likely of early generations at RNP. Despite no morphological signs of recent hybridization at BH, genotyping revealed a signature of admixture in one individual. Such site-specific patterns of introgression offer a glimpse into the different stages of natural hybridization *in situ* (Lamont et al., 2003).

**Morphological variation and cryptic hybrids**

The strong morphometric and ecological distinction between *L. myricoides* and *L. silaifolia* exceeds that found in most studies of naturally hybridizing plant species (Maguire et al., 2002;
Viscosi et al., 2009). Genetically identified pure species could be clearly distinguished by morphometric studies; however, when such approaches were used to interpret hybridizing populations, they masked the true extent of genetic diversity, particularly in the NSF hybrid swarm. Lomatia hybrids had highly variable morphologies, likely the results of random segregation, novelty and genetic recombination amongst multiple hybrid generations, as well as the gradual decay of linkage disequilibrium over time (Rieseberg and Ellstrand, 1993; Arnold and Martin, 2010). As in an increasing number of studies, the majority of Lomatia hybrids expressed greater phenetic similarity to either L. myricoides or L. silaifolia rather than being strictly intermediate in all characters (cf. Lihová et al., 2007; Field et al., 2009). Leaf shape in Lomatia may be influenced by diversifying selection towards the two parental character states (Oberprieler et al., 2010), explaining the contrast between the slightly bimodal morphometric dataset and the continuum of genetic diversity in hybrid populations.

For the purposes of identifying hybrids in the field, petiole length relative to overall leaf length and the number of leaf lobes are likely to be the most reliable morphological characters, although it should be noted that all species of Lomatia have highly variable morphology. L. silaifolia potentially most of all (Weston and Crisp, 1994). The parental extremes of morphometric variation masked the hybrid origin of many Lomatia plants, particularly in the complex hybrid swarm at NSF. Hybrids that were misidentified in the field as pure species exhibited high levels of genetic admixture and were likely to be later-generation backcross hybrids resembling the parental species to which they were genetically most similar. This explains the low correlation between morphometric and molecular patterns of variation within the hybrid swarm. Interestingly, where species connectivity was not apparent at BH, intermediate morphotypes had disappeared, masking the introgressed genes that remained in the population.

The large proportion of plants incorrectly assigned in the field and the presence of cryptics genetically close to one parent but morphologically resembling the other pointed to complex patterns of introgression between L. myricoides × L. silaifolia hybrids. While the examination of morphological character traits will always be an important component of hybridization studies, these findings highlight the value of molecular tools when identifying gene flow among closely related but strongly differentiated lineages (Minder et al., 2007; Marques et al., 2010).

There is great potential with next-generation sequencing (NGS) technologies to trace the extent of genome-wide introgression (Lai et al., 2012; Twyford and Ennos, 2012), identify introgressed genes and determine the evolutionary adaptive effects of introgressed genomic regions (Song et al., 2011; Heliconius Genome Consortium, 2012). The decreasing costs, high throughput and extensive coverage of NGS technologies make them valuable for non-model species and those with little available genomic data, such as Lomatia (Twyford and Ennos, 2012), where traditional approaches involve significant time and costs to obtain the large numbers of markers required in population genetic studies. The analysis of plastid markers alongside the genotyped nuclear markers in this study is a priority for future research and will greatly improve inferences regarding the direction of introgression and the evolution of reproductive barriers in this hybrid zone.

Fire dependency of reproductive isolation between L. myricoides and L. silaifolia

We hypothesize that hybridization between L. myricoides and L. silaifolia is restricted to certain stochastic events as F1 hybrids are rare. Herbarium records suggest that, despite their evident reproductive compatibility, broadly overlapping flowering seasons and substantial area of sympathy, morphological evidence for hybridization is lacking at most sites where L. myricoides and L. silaifolia co-occur.

One plausible explanation for the low incidence of F1 hybrids is temporal reproductive isolation imposed by the timing of flowering after fire. Both Lomatia species resprout from lignotubers after fire but L. silaifolia concentrates its reproductive effort in the first year or two following a fire (Denham and Whelan, 2000), whereas L. myricoides is more sensitive and is unlikely to flower in the years immediately following fire unless plants are not burnt. Fire records at the study sites corroborate this theory. Early-generation hybrids at RNP may be the result of a recent fire event, whereas opportunities for F1 formation at NSF are likely to have been fewer since no fires had been through the area for at least a decade. As expected, no L. silaifolia were observed to flower at NSF in the year of this study (E. J. McIntosh, pers. observ.).

Our findings of bi-directional gene flow between the parental species at NSF do not support our expectation that gene flow is primarily asymmetrical from L. myricoides into hybrids. Therefore, the hypothesis that fire is a mechanism for producing asymmetry has been retracted, but the way in which fire shapes patterns of hybridization between Lomatia requires further study.

Stochastic fire events are an important feature in the occurrence of hybridization of other species in Australian sclerophyllous environments, such as between Banksia robur and B. oblongifolia (Usher et al., 2010) and Kunzea rupestris and K. capitata (Tierney and Wardle, 2008). In the latter study, Kunzea hybrids did not resprout after fire, and this lower fitness relative to parental plants appeared to restrict the evolutionary potential of hybrids, a trait that should be further investigated in Lomatia hybrids. The requirement for fire to stimulate flowering of L. silaifolia is likely to lead to the re-establishment of reproductive isolating barriers between pure species following F1 formation, and our findings suggest backcrosses may occur readily following these initial hybridization events (Broyles, 2002). As initial genetic bridges, F1 hybrids allow introgressive hybridization to subsequently become an important evolutionary force in a population (Ellstrand et al., 2007).

Transient hybrid swarms or ongoing gene exchange via introgression?

Hybridization between L. myricoides and L. silaifolia is unlikely to be a recent phenomenon where these species are sympatric. Porous species barriers characterize the entire genus; six of the nine Australian native Lomatia species have been reported to hybridize, and both L. myricoides and L. silaifolia hybridize with other Lomatia species elsewhere over their broad geographic ranges (Milner et al., 2012). Furthermore, the high level of
Gene flow detected between parental species at NSF, as well the presence of introgression at BH despite no morphologically identifiable hybrids, hints at a pattern of recurrent introgression (potentially over many generations) between *L. myricoides* and *L. silaifolia* across their shared geographic region. Such findings add to growing research suggesting that even strong barriers may not be sufficient to prevent gene flow between species (Steeconhi et al., 2004; Sambatti et al., 2012).

Genetic distinction between *L. myricoides* at separate sites was unexpectedly revealed in this study. The pattern was not present amongst sampled *L. silaifolia* and could be a consequence of the greater habitat specificity of *L. myricoides*. Site-by-site differences in *L. myricoides* match the findings of Milner et al. (2013) that cpDNA gene phylogenies of Australian *Lomatia* species are more congruent with geography than taxonomy, confirming that geography has been important in the evolution of the genus. Exploration of within-species genetic structure was not an objective of this study but will be the focus of in-depth future studies, including both nuclear and chloroplast markers, as in recent investigations of the closely related *Telopea* species (Rossetto et al., 2011, 2012).

**Species cohesion in the face of permeable species boundaries**

Our findings indicate a need to consider the processes by which species maintain distinct morphologies, genotypes, ecological preferences, phenologies and broader geographic distributions, despite ongoing interspecific exchanges of genetic material. Species cohesion in *Lomatia* is expected to be maintained despite the likelihood of continued gene flow and introgression wherever the species co-occur (Usher et al., 2010). Potential explanations include selective pressures maintaining species-specific character traits, genomic filters reducing introgression at key genomic sites, and adaptive introgression being triggered only when environmental conditions change (Reed et al., 2011).

Gene flow can be ongoing even in cases where selective pressures and isolating barriers are strong (Ito et al., 2008; Sambatti et al., 2012). The distinct environmental, phenotypic and genotypic characteristics of pure *L. myricoides* and *L. silaifolia* may constitute two adaptive peaks maintained by selection. We observed very restricted intermediate habitats compared with the preferred habitats of the two parental species (unlike Rieseberg et al., 1998; Lihová et al., 2007). Microclimatic temperature measurements proved insufficient to quantify this habitat transition (data not shown); however, our observations suggested the transition was sharp, and at BH in particular *L. myricoides* was tightly restricted to a steep-sided, wet creekline in an otherwise dry sclerophyll vegetation type, where *L. silaifolia* inhabited the higher, drier slopes. Hybrids were found in both habitats but this warrants further investigation, particularly if later-generation backcrosses are more likely to be restricted to the characteristic parental habitat types than earlier-generation hybrids. This ecological barrier is suspected to contribute to the rapid reversion of hybrid populations to their parental character state due to limited opportunities for hybrid/intermediate advantage (Arnold, 1997).

Strong genomic filters may facilitate continued gene flow between species without the danger of assimilation, due to the powerful effect of selective forces acting to maintain species cohesion, such as stabilizing selection or the spread of advantageous alleles (Martinsen et al., 2001; Palma-Silva et al., 2011). This ‘porous view’ of species boundaries (Muir et al., 2012), involves the maintenance of conserved genomic regions, preserving intraspecific cohesion despite the presence of introgressed genes at unlinked or more distantly linked chromosomal regions (Mallet, 2005). This is evident between sympatric *Pitcairnia* species, in which asymmetrical introgression rates of single loci suggested that Bateson–Dobzhansky–Muller incompatibilities contribute to the maintenance of species integrity (Palma-Silva et al., 2011).

**Conclusions**

Morphological and ecological distinctions between plant species can be maintained despite ongoing gene flow via natural hybridization. Localized gene flow and introgression are expected to be ongoing between *L. myricoides* and *L. silaifolia* and their hybrids wherever they occur in sympaternity, due to the permeability of this species barrier. Future studies will include the use of NGS technologies and detailed analyses of plastid data to better characterize the extent and direction of introgression in *L. myricoides* and *L. silaifolia* and to gain insight into how less porous regions of the genome contribute to maintaining the integrity of parental *Lomatia* species.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Supplementary Data Figure S1: bar plots depicting Bayesian admixture proportions estimated using NewHybrids with samples from three study sites.

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**LITERATURE CITED**


