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Asymmetrical introgression between two *Morus* species (*M. alba*, *M. rubra*) that differ in abundance

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Abstract

Asymmetrical introgression is an expected genetic consequence of hybridization when parental taxa differ in abundance; however, evidence for such effects in small populations is scarce. To test this prediction, we estimated the magnitude and direction of hybridization between red mulberry (*Morus rubra* L.), an endangered species in Canada, and the introduced and more abundant white mulberry (*Morus alba* L.) using nuclear (randomly amplified polymorphic DNA) and cytoplasmic (chloroplast DNA sequence) markers. Parentage of 184 trees (n = 42 using cpDNA) from four sympatric populations was estimated using a hybrid index and related to six morphological characters and population frequencies of the parental classes. Overall, the frequency of nuclear hybrids was 53.7% (n = 99) and ranged from 43% to 67% among populations. The parental and hybrid taxa differed with respect to all of the morphological traits. Sixty-seven percent of all hybrids contained more nuclear markers from *M. alba* than *M. rubra* (hybrid index $\bar{x}$ = 0.46); among populations, the degree of *M. alba* bias was correlated with the frequency of *M. alba*. In addition, the majority of hybrids (68%) contained the chloroplast genome of white mulberry. These results suggest that introgression is bidirectional but asymmetrical and is related, in part, to the relative frequency of parental taxa.

Keywords: asymmetric hybridization, chloroplast DNA, gene flow, genetic assimilation, Moraceae, small populations

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Introduction

Hybridization between populations that differ in abundance can have a number of genetic consequences (Ellstrand 1992; Ellstrand & Elam 1993; Levin *et al*. 1996; Rhymer & Simberloff 1996; Arnold 1997). These effects include the formation of hybrid taxa, which have associated effects on fitness (Rieseberg & Carney 1998; Burke & Arnold 2001), and introgression, which results from backcrossing of hybrids to parental taxa and other hybrids (Arnold 1992; Rieseberg & Wendel 1993). Although these genetic effects are common to all hybridizing taxa, the quantitative impacts of hybridization are particularly severe on parental taxa in small populations. The effects will be disproportionately large, because, all else being equal, hybrid fertilizations constitute a larger proportion of the total fecundity of small populations, and hybrids will backcross differentially to the common parental taxa. Under some circumstances, these processes can lead to local extinction of the rare taxa.

The term 'genetic assimilation' refers to the loss of a rare taxon (or genotype) through asymmetrical introgression with a more numerous congener (Ellstrand 1992; Ellstrand & Elam 1993; Levin *et al*. 1996; Rhymer & Simberloff 1996; Arnold 1997). Through unidirectional backcrossing of partially fertile hybrids to the more abundant parent (Anderson 1948, 1949; Huxel 1999; Wolf *et al*. 2001; Buerkle *et al*. 2003; Haygood *et al*. 2003), asymmetrical introgression leads to a disproportionately high number of hybrids with the nuclear and cytoplasmic composition of the more abundant parent. Over time, such uneven gene flow facilitates chloroplast swapping and contributes to the loss of parental nuclear genotypes (Harrison 1990; Arnold 1992,

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1997; Rieseberg & Brunsfeld 1992; Rieseberg & Wendel 1993; Ennos *et al*. 1999). This process is the basis for a common conservation concern that species at risk will be necessarily 'swamped out' by more common congeners (Levin *et al*. 1996; Rhymer & Simberloff 1996; Vilà *et al*. 2000; Mooney & Cleland 2001; Ellstrand 2003a, b; Potts *et al*. 2003).

The genetic effects of hybridization have been studied in a limited number of rare plant genera (Ellstrand & Elam 1993) and, in several of these, genetic assimilation has been linked to the risk of decline (Brochman 1984; Rieseberg *et al*. 1989; Liston *et al*. 1990; Rieseberg & Gerber 1995; Smith *et al*. 1996; Daehler & Strong 1997; Cogolludo-Augustín *et al*. 2000). Unfortunately, an accurate assessment of asymmetric introgression is lacking from many of these analyses largely because of the limited number of hybrids available for study due to plant rarity and the lack of information on the genetic composition of hybrids based on both nuclear and cytoplasmic markers. Moreover, a relationship between the magnitude of introgression and rarity has never been demonstrated. Testing this association is important because asymmetrical introgression may have many other causes related to differential gamete production, fertilization and offspring survival (Tiffin *et al*. 2001), which may account for its presence in a number of hybridizing plant taxa (Harrison 1990; Reiseberg & Brunsfeld 1992; Rieseberg & Wendel 1993; Arnold 1997; Vilà *et al*. 2000; Mooney & Cleland 2001; Abbott *et al*. 2003; Ellstrand 2003a, b).

Red mulberry (*Morus rubra* L., Moraceae) is a wind-pollinated, dioecious, understorey tree, native to eastern North America. At the northern limits of its geographical range, in southern Ontario, it is restricted to six small populations (Fig. 1) (Wunderlin 1997; Ambrose & Kirk 2004). Hybridization with the introduced and more abundant white mulberry (*Morus alba* L.) is considered a major threat to the remaining populations (Ambrose & Kirk 2004). The hypothesis that hybridization is leading to the decline of red mulberry is based on the presence of individuals with intermediate leaf morphology in sympatric populations. However, their parentage has not been confirmed using molecular techniques and the likelihood of genetic assimilation

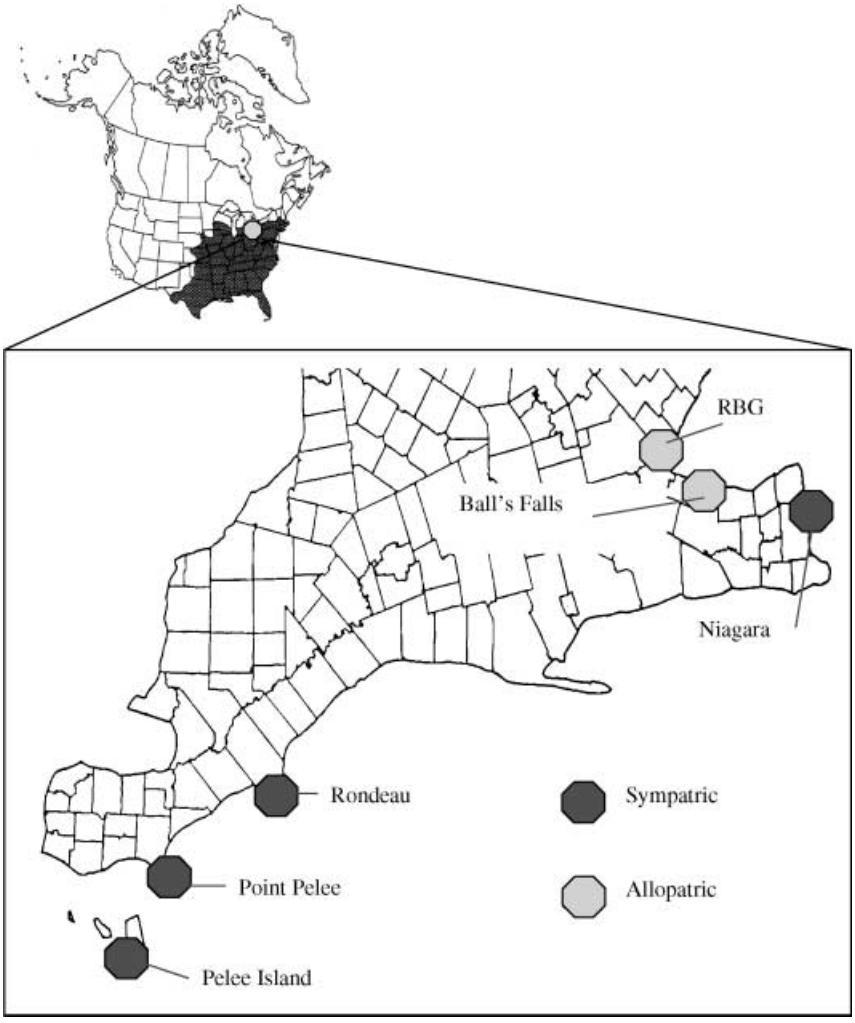

Fig. 1 Distribution of red mulberry populations in eastern North America and location of populations that contain five or more trees in southern Ontario, Canada. Two allopatric populations (hatched octagons) and four sympatric populations (solid octagons) with white mulberry used in this study are indicated.

has not been assessed. Here, we test for evidence of hybridization and asymmetric introgression using a combination of nuclear and cytoplasmic genetic markers and address the following specific questions: (i) What is the frequency of hybrids formed between red and white mulberry in natural populations? (ii) Do hybrids show evidence of asymmetrical introgression? (iii) Is the genetic composition of hybrids related to morphology? and (iv) Is the frequency and genetic composition of hybrids related to the numerical asymmetry of the parental species.

Materials and methods

Study site and sampling

The magnitude of hybridization between *Morus rubra* and *Morus alba* was estimated by sampling trees of unknown parentage from four sympatric populations (both species within 25 m of each other) in southern Ontario (Fig. 1). From each population, we sampled all putative individuals of *M. rubra* and approximately 10 putative *M. alba* or hybrid trees within a 25-m radius of each *M. rubra* tree (Table 1). This stratified sampling design ensured that sufficient numbers of the rare taxon would be included in our analysis while providing a minimum estimate of the frequency of white/hybrid mulberry in close proximity to red mulberry; however, the sampling bias applies equally across populations and is not likely to affect the relative differences of taxa among populations. The 25-m radius was not based on a known pollen dispersal distance, but because pollen dispersal is usually leptokurtic, this plot size likely captures the area of most frequent gene flow. In fact, an additional experiment showed that removal of white/hybrid mulberry from these plots had a significant effect on hybridization rates (Burgess *et al.*, unpublished).

To serve as genetic reference material, we sampled known *M. rubra* from two allopatric populations in southern Ontario and approximately 10 *M. alba* trees located a minimum of 0.5 km away (Table 1). *M. rubra* and *M. alba* leaf tissue was also sampled outside the southern Ontario range from selected arboreta, herbaria, and historical sites (Table 1). Mulberry trees were classified initially using a suite of leaf characters (measures of leaf size, shape and texture) traditionally used as field markers.

Leaf tissue was collected from each sampled individual and immediately stored at –80 °C for future genetic analysis. In addition, one medium-height leaf was randomly collected from each of the four cardinal directions (north, south, east, and west) on each tree in the sympatric populations for morphometric analysis. Of the trees of unknown parentage, all putative *M. rubra* and a random subsample of the putative *M. alba* and hybrid samples (≈ 25% of those collected around each putative red mulberry tree) were included in the genetic and morphological analysis ($N = 184$).

DNA isolation and amplification

Total genomic DNA was isolated from ≈ 80 mg of frozen leaf material using QIAGEN DNeasy Plant Mini Extraction kits yielding ≈ 30 ng/μL of DNA. Tissue was homogenized using a FastPrep Instrument (BIO 101). DNA was amplified in 10-μL reaction mixtures containing 4.4 mmol/L $MgCl_2$, 500 μmol/L dNTPs, 0.6 μmol/L RAPD primer, 25 ng (5 ng/μL) genomic DNA, Stoffel reaction buffer (10 mmol/L Tris-HCl, pH 8.3, 10 mmol/L KCl) and 0.2 unit/μL Ampli*Taq* DNA polymerase Stoffel fragment (PerkinElmer). Amplifications were performed in a PTC-100 Thermocycler (MJR Research) using the reaction protocol of DeVerno *et al*. (1998), modified with the 'touchdown' polymerase chain reaction (PCR) protocol of Gallego & Martinez (1997). Amplification products were separated electrophoretically at 100V for 1.5 h using 2% agarose gels with a Tris–boric acid–ethylenediamine tetraacetic acid (TBE) buffer

Table 1 Sources of mulberry leaf material for genetic analysis. Shown are the numbers of individuals sampled at each site for both RAPD and cpDNA (in brackets) analysis. (A) Reference leaf material was derived from allopatric populations*, historical sites, arboreta and herbaria. (B) Trees of unknown parentage were sampled in four sympatric populations in southern Ontario, Canada

Samples	Location	*M. rubra*	*M. alba*	Total
A) Reference	Royal Botanic Gardens (Niagara Escarpment Properties), ON, Canada*	7 (5)	9 (4)	16
	Ball's Falls Conservation Area, ON, Canada*	4 (1)	—	4
	Kew Herbarium, London, UK	3 (1)	1 (1)	4
	Morton Arboretum, IL, USA	1 (1)	4 (1)	5
	Holden Arboretum, OH, USA	3 (1)	—	3
	Shakertown Historic Site, KY, USA	—	2 (1)	2
	Arnold Arboretum, MA, USA	—	3 (1)	3
B) Unknown	Niagara Glen, Niagara Parks Commission, ON, Canada	—	—	21 (2)
	Rondeau Provincial Park, ON, Canada	—	—	44 (3)
	Point Pelee National Park, ON, Canada	—	—	63 (11)
	Fish Point Provincial Reserve, Pelee Island, ON, Canada	—	—	56 (9)

(45 mM Tris base, 45 mM boric acid, 1 mM EDTA) and visualized by ethidium bromide fluorescence (0.1 μg/mL) and photographed using a Bio-Rad ChemiDoc Gel Documentation System and Quantity One Quantification Software (Bio-Rad Laboratories).

RAPD analysis

Initially, reference samples of *M. rubra* (n = 18) and *M. alba* (n = 19) (Table 1) were screened using 100 randomly amplified polymorphic DNA (RAPD) primers (Nucleic Acid — Protein Service Unit, Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada — primers #1–100). Five primers [#18 (GGGCCGTTTA); #28 (CCGGCCTTAA); #13 (CCTGGGTGGA); #53 (CTCCCTGAGC); and #55 (TCCCTCGTGC)] were selected that produced clear reproducible RAPD fragments that contained species-specific markers. In total five RAPD fragments were diagnostic of white mulberry and four of red mulberry. Because an analysis based on the species-specific markers alone would provide limited information on the genetic composition of hybrid taxa found in sympatric populations, 34 additional fragments that were not species specific, but still informative, were scored for all reference material (total of 43 fragments). The reproducibility of all RAPD fragments (species specific and polymorphic) was confirmed with triplicate amplifications across three DNA concentrations (1/20: 1/10: 1/5 dilution series with controls) for all reference samples. To assess the magnitude and direction of hybridization, PCR products from individuals sampled in the four sympatric populations were then scored for the presence/absence of species-specific markers as well as the polymorphic fragments (see Appendix I for an example).

The parentage of each individual sampled was estimated using a hybrid index, modified from Hardig *et al.* (2000) (M. Morgan — program available upon request). The hybrid index of a sample of unknown parentage was estimated as a proportion (0–1) using a maximum-likelihood estimator, given the genetic constitution of the reference populations. For this analysis, data were grouped into three subsets: reference subset 1 (S1), which comprised *M. rubra* of confirmed provenance from allopatric populations, arboreta and herbaria (n = 21); reference subset 2 (S2) consisting of *M. alba* of confirmed provenance from allopatric populations, arboreta and herbaria (n = 24); and subset 3 (S3), containing all mulberry of unknown parentage from the four sympatric populations (n = 184). A small sample of red and white mulberry from sympatric populations (n = 8), whose parentage was confirmed with species-specific RAPD markers and cpDNA (Burgess *et al.*, unpublished data) were also included in S1 and S2, respectively, to provide information on the genetic characteristics of parental taxa on a local scale.

Mean standardized hybrid index scores were estimated for all reference and unknown samples. The upper 97.5% and lower 2.5% confidence limits around each standardized hybrid index score were estimated from 10 000 bootstraps using individual loci as the unit of replacement. Because estimates are based on a probability of assignment to a particular class, standardized hybrid index scores (and their confidence intervals) range between 0 and 1, indicating white and red mulberry, respectively. Index values for subsets S1 and S2 provided reference points to which all unknown samples in S3 were compared. Individuals were identified as hybrids when their lower confidence limits exceeded the upper limit for S1 and their upper confidence limits were less than the lower limit for S2.

Variation in hybrid frequencies and the mean standard hybrid index of hybrids among populations were tested using a χ^2 likelihood ratio and a one-way ANOVA with a Tukey HSD comparison of means, respectively. To meet the assumptions of normality for the F-test, standard hybrid indices for hybrids were square-root transformed (reported as back-transformed means in the Results). Correlation analyses were performed to examine relationships between *M. alba* frequency and the mean standard hybrid index of hybrids across populations. Because we were interested in the effect of parental species abundance on hybrid composition, the frequency of *M. alba* was calculated as the proportion of white + red mulberry (excluding hybrids) in each population. All statistical analyses were performed using JMP statistical software version 5.0 (SAS Institute 2002).

Chloroplast DNA sequence

To determine the direction of hybrid matings, ribulose 1,5-biphosphate carboxylase (rbcL) gene was sequenced in 42 red, white and hybrid mulberry plants from allopatric and sympatric populations using DNA extracted for the RAPD analysis. We included nine red and eight white mulberry samples from allopatric populations and 25 hybrid samples from sympatric populations, identified from nuclear hybrid index values.

Forward and reverse primers were designed to amplify a polymorphic region of the rbcL gene of the chloroplast genome for red and white mulberry. Primer design was based on the sequences of Soltis *et al.* (1990) and Albert *et al.* (1992) for the *M. alba* rbcL gene (GenBank GI: 7240336) and the sequences of Soltis *et al.* (1993) and Morgan *et al.* (1994) for the *M. rubra* rbcl gene (GenBank GI: 533039). These sequences were aligned using BLASTN 2.2.4 (NCBI 2002). Two conserved regions flanking a sequence 832 bp in length were identified as suitable for the design of forward and reverse primers. Primers, 25 bp in length, were designed to have low GC content relative to AT content (% GC = 40%) in both forward and reverse compliment priming sequences. Melting points were calculated using

the '4 × GC + 2 × AT' = Tm –5 °C method (Sambrook *et al*. 1989) confirming a Tm = 67 °C for both the forward primer: 5'-GGTTATCCGCTAAGAATTACGGTAG-3' and the reverse primer: 5'-GCTAGTTCAGGACTCCATTTACTAG-3'. Both the forward and reverse primers were checked for primer–primer and self-priming interactions as well confirmed for homologous priming using BLASTN version 2.2.4 (NCBI 2002).

All amplifications were performed in a PTC-100 Thermocycler (MJR Research). Amplifications of template DNA were performed in 50-μL reactions containing 5.0 μL 10× PCR buffer; 3.0 μL $MgCl_2$ (25 mM); 0.4 μL dNTP mix (25 mM); 1.0 μL forward primer (20 μM); 1.0 μL reverse primer (20 μM); 0.5 μL BSA (5 mg/mL); 0.4 μL *Taq* polymerase (5 U/μL); 1 μL of template DNA (30 ng/μL) and sterile ddH_2O. PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and extension for 1 min at 72 °C; final extension at 72 °C; hold at 4 °C. PCR products were cleaned for cycle sequencing using QIAquick PCR Purification Kits (QIAGEN). Cycle sequencing of cleaned PCR product was performed in 10-μL reactions containing 4.0 μL dilute (2:1) BDV3.1 (BigDye; Applied Biosystems); 1.5 μL sequencing primer (2 μM); 0.7 μL of PCR product (70 ng/μL) and sterile ddH_2O. Cycle sequencing reaction parameters were as follows: initial denaturation at 96 °C for 2 min; 30 cycles of denaturation at 96 °C for 30 s, primer annealing and extension at 60 °C for 4 min; hold at 4 °C. Sequencing was performed at the University of Guelph Molecular Supercentre (College of Biological Sciences, Guelph, Ontario, Canada) on an ABI PRISM 377 DNA Sequencer (Applied Biosystems) using BigDye Terminator (version 3.1) Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed with DNA Sequencing Analysis Software version 3.4 (Applied Biosystems). An 802-bp region of the rbcL gene was sequenced for all 42 samples for both reverse and forward primers, edited and aligned using SEQUENCHER version 3.0 (Gene Codes). Sequences were then converted to MACCLADE format in CLUSTAL_X (Thompson *et al*. 1997), and aligned using MACCLADE 4 (Sinauer Associates).

A chi-squared test was conducted to determine if the proportion of hybrid mulberry containing the red or white mulberry rbcL sequence differed significantly from a 1:1 ratio. This test was performed using JMP statistical software (SAS Institute 2002).

Morphometric analysis

Each of four leaves per individual was scanned and images imported into ©NORTHERN ECLIPSE image analysis software (Empix Imaging). Each leaf was measured for area, perimeter, the number of lobes, and the sinus depth from the tip of the first lobe. The values were then averaged for each individual. The density of abaxial and adaxial trichomes was also counted in two 1 × 10 micrometre regions located halfway along the midvein of each leaf. Mean trichome density per tree was calculated as the mean of four averages.

The leaf characters for red, white, and hybrid mulberry plants were compared within and among populations using univariate (ANOVA, Tukey–Kramer multiple comparison) and multivariate (MANOVA) analyses. A canonical discriminant function analysis was performed to graphically depict the MANOVA. Morphological characteristics of hybrids were also regressed against the hybrid index scores from the molecular analysis to determine whether hybrid morphology was related to parentage. All statistical analyses were performed using JMP software (SAS Institute 2002).

Results

RAPD analysis

In total, 43 polymorphic RAPD fragments were scored in 225 individuals. The *Morus rubra* reference plants (S1) had a mean standardized hybrid index of 0.89 (n = 21; 2.5% CL = 0.79; 97.5% CL = 0.93), whereas the *Morus alba* reference material (S2) had a mean index of 0.09 (n = 24; 2.5% CL = 0.05; 97.5% CL = 0.20). The 184 individuals of unknown parentage (S3), sampled from four sympatric populations, had a mean standardized hybrid index of 0.50 (2.5% CL = 0.43; 97.5% CL = 0.57).

Of the 184 individuals sampled from the four sympatric populations, 29% (n = 53) were classified as *M. rubra*, 18% (n = 33) were *M. alba* and 53% (n = 98) were hybrids. The relative frequency of hybrids differed significantly among populations (χ^2 = 13.46, d.f. = 6, P < 0.05) and ranged from 67% in Point Pelee to 43% in Rondeau (Fig. 2). The mean

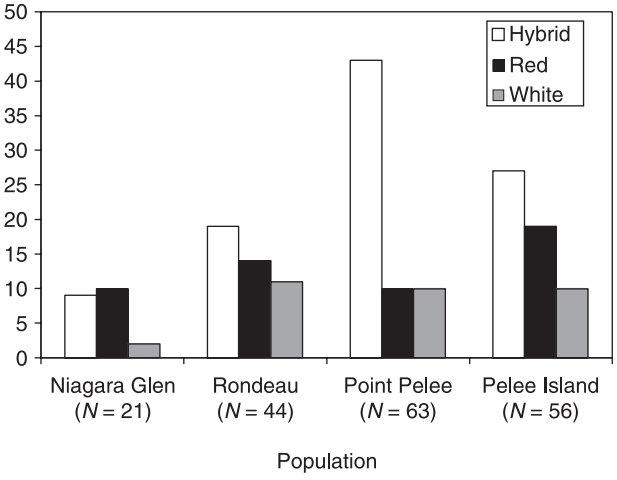

Fig. 2 Frequency of red, white and hybrid mulberry among four sympatric populations in southern Ontario, Canada, based on RAPD analysis. Hybrid frequency differed significantly among populations (χ^2 = 13.46, d.f. = 6, P < 0.05).

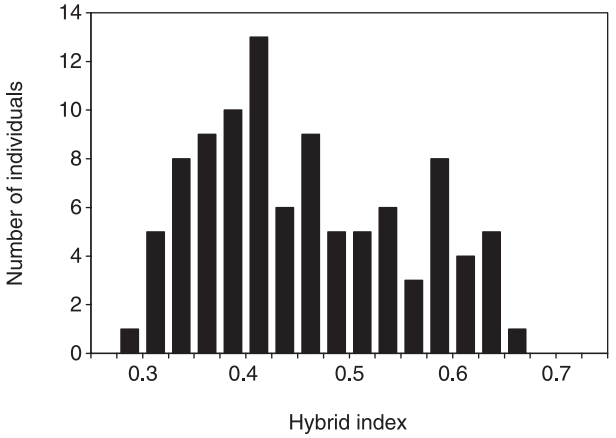

Fig. 3 Distribution of standardized hybrid index scores for 98 hybrid mulberry from four sympatric populations in southern Ontario, Canada. Values below and above 0.5 reflect an excess of white and red mulberry genome, respectively.

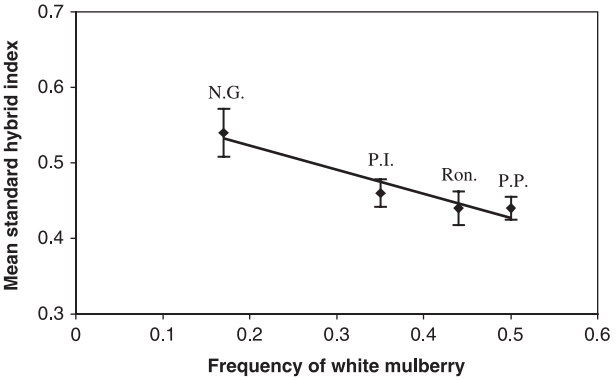

Fig. 4 The relationship between the frequency of white mulberry (as detected from the RAPD analysis) and the mean standard hybrid index of hybrids in four sympatric populations of red and white mulberry in southern Ontario. Linear regression: $R^2 = 0.93$; $F_{1,2} = 25.93$, $P < 0.05$. Population means and their standard errors are depicted for Niagara Glen (N.G.); Pelee Island (P.I.); Rondeau (Ron.); Point Pelee (P.P.). Means with different letters are significantly different based on a Tukey–Kramer post hoc comparison of means.

standardized hybrid index score for the 98 hybrids was 0.46 (97.5% CL = 0.48; 2.5% CL = 0.44; median = 0.44) (Fig. 3), indicating a disproportionately high proportion of *M. alba* genome. Expressed in different terms, 67% of all hybrids had an index score lower than 0.5, a proportion significantly different from a 1:1 ratio ($\chi^2 = 12.05$, d.f. = 1, $P < 0.001$). The mean hybrid index for hybrid individuals differed significantly among populations ($F_{3,94} = 2.9$, $P < 0.05$) (Fig. 4) and was negatively correlated with the population frequency of white mulberry when using population means ($R^2 = 0.93$; $F_{1,2} = 25.93$, $P < 0.05$) (Fig. 4) or when using raw hybrid index values ($R^2 = 0.07$; $F_{1,96} = 25.93$, $P < 0.01$). As the frequency of white mulberry increased, so did the proportion of white genome in hybrids.

cpDNA analysis

Reference samples of *M. rubra* and *M. alba* consistently differed from each other at three nucleotide sites (0.4% bp difference) (Appendix II). Trees from the sympatric populations, identified as *M. rubra* and *M. alba* from the RAPD analysis, had cpDNA sequences consistent with their respective reference samples. There was no sequence variation within these two species. Of the 25 hybrids that were identified from the RAPD analysis, 68% contained the *M. alba* rbcL sequence, while 32% contained the *M. rubra* sequence (Appendix III). The proportions were not significantly different from a 1:1 ratio ($\chi^2 = 2.72$, d.f. = 1, $P = 0.0992$).

Correlations among morphometric traits

Morus rubra, hybrid and *M. alba* plants (identified using RAPDs) differed with respect to all six morphological characters. Each taxon differed from each other for leaf area and perimeter (Fig. 5a–b; Table 2). For the characters number of lobes, sinus depth, and density of adaxial and abaxial trichomes, *M. alba* and hybrids were indistinguishable, but distinct from *M. rubra* (Fig. 5c–f; Table 2). *M. rubra* had significantly higher values than hybrid and *M. alba* plants for all morphological characters except the number of lobes, which was lower (Fig. 5c; Table 2). A MANOVA also showed significant differences among *M. rubra*, hybrid, and *M. alba* (Wilks lambda$_{10,180} = 0.33$, $P < 0.0001$). Multivariate means that best separated the three taxa in two-dimensional canonical space showed that *M. alba* and hybrid mulberry were more similar to each other than either was to *M. rubra* (93.4% of the variation was explained by canonical 1) (Fig. 6).

Individuals identified as hybrids exhibited substantial variation in morphology. Moreover, hybrid index scores

Table 2 Summary of ANOVA for differences in six leaf characters among red, white and hybrid mulberry. Results are for (A) area, (B) perimeter, (C) no. of lobes, (D) sinus depth, (E) density of adaxial trichomes, and (F) density of abaxial trichomes

Variable	d.f.	MS	*F*
A) Area† (mm)	2	20.56	87.46**
B) Perimeter† (mm)	2	3.51	48.88**
C) No. of lobes‡	2	5.41	23.96**
D) Sinus depth† (cm)	2	12.40	8.25*
E) Density of adaxial trichomes‡	2	3.84	11.20**
F) Density of abaxial trichomes‡	2	118.02	97.91**

*$P < 0.001$; **$P < 0.0001$.
†Data were log transformed; ‡data were arc sinh transformed.

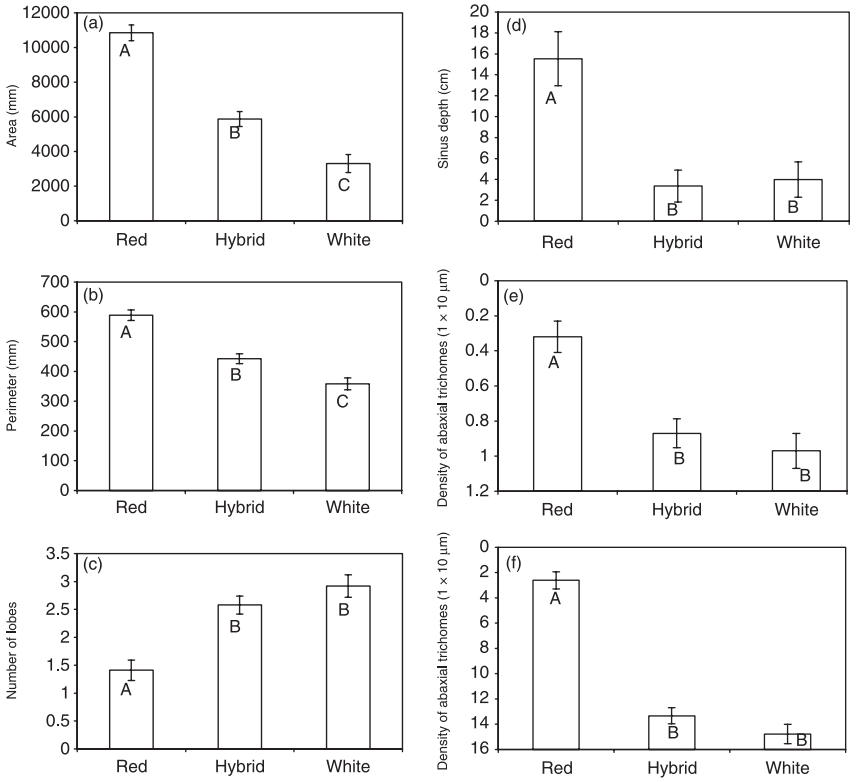

Fig. 5 Mean (± SE) values for red, hybrid, and white mulberry leaves with respect to six morphological characters: (a) area; (b) perimeter; (c) number of lobes; (d) length of sinus; (e) density of adaxial trichomes; and (f) density of abaxial trichomes. Differences were compared using a Tukey–Kramer post hoc comparison of means. Means with different letters are significantly different.

for hybrids were significantly related to area ($R^2 = 0.25$; $F_{1,67} = 21.82$, $P < 0.0001$), perimeter ($R^2 = 0.15$; $F_{1,67} = 11.69$, $P < 0.001$), density of adaxial trichomes ($R^2 = 0.08$; $F_{1,66} = 5.33$, $P < 0.05$) and density of abaxial trichomes ($R^2 = 0.30$; $F_{1,66} = 27.91$, $P < 0.0001$) (Fig. 7). When all variables were combined in a multiple regression, only the density of abaxial trichomes explained a significant proportion of variation in hybrid index ($F_{4,63} = 10.54$, $P < 0.0001$, $R^2 = 0.4$).

Discussion

Asymmetrical introgression has been documented in a number of studies of hybridization between plant species (Harrison 1990; Rieseberg & Brunsfeld 1992; Rieseberg & Wendel 1993; Arnold 1997). However, the evidence for such an effect is often incomplete and its causes are rarely attributable to the uneven abundance of parental taxa. We examined the patterns of hybridization between small populations of *Morus rubra* and the introduced *Morus alba*. These two species are widely distributed in North America, but at the northern limit of its range, in Canada, red mulberry is endangered and can vary from abundant to rare in local populations. To test for asymmetrical introgression, we determined whether hybridization had occurred, whether the genetic composition of hybrids was more similar to the abundant parent (i.e. *M. alba*) and whether the composition of hybrids was related to the local frequency of *M. alba*.

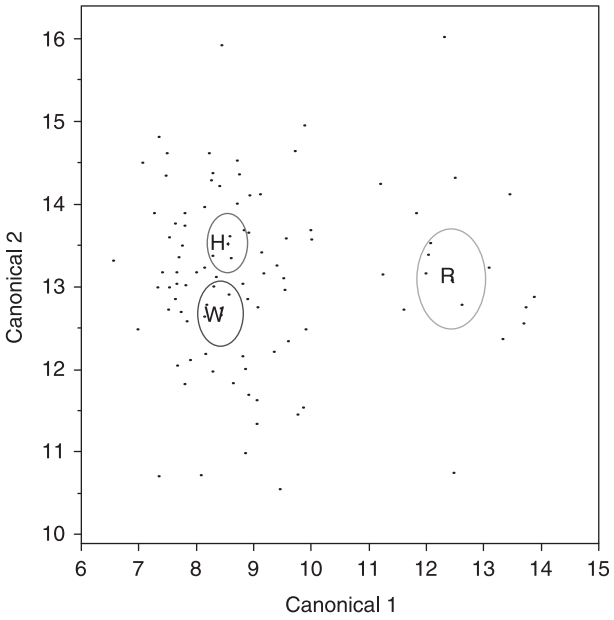

Fig. 6 Two-dimensional canonical plot displaying the points and multivariate means that best separate red (R), hybrid (H), and white (W) mulberry based on morphological analysis. Canonical variable 1 is correlated with the density of adaxial and abaxial trichomes. Leaf area, perimeter, number of lobes and sinus depth are correlated with canonical variable 2. Circles depict 95% confidence limits of the multivariate means for each taxonomic class. A MANOVA confirmed significant differences among red, hybrid, and white mulberry (Wilks lambda$_{10,180} = 0.33$, $P < 0.0001$).

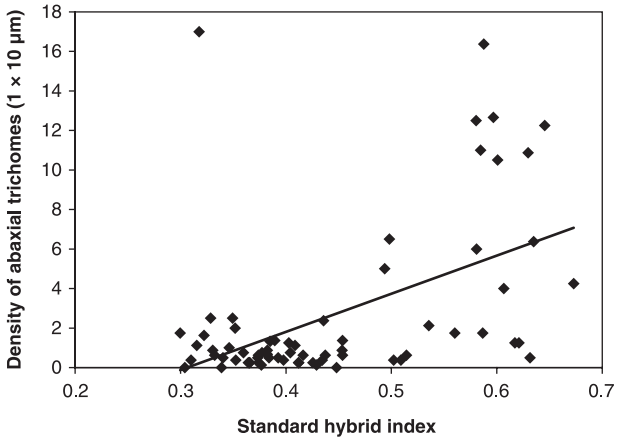

Fig. 7 Relationship between the number of abaxial trichomes of leaves and mean standard hybrid index scores for hybrid mulberry. The density of abaxial trichomes is related to parentage among hybrid mulberry ($R^2 = 0.30$; $F_{1,66} = 27.91$, $P < 0.0001$).

Based on the RAPD analysis, hybrid *Morus* was found in all four sympatric populations, and, in fact, averaged 53% of all trees sampled. Surprisingly, this estimate is high relative to most studies of rare plant taxa (Stace 1975; Ellstrand & Elam 1993; Rieseberg & Wendel 1993; Levin *et al*. 1996; Rhymer & Simberlof 1996; Arnold 1997). It is possible that this value is an overestimate of the frequency of hybrids (and underestimate of whites) because we restricted sampling to 25 m around each putative *M. rubra* in our sampling scheme. On the other hand, our estimate represents only those hybrids that were successful at establishing; hybridization rates for *Morus* may be even higher at the time of fertilization. The discrepancy with other studies may be more related to the fact that many hybridization studies involving rare plants are based on morphological comparisons rather than genetic markers. Morphological data can be notoriously difficult to interpret because of the high degree of variability within individuals (Rieseberg & Ellstrand 1993; Rhymer & Simberloff 1996; Rieseberg *et al*. 2000).

Even when molecular markers have been used to investigate hybridization with rare taxa, relatively few hybrids have been detected (Liston *et al*. 1990; Rieseberg 1991; Rieseberg & Gerber 1995; Daehler & Strong 1997). These low estimates are often associated with rarity itself and the difficulty in acquiring enough samples to evaluate the likelihood of hybridization. For example, hybridization rates in two *Spartina foliosa* populations (16% and 13%) are based on the detection of only seven and four hybrids, respectively (Daehler & Strong 1997). Similarly, only four hybrids formed the basis of hybridization rates in *Lotus scoparius* ssp. *traskiae* (10.5%) (Liston *et al*. 1990). This problem highlights the difficulty of using extremely rare species to study hybridization, and stresses the importance of systems such as *Morus*, which are not globally rare but vary widely in local abundance, for understanding the genetic effects of parental abundance.

Three independent tests for asymmetric introgression were used in this study: nuclear genetic variation based on RAPD analysis, cytoplasmic genetic variation based on cpDNA sequence, and morphological variation based on leaf characters. The RAPD analysis provided a measure of the nuclear composition of hybrids and showed that 67% of all hybrids found had a hybrid index score less than 0.5. The mean hybrid index for hybrids (0.46) was statistically less than 0.5. These results suggest that some of the hybrids are not F_1 crosses; rather they are later generation backcrosses that contain high proportions of *M. alba* genome. This conclusion was corroborated by the cpDNA analysis, which indicated that *M. rubra* and *M. alba* had fixed nucleotide differences in the rbcL gene and that most (although insignificantly) hybrid individuals exhibited the *M. alba* haplotype. Similarly, the morphological comparisons indicated that leaves of hybrid *Morus* more closely resembled those of *M. alba*. The presence of later generation hybrids is consistent with historical records, suggesting that white mulberry was introduced in the early 1600s, and the observation that generation times in this species are relatively short (< 15 years) (Cobb 1833; Rehder 1940).

The genetic bias of hybrids towards the *M. alba* parent may have arisen due to asymmetrical backcrossing to the abundant parent, which is expected when one parent is less common than the other (Ellstrand & Elam 1993). White-biased genomes may also have arisen due to the differential survival of hybrids with predominantly *M. alba* genome, perhaps because their fitness is higher than those with predominantly *M. rubra* genome. This possibility cannot be excluded based on our genetic analysis, and requires information on rates of hybridization at the time of fertilization and the relative fitness of hybrids and parents in their natural environments. In a glasshouse comparison, offspring from reciprocal *M. alba* × *M. rubra* crosses had different growth rates although no backcross treatments were conducted (Burgess & Husband 2004). Although we cannot discount the role of selection in creating genetic asymmetries in hybrids, such a mechanism cannot account for the fact that population hybrid index scores for hybrids were related to the frequency of *M. alba* plants. This lends some support to the idea that asymmetrical introgression is, at least in part, due to different parent abundances and asymmetrical backcrossing to the most abundant parent.

Asymmetrical introgression is not uncommon in hybridizing plant taxa, and has been demonstrated in numerous genera (Edwards-Burke *et al*. 1997; Wolfe *et al*. 1998; Hardig *et al*. 2000; Broyles 2002; Petit *et al*. 2003; Sweigart & Willis 2003; Palme *et al*. 2004). It has also been documented

in a number of cultivated species that hybridize with wild/weedy congeners (Ellstrand & Hoffman 1990; Abbott 1992; Ellstrand 1992, 2001, 2003a, b; Snow & Palma 1997; Ellstrand *et al*. 1999; Vilà *et al*. 2000; Mooney & Cleland 2001; Abbott *et al*. 2003; Potts *et al*. 2003). Although numerous hybridization studies do involve numerically asymmetric parental taxa (Brochmann 1984; Meyn & Emboden 1987; Sale *et al*. 1996; Smith *et al*. 1996; Albert *et al*. 1997; Galen *et al*. 1997; Gallagher *et al*. 1997; Holderegger 1998; Cogolludo-Agustín *et al*. 2000; Bleeker & Hurka 2001; Broadhurst *et al*. 2001; Caraway *et al*. 2001; Bleeker 2003) surprisingly few have adequately explored the relationship between local abundance and patterns of asymmetric introgression.

To our knowledge, only three studies have interpreted hybrid composition in terms of the local abundance of parental taxa (Levin 1975; Nason *et al*. 1992; Carney *et al*. 2000). Nason *et al*. (1992) analysed the spatial distribution of hybrids in *Iris* and showed how higher gene flow rates from the numerically superior *Iris hexagona* into *Iris fulva* can increase the distribution and abundance of *I. hexagona*-like hybrids and lead to asymmetrical introgression of the more abundant taxon into the genome of the less common. Similarly, Carney *et al*. (2000) documented asymmetric introgression between a local population of the rare *Helianthus bolanderi* and the more common *Helianthus annuus* over a 50-year period. Here, the population, which previously consisted of *H. bolanderi*, is now almost entirely composed of *H. annuus*-like hybrids and the more common parent, *H. annuus*. Although based on results from single populations, these results seem to parallel our results in *Morus* where the frequency of white-like hybrids is high across populations and the hybrid index of hybrids is negatively associated with increasing frequency of white mulberry.

What are the implications of asymmetric introgression for the persistence of red mulberry? In mulberry, as with other rare species, the conventional view is that hybridization with a more common congener will necessarily lead to genetic assimilation and local extinction (Ellstrand 1992; Levin *et al*. 1996). Indeed, our results are consistent with the process of genetic assimilation, in that red nuclear genome is now occurring in a white mulberry background, more often than the reverse. However, the likelihood of extinction will also depend on the extent of this process and its demographic impacts on the remaining red mulberry. Neither can be assessed with the data in our study. Especially critical to this evaluation, we require information on the extent to which hybridization reduces the formation (fertility cost) and recruitment (establishment cost) of red mulberry. These impacts will rest not only on the rates and directions of hybridization but also on ecological parameters such as the degree of pollen limitation and habitat differentiation between red and white/hybrid mulberry. Our results provide compelling evidence for asymmetrical introgression that is related to the abundance of parental taxa; however, we believe a combined ecological and genetic approach will be necessary to provide a comprehensive assessment of the long-term impacts of hybridization on taxa in small populations.

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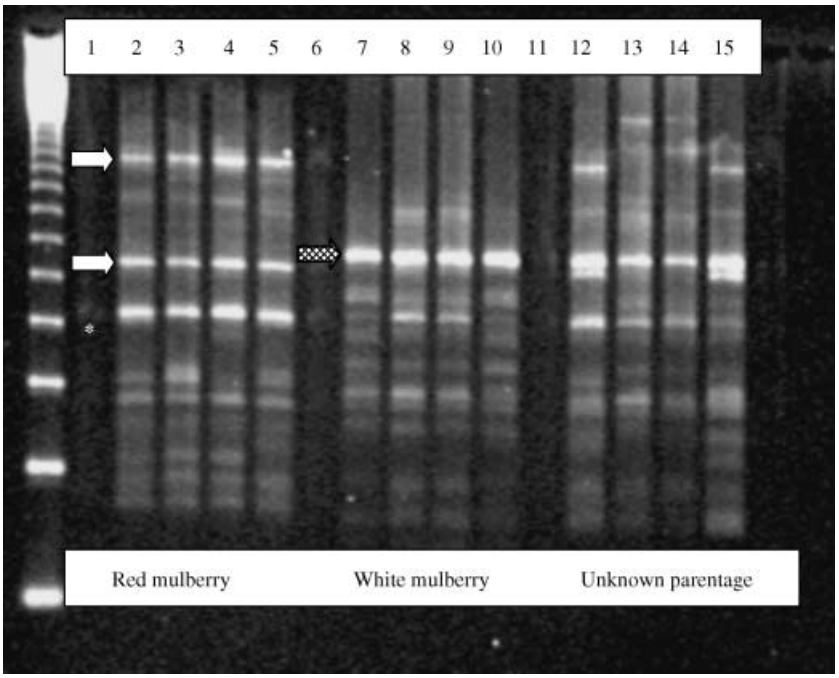


Appendix I Species-specific and poly-morphic RAPD fragments (UBC primer #28) for red and white mulberry reference samples and four samples of unknown parentage from sympatric populations. Far left lane contains a 123-bp standard. Lanes 2–5 show two species-specific markers for red mulberry 665 bp and 1170 bp in length (white arrows). Lanes 7–10 show one species-specific marker for white mulberry 695 bp in length (hatched arrow). RAPD fragments 500 bp in length were polymorphic to red and white mulberry (asterisk). Samples of unknown parentage (lanes 12 through 15) were scored for the presence/absence of these markers.

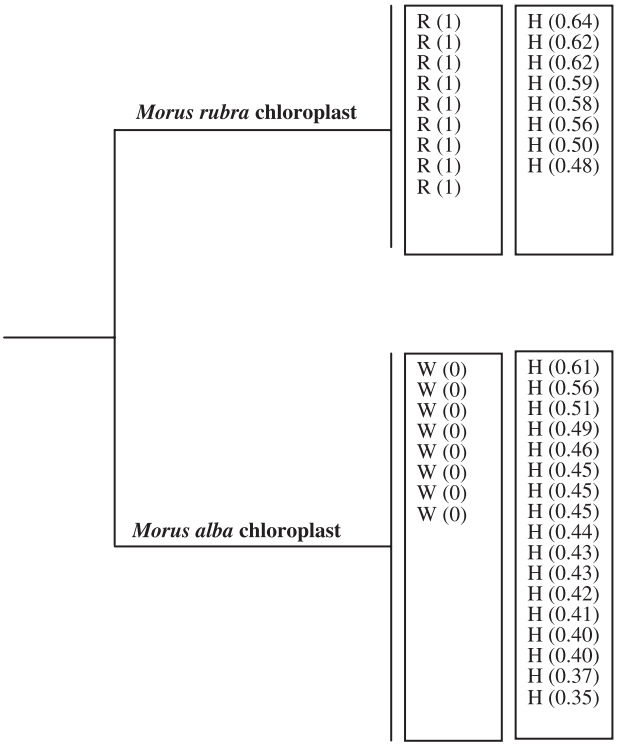
***Morus rubra* L.**

5′-TTACGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATTTTACCAAAGATGATGA
GAACGTGAATTCCCAACCCTTTATGCGTTGGAGAGATCGTTTCTTATTTTGTGCAGAAGCA
ATTTATAAATCACAGGCTGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCA
GGTACATGTGAAGAAATGAT**C**AAAAGGGCTGTATTTGCCAGAGAATTGGGAGTTCCTATC
GTAATGCATGATTACTTAACAGGAGGATTCACTGCAAATACTA**G**CCTGGCTCATTATTGTC
GAGATAATGGTCTACTTCTTCACATCCATCGTGCAATGCATGCAGTTATTGATAGACAGAA
AAATCATGGTATGCACTTTCGCGTACTAGCTAAAGCCTTACGTATGTCTGGTGGAGATCAT
ATTCACGCAGGTACTGTAGTAGGGAAACTTGAAGGGGAAAGAGAAATCACTTTAGGCTTT
GTTGATTTACTACGTGATGATTTTATTGAAAAAGATCGAAGTCGTGGTATTTATTTCACTC
AAGATTGGGTTTCTCTACCAGGTGTTCTGCCCGTGGCTTCAGGGGGTATTCACGTTTGGCA
TATGCCTGCTTTGACCGAGATCTTTGGAGATGATTCCGTACTACAATTCGGTGGAGGAACT
TTAGGACATCCTTGGGGAAATGCACCCGGTGCCGTAGCTAATCGAGTAGCTCTAGAAGCA
TGTGTAAAAGCTCGTAATGAGGGACGTGATCTTGCT**C**TTGAGGGTAATGAAATTATTCGT
GAGGCTAGTAAATGG**-3′** [802 bp]

***Morus alba* L.**

5′-TTACGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATTTTACCAAAGATGATGA
GAACGTGAATTCCCAACCCTTTATGCGTTGGAGAGATCGTTTCTTATTTTGTGCAGAAGCA
ATTTATAAATCACAGGCTGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCA
GGTACATGTGAAGAAATGAT**G**AAAAGGGCTGTATTTGCCAGAGAATTGGGAGTTCCTATC
GTAATGCATGATTACTTAACAGGAGGATTCACTGCAAATACTA**C**CCTGGCTCATTATTGTC
GAGATAATGGTCTACTTCTTCACATCCATCGTGCAATGCATGCAGTTATTGATAGACAGAA
AAATCATGGTATGCACTTTCGCGTACTAGCTAAAGCCTTACGTATGTCTGGTGGAGATCAT
ATTCACGCAGGTACTGTAGTAGGGAAACTTGAAGGGGAAAGAGAAATCACTTTAGGCTTT
GTTGATTTACTACGTGATGATTTTATTGAAAAAGATCGAAGTCGTGGTATTTATTTCACTC
AAGATTGGGTTTCTCTACCAGGTGTTCTGCCCGTGGCTTCAGGGGGTATTCACGTTTGGCA
TATGCCTGCTTTGACCGAGATCTTTGGAGATGATTCCGTACTACAATTCGGTGGAGGAACT
TTAGGACATCCTTGGGGAAATGCACCCGGTGCCGTAGCTAATCGAGTAGCTCTAGAAGCA
TGTGTAAAAGCTCGTAATGAGGGACGTGATCTTGCT**G**TTGAGGGTAATGAAATTATTCGT
GAGGCTAGTAAATGG**-3′** [802 bp]

Appendix II Sequences for red (*Morus rubra* L.) and white (*Morus alba* L.) mulberry based on an 802-bp region of the ribulose 1,5-biphosphate carboxylase (rbcl) gene of the chloroplast genome. Species-specific divergent sites are highlighted in bold.



Appendix III Neighbour-joining tree depicting the results of chloroplast DNA analysis for reference samples from nine red (*Morus rubra*) and eight white (*Morus alba*) mulberry and 25 hybrid samples from sympatric populations. Numbers in brackets indicate hybrid indices for red (R), white (W) and hybrid (H) based on a maximum-likelihood estimation of RAPD fragments.