Molecular and morphological evidence for and against gene flow in sympatric apomicts of the North American *Crepis* agamic complex (Asteraceae)[1]

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Abstract: The study of sympatric populations of closely related plant species often reveals evidence of hybridization. Mechanisms that reduce outcrossing (e.g., selfing, apomixis) may allow co-occurrence without gene flow. In this study, we describe patterns of genetic variation in two contact zones, each comprising three closely related morphological types, that key to three distinct species in the North American *Crepis* agamic (apomictic) complex. We used RAPD markers to characterize individuals from two sites: one in northern California (Sardine Lookout) and another in northwestern Oregon (Summit Road). At Sardine Lookout, we discerned a total of four multilocus genotypes, two in one species, and one each in the other two species. Our findings suggest that distinct morphological types are maintained by absolute barriers to gene flow at this site. At Summit Road, we found greater genotypic diversity, with a total of 24 genotypes across 30 individuals. One of the morphological types was clearly genetically differentiated from the other two, with no variable markers shared with other species at this site. The two remaining species showed evidence of gene flow, with no unique markers discerning them. Morphological data tend to support this conclusion, with univariate and multivariate analyses indicating a pattern of variation spanning the two species. Taken together, these patterns suggest that contact zones need not represent hybrid zones, and that apomixis can serve as an effective barrier to gene flow that may allow for stable coexistence of close relatives.

Key words: apomixis, contact zone, gene flow, hybridization, multivariate analysis, RAPD.

Résumé : L'étude de populations sympatriques d'espèces végétales apparentées montre souvent des signes d'hybridation. Les mécanismes qui tempèrent les croisements interspécifiques (autofécondation, apomixie) peuvent permettre une cooccurrence sans flux de gènes. Les auteurs décrivent des patrons de variation génétique dans deux zones de contact, chacune comportant trois types morphologiques étroitement apparentés, indiquant trois espèces distinctes dans le complexe nord-américain des *Crepis* agames (apomictique). Les marqueurs RAPD ont servi à caractériser les individus de deux sites: un situé dans le nord de la Californie (Sardine Lookout) et l'autre dans le nord-ouest de l'Oregon (Summit Road). À Sardine Lookout, on distingue un total de quatre génotypes à lieux multiples, deux dans une espèce, et un dans chacune des deux autres espèces. Ces constatations suggèrent que les types morphologiques distincts soient maintenus par des barrières absolues au flux de gènes sur ce site. À Summit Road, on trouve une plus grande diversité génotypique, avec un total de 24 génotypes pour les 30 individus. Un des types morphologiques montre une nette différenciation génétique par rapport aux deux autres, sans partager de marqueurs de variation avec les autres espèces de ce site. Les deux autres espèces résiduelles montrent des indications de flux de gènes, aucun marqueur ne pouvant les distinguer. Les données morphologiques semblent supporter cette conclusion, les analyses univariées et multivariées indiquant un patron de variation recouvrant les deux espèces. Pris dans leur ensemble, ces patrons suggèrent que les zones de contact ne représentent pas nécessairement des zones d'hybridation, et que l'apomixie pourrait servir comme barrière efficace contre le flux de gènes assurant ainsi une coexistence stable d'espèces étroitement apparentées.

Mots-clés : apomixie, zone de contact, flux de gènes, hybridation, analyse multivariée, RAPD.

[Traduit par la Rédaction]

Introduction

Over the last 20 years, molecular phylogenetic studies have become a primary focus of systematics research, providing rich new insights into relationships among diverse taxa scattered across land-plant phylogeny (e.g., Soltis et al. 2005). Nevertheless, phylogenetic studies at or near the level of species pose distinct methodological and biological chal-

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lenges (e.g., Hughes et al. 2006; Maddison and Knowles 2006). For example, groups in which speciation has been recent or rapid are less likely to have achieved the state of reciprocal monophyly, so that different gene trees, or gene and species trees may be at odds (Felsenstein 2004; Maddison and Knowles 2006). Parallel evolution (Levin 2001), mating system changes (Fishman and Wyatt 1999), and recurrent ploidy shifts (Soltis and Soltis 2005) can also produce species that are polyphyletic. Hybridization and introgression, related phenomena that act to break down phylogenetic signal following divergence, also hamper the recovery of phylogenetic relationships (Linder and Rieseberg 2004). In all of these situations, indeed in nearly all phylogenetic studies of closely related groups of species, an understanding of the processes affecting the distribution of genetic variation, and hence the clarity of phylogenetic signal, will be critical to the detection and interpretation of phylogenetic patterns.

Among the most challenging groups of species for systematic study are agamic complexes. In these groups hybridization, polyploidy, and apomixis (asexual seed formation; Richards 2003) may combine to produce often-bewildering patterns of morphological complexity. While hybridization and polyploidy alone can generate complexity, the additional occurrence of apomixis has at least two implications. First, unlike sexual polyploids, asexual polyploids will not experience the homogenizing effects of regular gene flow within ploidy levels; second, apomixis can multiply hybrid genotypes, resulting in the establishment of a wide diversity of phenotypes across the landscape. Occasional bouts of sexual reproduction between divergent apomicts or between apomicts and sexuals will generate further diversity.

That many apomicts remain capable of occasional sex is evident from studies of apomictically reproducing populations. Studies aimed at detecting evidence of recombination in clonal lineages (Burt et al. 1996; Mes 1998; Thompson 2007) typically find evidence of occasional recombination in apomicts (Mes 1998; Van der Hulst et al. 2000, 2003; Chapman et al. 2004; Kjølner et al. 2006; but see Thompson et al. 2008). The inference of occasional sex in apomictic lineages is in general agreement with limited results of molecular phylogenetic studies of agamic complexes (Whitton 1994; Holsinger et al. 1999; Wittzell 1999; Dobes et al. 2007; Noyes 2007). The results in *Taraxacum* (Wittzell 1999) are particularly illustrative: phylogenetic analysis of plastid DNA sequence variation reveals widespread patterns of intersectional plastid introgression.

The North American species of *Crepis* provide a classic example that served as an early model of the complex patterns of variation that are possible in an agamic complex (Babcock and Stebbins 1938; Stebbins 1950; Grant 1981). This group includes nine species that range from California northward to British Columbia, and eastward to Nebraska. Seven of these include both diploid sexual populations and polyploid populations, with the majority of the latter being apomictic. The remaining two species, *Crepis intermedia* Gray and *Crepis barbigera* Leib., are termed agamospecies according to Babcock and Stebbins (1938); these are known only as polyploid apomicts that combine the morphological characteristics of various diploids. Beyond the recognition of the two agamospecies, the taxonomy of the entire complex is admittedly artificial, as boundaries are blurred by the overwhelming diversity of intermediate forms that are presumed to have originated multiple times. However, diploid elements of the complex are well characterized and easily distinguished from one another, both morphologically and ecologically (Babcock and Stebbins 1938).

Apomictic polyploids in *Crepis* are considered facultatively apomictic, producing the majority of their seed asexually, while retaining the ability to produce sexual offspring (Stebbins and Jenkins 1939). Apomixis in *Crepis* is aposporous, with the embryo sac derived from a somatic cell of the nucellus, and the embryo developing from the unreduced egg cell by parthenogenesis (Richards 2003). It is likely that endosperm development occurs without fertilization (autonomously) as *Crepis* is self-incompatible and endosperm development begins before the flower heads open (Stebbins and Jenkins 1939).

Studies of plastid DNA (cpDNA) restriction site variation (Whitton 1994; Holsinger et al. 1999) and sequence variation (Whitton et al. 2008; C.J. Sears, unpublished data, 2007) support Babcock and Stebbins' (1938) view of the tangled history of the agamic complex. Within most of the agamic complex, there is little correspondence between cpDNA haplotype and taxonomic species. In addition, the restriction-site survey included collections from five sites where more than one species was growing sympatrically; surveys of bulked leaf samples from each species found no evidence of introgression at any of these sites. This pattern was somewhat surprising given the introgressive patterns found throughout the range of the complex, and suggests that the broad-scale pattern of cytoplasmic introgression may represent multiple origins of polyploids rather than ongoing gene flow among apomicts. However, because only cpDNA was examined, and because bulked leaf samples were analyzed, this earlier survey may have overlooked ongoing or recent hybridization. In addition, studies of cpDNA provide no indication of the potential for sexual reproduction within apomicts.

In this study, we examine patterns of genetic variation at two contact zones that each contain three species of *Crepis*. Our aims were to assess evidence for sexual reproduction within each species and to determine whether there is evidence of ongoing gene flow between species at these sites, i.e., whether the contact zones in fact represent hybrid zones. We analyzed pollen stainability as an indicator of potential male fertility, and variation in morphological traits (at one site) and RAPD markers for evidence of recombination and gene flow.

Materials and methods

Sources of materials

Two contact zones at which individuals of three *Crepis* species occurred were selected for this study (Table 1). Field collected leaf samples from both locations were flash-frozen and stored at –70 °C until used in DNA extraction.

The Sardine Lookout contact zone was collected in 1991 as part of an earlier broad scale study of the North American *Crepis* agamic complex. This site is located in Sierra County, California, along the road to Sardine Lookout. In the field, two morphotypes were initially distinguished at this site, with bulk and individual leaf samples and herba-

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Table 1. Collection information, pollen data and summary of genotyping data for six taxa in two *Crepis* contact zones.

Contact zone and taxon (collection number)*	No. of samples (*N* for pollen)†	Mean pollen stainability %‡ (SE)	No. of unique genotypes
Sardine Lookout, Sierra County, California	27		4
C. bakeri (153A)	4 (1)	68.0	1
C. occidentalis subsp. *occidentalis* (153A)	15 (3)	67.2 (8.3)	1
C. acuminata (153B)	8 (1)	0	2
Summit Road, Union County, Oregon	30		24
C. occidentalis subsp. conjuncta (203A)	9	11.1 (7.4)	9
C. barbigera (203B)	8	18.8 (8.0)	5
C. intermedia (203C)	13	32.5 (6.3)	10

*Collection numbers are those of J. Whitton, and refer to specimens deposited at UBC.

†No. of individuals used to assess pollen stainability, if different from sample size for molecular marker variation.

‡Individuals in which no pollen was detected are counted as having 0% stainability. Remaining values are percentages, even in cases where fewer than 200 grains were counted (1 individual each of 203A and 203C, 2 individuals of 203B).

rium vouchers collected in two sets, labelled 153A and 153B. Bulk leaf samples were used for broad-scale examination of cpDNA RFLP variation (Whitton 1994; Holsinger et al. 1999). Herbarium vouchers were later identified using keys in Babcock and Stebbins (1938) and the *Flora of North America* (Bogler 2007), and identifications were confirmed by comparison with the herbarium vouchers from JEPS cited in Babcock and Stebbins (1938). The collected samples were found to include not two but three distinct species, as 153A was a mixed collection. Among the five vouchers taken from this site, one was identified as *Crepis bakeri* Greene (herbarium voucher labelled 153A-3), three were *Crepis occidentalis* Nutt. subsp. *occidentalis* (vouchers 153A-1,-2 & -4), and one was *Crepis acuminata* Nutt. (voucher 153B) (Table 1). Previous studies of plastid DNA restriction site variation using the two bulk samples from this population detected polymorphism in bulk sample 153A, consistent with its identification as a mixed sample (Whitton 1994; Holsinger et al. 1999).

The Summit Road contact zone is located in Union County, Oregon, 14 miles (22.5 km) west of Elgin along Summit Road. At this location, three morphological types co-occur. Corresponding leaf samples and herbarium vouchers were taken from a total of 33 individuals at this site. Individuals were tentatively assigned to groups (labelled 203A, 203B, and 203C) in the field, but each voucher was later identified in the herbarium as above. After herbarium designation we identified these as follows: 10 *Crepis occidentalis* Nutt. subsp. *conjuncta* Babcock & Steb. (all labelled 203A in the field), 8 *C. barbigera* (including all 203B and some 203C), and 15 *C. intermedia* (all labelled 203C in the field) (Table 1). Morphological partitioning of samples of 203B and 203C was difficult for a number of reasons. First, these individuals, especially those assigned to *C. barbigera*, are not typical representatives of the taxon. Second, while keys suggest unambiguous assignment to *C. barbigera* or *C. intermedia*, the overall similarity in their habit suggests that they may be conspecific or represent a hybrid swarm. However, it should be noted that this level of taxonomic ambiguity is not unusual in the *Crepis* agamic complex, though in most cases this is because of between- rather than within-population, variation.

Pollen stainability

We assessed pollen stainability for all herbarium vouchers. Pollen fertility often is dramatically lower in apomicts (Richards 2003) and can be a useful indirect indicator of apomixis, as well as indicating the potential for sexuality through male function (Nybom 1985). Pollen stainability in lactophenol (cotton blue) was used as an indication of male fertility (Maneval 1936; Nybom 1985; Mayer 1991). Anthers were placed on a glass slide with a drop of water, torn with a dissecting needle to release pollen grains, covered with a cover slip and allowed to hydrate for 30 min. A single drop of lactophenol was then added and the pollen grains stained for 10 min prior to examination under a compound microscope at 400× magnification. When available, a minimum of 200 pollen grains was scored, with only regularly shaped pollen with deeply, fully staining cytoplasm counted as viable. Irregularly shaped grains and those with unstained or condensed cytoplasm were counted as nonviable.

Molecular markers

DNA was isolated following the CTAB protocol of Doyle and Doyle (1987) from approximately 1 g of dried leaf material. An aliquot of each sample was further purified using an Elu-Quick kit (Whatman Inc., Florham Park, N.J.), according to the manufacturer's protocol. Purified DNA samples were quantified on a DNA fluorometer (Hoefer Scientific San Fransisco, Calif.) and standardized to 10 ng/μL by dilution with TE buffer (10 mmol/L Tris, 1 mmol/L EDTA).

Based on screening of four to five individuals per population with UBC RAPD primer set 1 (primers 1–100; University of British Columbia NAPS Facility, Vancouver, B.C.), 20 primers that yielded clear, reproducible banding patterns were selected for surveys of all individuals. Each of the 20 primers selected produced one to five scorable bands. Repeatability of amplification patterns was assessed for all markers that appeared at low frequency, or that were absent in only one or two individuals per morphotype. This was done by repeating the amplification using both half and twice the original amount of DNA template for the individuals in question and for two individuals originally scored as

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possessing the band in question. Homology of bands of similar sizes that occurred in different morphotypes at a single locality was assessed by gel excising and purifying the fragment from two individuals per morphotype (where the frequency of the band allowed) using the Elu-quick method (Whatman Inc.). Aliquots of the purified bands were then digested with three restriction enzymes (*HeaII*, *HinfI*, *TaqαI*, New England Biolabs, Pickering, Ont.) and the products separated on 1.5% agarose gels in 0.5× TBE buffer. Concordant patterns of digested fragments were taken as evidence of band homology. It should be noted that RAPD profiles from the two localities were not compared, i.e., homology was only assessed within each locality.

Analysis of molecular marker data

Parsimony analysis

In the absence of meiosis and recombination, genomes of individuals sharing a most recent common ancestor act as a single linkage group (Richards 2003). Genetic variation in such a lineage arises solely from mutation, and as a result, relationships among genotypes can be represented as a bifurcating tree (in the absence of homoplasy among such closely related genotypes). If recombination has occurred since divergence from the most recent common ancestor, covariation among loci and between individuals will be reduced and parsimony analysis of multilocus genotypes will tend to produce a comb-like structure, e.g., in strict consensus trees (Burt et al. 1996; Clement et al. 2000).

Binary RAPD data sets were imported into MacClade, version 3.05 (Maddison and Maddison 1992) and unique genotypes identified and duplicates removed from each data set. The data sets were analyzed using PAUP*, version 4.0b (Swofford 2003) under the maximum parsimony criterion with heuristic search using 1000 replicates and tree bisection–reconnection (TBR) branch swapping on best trees only. The number of most parsimonious trees, their length and homoplasy index (HI) was recorded.

We tested for significant phylogenetic structure using permutation tail probability (PTP) tests (Faith and Cranston 1991), implemented in PAUP* using 100 permutations of the data. Each randomized data set is subjected to parsimony analysis and the set of trees is used to generate a null distribution of tree lengths against which the original tree length is tested for evidence of significantly shorter tree length.

Character compatibility analysis

Character compatibility analysis has become a common method for inferring the role of recombination in producing patterns of genotypic diversity among multilocus genotypes in asexual lineages (Mes 1998; Ceplitis 2001; Eckert et al. 2003; Houliston and Chapman 2004; Thompson et al. 2008). Based on the character compatibility approach originally described for detecting phylogenetic signal (Le Quesne 1969), character compatibility analysis rests on the principle that pairs of loci should have fully compatible variation in the absence of recombination (and recurrent mutation). For example, if the ancestral condition for a pair of binary loci is (00), then assuming the lineage is clonal, only two of the remaining three combinations of character states might be expected (e.g., 01 and 11). Observing the fourth combination (in this case 10) is evidence of incompatibility, and suggests the action of recombination. In the absence of information about ancestral character states, incompatibility is nonetheless inferred when all four combinations of two binary character states are observed. In compatibility analysis, the number of incompatibilities (the matrix incompatibility count, MIC) between each pair of multilocus genotypes is tallied. Genotypes with the highest MIC values are then successively removed until a fully compatible set of genotypes remains, providing insight into the relative role of recombination.

We conducted compatibility analysis in a subset of species (see below) using the JACTAX program in PICA version 4.0 (Wilkinson 2001). The contribution of each genotype to overall matrix incompatibility is determined by genotype jack-knifing. This involves successive removal of genotypes with the highest MIC and their replacement with a resampled genotype. In this way the contribution of each genotype to the overall MIC is determined. The genotype with the highest MIC is removed and the analysis repeated until the MIC = 0. Phylogenetic analysis and PTP were rerun on the fully compatible set of genotypes in PAUP* as described above.

Analysis of morphological variation at Summit Road

Results of the molecular marker analysis (see below) support the view that gene flow among species only occurs at the Summit Road locality. We therefore sought to further characterize patterns of variation with an analysis of morphological variation at this site, using the 33 voucher specimens collected along with DNA samples (note that the final molecular data set includes only 30 samples; 3 samples were discarded because of poor yield or poor quality). Nineteen quantitative characters were scored on all vouchers of the three species present at this site (Table 2). Means and standard deviations were calculated for all variables for each taxon, and compared using analysis of variance (ANOVA). Tukey HSD post hoc tests were conducted to test for significant differences between the means ($p < 0.05$).

Multivariate analysis was used to summarize pattern of variation over all traits. Principal components analysis (PCA) was conducted on all three species in SYSTAT version 4.0 software (Systat Software Inc., Richmond, Calif.).

Results

Sardine Lookout

Pollen stainability

Because only five vouchers were available from Sardine Lookout, it is difficult to draw conclusions about potential pollen fertility at this site. Nonetheless, some stainable pollen was obtained from four of five voucher specimens, ranging from 52%–69% stainability (mean 67%–68%). Pollen was not detected in sampling from 10 florets of the single voucher of *C. acuminata* (Table 1).

Genotypic diversity

We detected a total of four genotypes based on 90 variable markers in 27 samples collected from the Sardine Lookout site. The set of 19 plants originally collected as

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Table 2. Results of ANOVA on morphological variation in voucher specimens from three species present at the Summit Road site.

Character	*C. occidentalis* subsp. *conjuncta* (*N* = 10)	*C. intermedia* (*N* = 15)	C. *barbigera* (*N* = 8)
Plant height (mm)*	162.8±24.5 (130–218)a	525.0±78.6 (420–632)b	426.0±46.1 (368–490)c
No. of outer involucral bracts*	3.9±0.8 (3–5)a	4.7±0.7 (4–6)b	5.9±0.4 (5–6)c
No. of achenes*	10.1±2.1 (7–13)a	12.9±3.1 (8–19)b	20.8±3.0 (17–27)c
Height to first branch in stem (mm)*	38.8±17.6 (15–77)a	340.3±75.2 (195–455)b	157.8±77.5 (85–355)c
No. of inner involucral bracts*	7.8±0.4 (7–8)a	8.5±1.4 (7–12)a	11.6±1.2 (9–13)b
Lobe length (mm)*	14.7±3.5 (8–20)a	16.9±5.5 (5–28)a	25.9±12.5 (2.5–43)b
Petiole length (mm)*	37.6±14.5 (23–75)a	72.7±27.3 (5–115)b	69.6±37.8 (7–130)b
No. of heads per stem*	8.3±1.57 (5–10)a	13.0±5.8 (6–27)b	10.5±1.5 (8–13)b
Blade length (mm)*	85.0±14.6 (66–118)a	123.5±31.0 (75–196)b	119.5±45.0 (13–155)b
Lobe width (mm)*	5.4±1.6 (3.0–8.0)a	2.8±0.9 (1.2–4.5)b	2.4±0.3 (2.0–3.0)b
Ligule length (mm)*	36.75±27.9 (10–72)a	60.2±16.2 (11–76)b	55.6±5.2 (47–65)b
Leaf blade width (mm)*	6.0±2.2 (4–11)a	12.2±3.3 (8–18)b	7.4±4.9 (4.7–19)a
Length of inner involucral bracts (mm)*	12.3±1.1 (10.5–14.0)a	10.7±1.1 (8.6–12.4)b	12.2±0.7 (11.3–13.0)a
Length of outer involucral bracts (mm)*	3.5±0.9 (2.3–5.0)a	2.3±0.5 (1.8–3.2)b	3.4±0.7 (2.9–5.0)a
Pappus length (mm)*	8.5±0.48 (8–9)a	7.6±1.0 (6.2–10.0)b	8.0±0.52 (7.2–9.0)a
Corolla tube length (mm)*	6.6±1.5 (5.2–9.6)a	4.8±0.9 (3.0–6.0)b	5.8±1.1 (4.5–7.9)ab
Ligule width (mm)	3.5±0.6 (2.5–4.2)	3.3±0.4 (2.6–4.2)	3.4±0.6 (2.3–4.2)
Theca length (mm)	4.2±1.4 (2.3–5.4)	4.4±1.3 (2.3–6.5)	3.7±0.7 (3.0–5.1)
Stigmatic lobe length (mm)	2.5±0.4 (1.9–3.4)	2.8±0.5 (1.8–3.6)	2.7±0.4 (2.1–3.2)

Note: Different letters following the values indicate significantly different means.
*Traits with significant difference according to ANOVA ($p < 0.05$)

153A included just two genotypes, one detected in 15 plants and the other in 4. These genotypes differ in the presence/absence of 44 markers. The morphological voucher identified as *C. bakeri* matched the genotype shared by four individuals, while vouchers of *C. occidentalis* subsp. *occidentalis* matched the alternative genotype. The eight samples of *C. acuminata* (153B) included two genotypes, one shared by seven individuals, and one detected only once. These two genotypes differ by five markers. Given the lack of variation within each taxon, phylogenetic and matrix incompatibility tests were not applied.

Summit Road

Pollen stainability

Individual plants and species at Summit Road varied in the production of stainable pollen (Table 1). In 12 of 30 vouchers examined, no pollen was detected, despite examination of at least 10 florets from open heads. Apparent pollen sterility was most prevalent in *C. occidentalis* subsp. *conjuncta*, with seven of nine plants producing no pollen, followed by *C. barbigera* with five of eight plants producing no pollen. All but one of the 13 samples of *C. intermedia* produced pollen, although mean pollen stainability was relatively low, at 32.5%.

Genotypic diversity and structure

After homology and repeatability assessment, 70 variable RAPD loci were included in the final data set for 30 individuals at the Summit Road location. These markers distinguished 24 multilocus genotypes in total, with each species including multiple genotypes, ranging from 9 genotypes in 9 samples of *C. occidentalis* subsp. *conjuncta*, to 10 genotypes in 13 samples of *C. intermedia*, and 5 genotypes in 8 samples of *C. barbigera*. All but three of the 28 markers present in *C. occidentalis* subsp. *conjuncta* were absent from the other two species. Samples of these later two shared numerous bands, including 26 fixed and 8 variable bands. Eight bands were unique to samples of one or the other of *C. intermedia* or *C. barbigera*, but only one of these was fixed in one species and absent in the other. Because of the pattern of shared polymorphism between samples of *C. intermedia* and *C. barbigera*, we pooled these samples in phylogenetic and character compatibility tests.

Parsimony analysis of nine samples of *Crepis occidentalis* subsp. *conjuncta* produced 24 most parsimonious trees of length 17 with an HI = 0.18. The strict consensus tree is completely unresolved (Fig. 1A), and the PTP test is nonsignificant ($p = 0.85$), indicating a lack of phylogenetic structure in these data. The strict consensus of 102 most parsimonious trees from the *C. barbigera* – *C. intermedia* data set has three resolved nodes, with a length of 32 steps and an HI = 0.46 (Fig. 1B). The PTP test for these data is significant, with $p = 0.01$.

Character compatibility analysis indicated that 4 of 9 genotypes of *C. occidentalis* subsp. *conjuncta* must be removed to reduce the MIC count to zero, and 6 of the 15 genotypes in the *C. barbigera* – *C. intermedia* samples. As expected, parsimony analysis of each reduced data set (after trimming incompatible genotypes) produces a single most parsimonious tree with an HI = 0 and several resolved nodes. However, when a PTP test is conducted on these reduced data sets, the tree for samples of *C. occidentalis* subsp. *conjuncta* still does not have significant phylogenetic structure ($p = 0.41$), while that for *C. barbigera* – *C. intermedia* does ($p = 0.05$). The lack of significance of the PTP test may reflect the lower number of polymorphic markers in this data set (Thompson et al. 2008).

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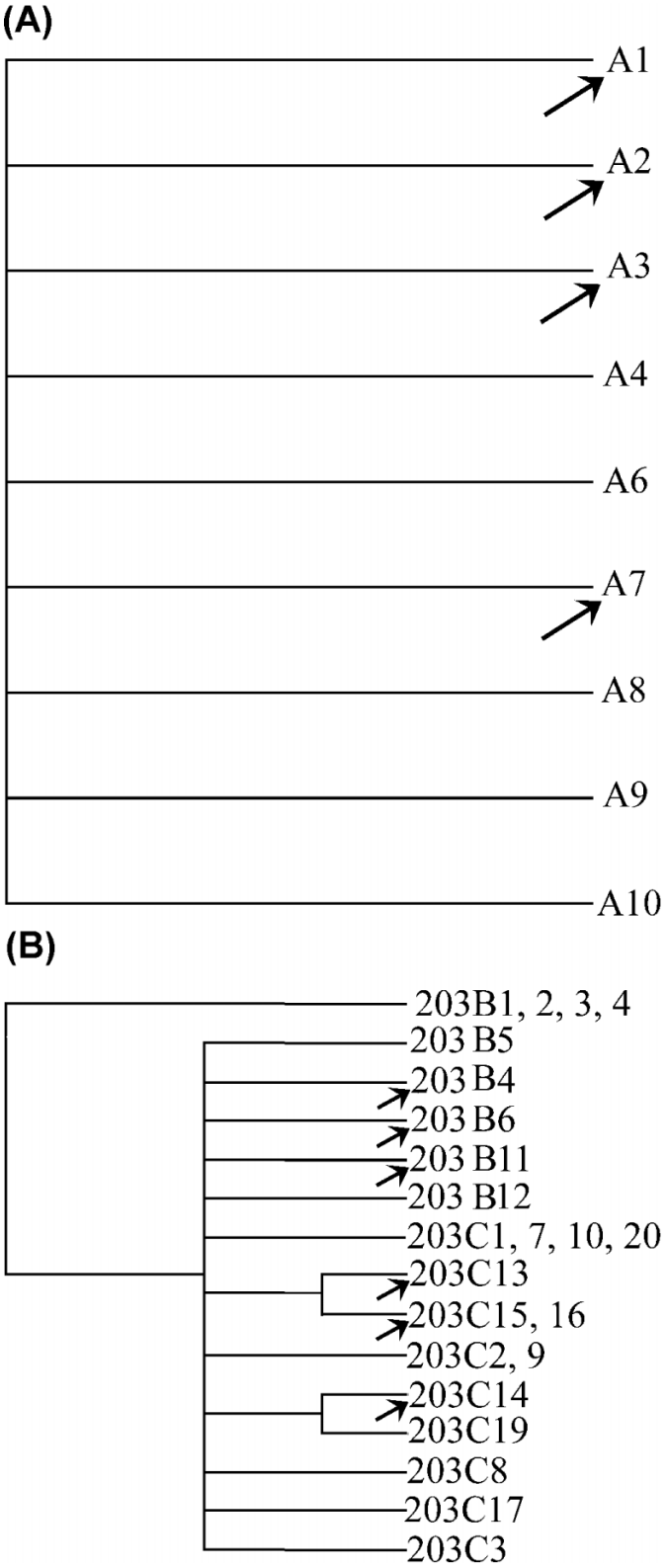
Fig. 1. Strict consensus trees from the analysis of unique multilocus genotypes. (A) *Crepis occidentalis* subsp. *conjuncta* (203A). (B) *Crepis barbigera* (203B) and *C. intermedia* (203C). Arrows indicate genotypes that when removed (after compatibility analysis) lead to most-parsimonious trees that are fully resolved.



Morphological analysis

A number of morphological traits displayed significantly different means across species at Summit Road. Plant height, number of outer involucral bracts, number of achenes per head, and height on stem of the first split differed significantly between all species ($p < 0.05$) at Summit Road. When the morphologically similar *C. barbigera* and *C. intermedia* are compared, an additional six character means differ significantly between these two taxa (Table 2). The number of inner involucral bracts and number of achenes are diagnostic characters in the keys used to place vouchers into groups (Babcock and Stebbins 1938; Bogler 2007).

The first two components of the PCA (Fig. 2) explain 45% of the variation in morphological traits measured (28% and 17% consecutively). The first PC axis separates the samples into two groups, one comprising all samples of *C. occidentalis* subsp. *conjuncta*, and the other including *C. barbigera* and *C. intermedia*. Plant height, petiole length, and blade length load heavily along this axis. The second PC axis provides separation of samples designated as *C. barbigera* and *C. intermedia*, with some overlap between clusters. Number of inner involucral bracts, number of achenes, and blade width are important variables along PC 2.

Discussion

Physical proximity of closely related species is often associated with hybridization, and it would seem reasonable to expect this in groups such as the agamic complex of *Crepis*, hypothesized to be built upon a reticulate history involving hybridization and allopolyploidy. However, in the cases reported here, we find that co-occurrence need not lead to gene flow, and that apomixis can indeed serve as an effective barrier to gene flow.

At Sardine Lookout, three distinct morphological types appear to be completely reproductively isolated from one another as a result of asexuality. Despite the availability of at least some pollen and overlapping flowering times, no gene flow and no evidence of recombination was detected. Patterns of molecular marker variation suggest that three distinct colonization events have resulted in co-occurrence of these closely related yet taxonomically distinct entities without reproductive interference. This outcome was partially predicted from results of an earlier broad-scale cpDNA survey (Whitton 1994; Holsinger et al. 1999), which reported that bulk samples of 153A (a mixed sample of *C. bakeri* and *C. occidentalis* subsp. *occidentalis*) and 153B (*C. acuminata*) differed by 17 cpDNA restriction-site mutations, with no evidence of within-sample polymorphisms for this set of mutations (which would be observed as co-dominance of shared restriction profiles in Southern blots).

The situation at Summit Road is somewhat more complex, both in the apparent occurrence of apomixis and the evidence of incomplete reproductive barriers in samples of *C. barbigera* and *C. intermedia*. While samples of *C. occidentalis* subsp. *conjuncta* are genetically and morphologically distinct from those of *C. barbigera* and *C. intermedia*, this latter set of samples is genetically diverse, with overlap-

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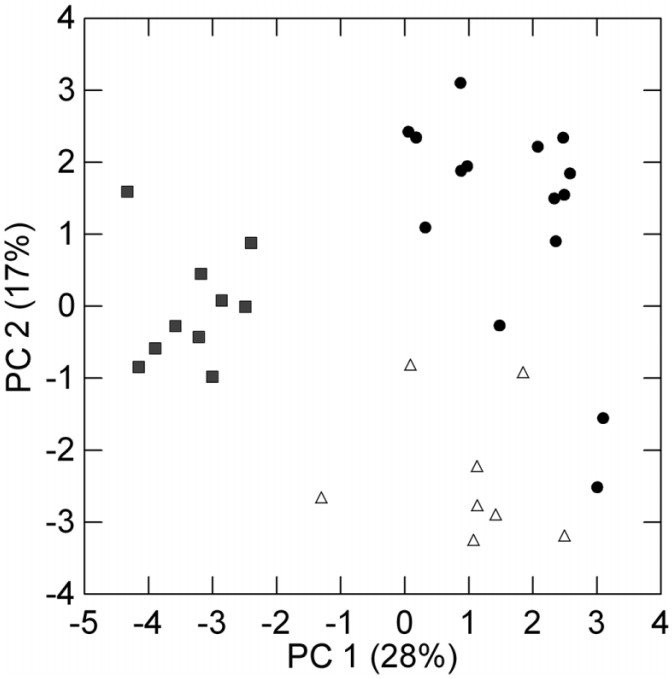
Fig. 2. Plot of Principal Components axes 1 and 2 from the analysis of morphological variation of vouchers from the Summit Road location. ■, *C. occidentalis* subsp. *conjuncta*; ●, *C. intermedia*; Δ, *C. barbigera*.



ping patterns of variation suggestive of possible gene flow. Results of the PCA of morphological data (Fig. 2) reveal that these two species form two nearly discrete, but partly overlapping clusters. Based on molecular data, both phylogenetic tests and compatibility analysis indicate that recombination has influenced patterns of genotypic diversity within this set of samples. This is intriguing because many of the vouchers examined appeared to produce no pollen, or produced pollen in quantities below the threshold of detection of our methods, a phenomenon that has been associated with autonomous apomixis, in which selection may not favor the retention of pollen fertility. Nonetheless, it appears that sufficient pollen is available for fertilization of some ovules to occur.

Other studies have found similar indications of recombination in apomictic populations, including apparently pollen-sterile populations. Thompson et al. (2008) found evidence of recombination in a population of Easter daisy (*Townsendia hookeri* Beaman) (Asteraceae) that has aborted anthers. In this case, it was hypothesized that gene flow may have involved unsampled sexual diploids, as apomictic *T. hookeri* is usually tetraploid, but both triploids and tetraploids were detected at this site.

Although our data suggest a role for recombination in this population, it should be noted that this does not appear to involve samples of *C. occidentalis* subsp. *conjuncta*, despite the fact that these too show evidence of recombination. Thus, it appears that not all taxa within the complex are capable of interbreeding, at least at the sampled sites.

We can envision two distinct scenarios that could yield the patterns involving the specimens identified as *C. barbigera* and *C. intermedia* at Summit Road. One possibility is that these two species have formed a hybrid zone at this site. Under this scenario, the two species independently colonized this site, with hybridization between the two types resulting in the establishment of the complex patterns of variation. An alternative scenario is that segregation of morphological variation among the offspring of an apomictic hybrid lead us to recognize two species at poles in the morphological spectrum. Numerous studies have shown experimentally that an F_2 population from an interspecific cross can recover complex phenotypes that resemble parental types in many or most of their key morphological features (e.g., Bradshaw et al. 1995; Nagy 1997). Given the hypothesized allopolyploid origin of both *C. barbigera* and *C. intermedia*, and the overlap in their range of phenotypes, a single round of recombination could produce a set of progeny that display the key characteristics of more than one parental species. A range of mechanisms could be involved in increased phenotypic variation among progeny, including segregation of allelic variants, changes in allele dosage, gene interactions or expression, with possible additional effects due to ploidy or aneuploid changes (Chen 2007). In the case of the interactions between *C. barbigera* and *C. intermedia*, both species are known only as polyploids, but both also are described as varying in ploidy. Chapman and Brown (2001) described a similar hypothesis for the "thawing of frozen variation" for the origin of variation in *Pilosella officinarum* F.W. Schultz & Sch. Bip. (Asteraceae) in New Zealand. They note that phenotypic variation in a range of *P. officinarum* localities is suggestive of segregation. Because of their probable relatively recent colonization history, and the generally low levels of genetic variation, observed phenotypic variation is best explained by the action of sex and recombination releasing variation held in asexual hybrid types. We cannot distinguish between these hypotheses for the origin of complex variation with the available data from Summit Road. It would be highly informative to examine morphological and molecular variation in sets of progeny from a number of maternal plants. However, members of the North American *Crepis* agamic complex are long-lived perennials with strong seed dormancy (C.J. Sears, personal observation) and we have yet to succeed in achieving high degrees of germination, with no individuals surviving to flowering.

In addition to providing an opportunity to investigate genetic interactions of close relatives, these sympatric localities raise a number of interesting questions about the ecological conditions and tolerances that allow coexistence. Do the apomicts of this complex represent stages in the frozen-niche variation model (Vrijenhoek 1979, 1984) that are sufficiently ecologically equivalent that coexistence depends mainly on colonization dynamics and demographic interactions (e.g., Hubbell 2005)? Or is it that apomicts are ecologically divergent, but more likely to co-exist because they are reproductively isolated from one another (Levin 1970; Johansson and Ripa 2006)? Sexually-reproducing close relatives may suffer from reproductive interference from heterospecific pollen, which could hamper coexistence (Armbruster and Herzig 1984; Kuno 1992). Studies aimed at uncovering the ecological tolerances of independently derived sympatric apomicts would greatly contribute to our understanding of the origin and maintenance of contact

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zones and provide insights into the factors that may limit sympatry.

Our results suggest that the complex phylogenetic patterns detected in broad-scale studies of agamic complexes do not necessarily imply that gene flow among apomictic lineages is ongoing wherever such lineages co-occur. However, this result is not necessarily surprising. Indeed, the time scale over which agamic complexes such as the North American *Crepis* have developed are vastly different (e.g., a hypothesized Pliocene origin for the North American *Crepis*, Babcock and Stebbins 1938), from the scale of tens to perhaps hundreds of years that we are likely sampling for the individual localities in our study. Nonetheless, as we have found here, the broad-scale patterns of complexity noted in phylogenetic studies may occasionally be reflected in the detection of local patterns of gene flow.

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