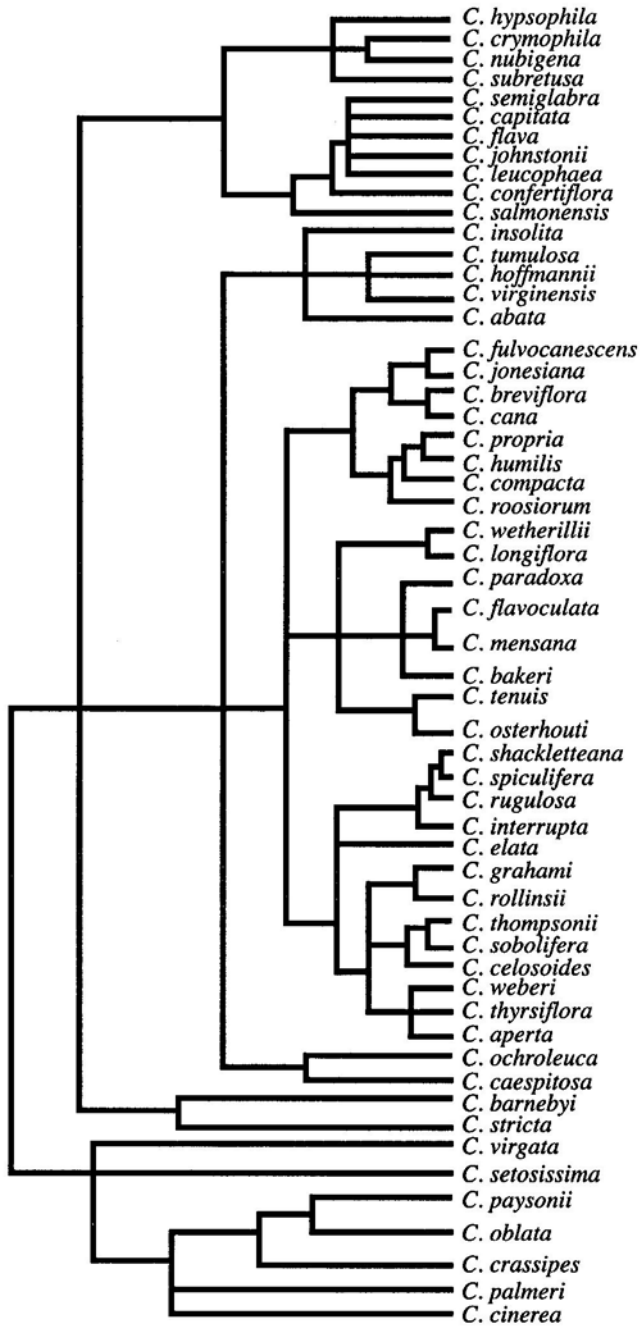
Figure 11. Relationships among species of the Oreocarya as proposed by Higgins (1971).

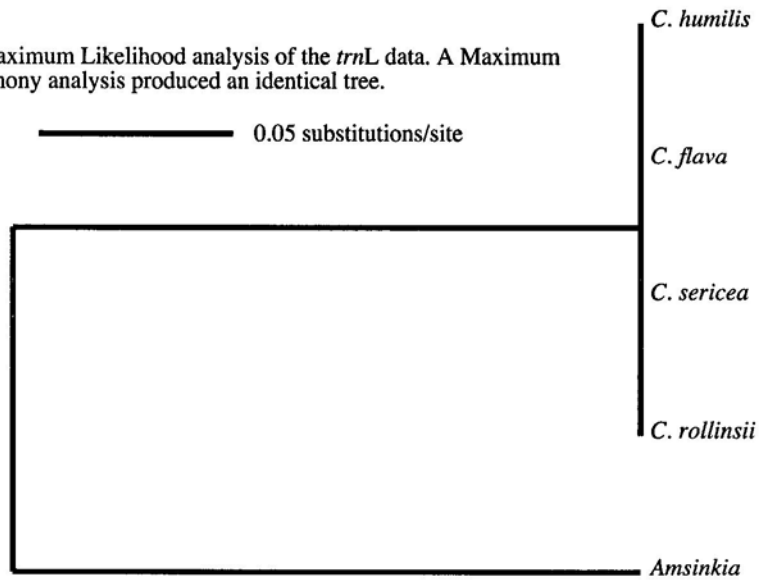


independently from several different perennial ancestors, while he presumes that the Oreocarya are monophyletic.

The primary objective of the study presented here was to use quantitative DNA data to develop a phylogeny of the perennial species of Oreocarya found in and around the Colorado Plateau region of eastern Utah, western Colorado, and northern Arizona. Additionally, several annual species, along with one species of *Amsinkia*, will be sampled to determine an appropriate outgroup for the Oreocarya, thereby testing the hypothesis that the annuals are derived from the perennials. Initially a series of introns and spacers were surveyed for their potential as phylogenetically informative markers. Rapidly evolving DNA in the ITS (internal transcribed spacer), ETS (external transcribed spacer), *trnL*, adh, *rps*, and *waxy* regions were examined among several perennial *Cryptantha* species. None of the regions surveyed showed variability adequate to resolve the relationships among species of Oreocarya (Figure 12, Figure 13, Table 6, Table 7). Since these commonly used species-level genetic markers proved to be non-informative, I reasoned that an approach more commonly used at the population level may be appropriate. Accordingly, I decided to use amplified fragment length polymorphism (AFLP) techniques. AFLPs typically provide a large number of variable characters suitable for resolving relationships at fine taxonomic levels (Hill *et al.* 1996, Kardolus 1998, Giannasi *et al.* 2001). Recent studies have also established the utility of AFLP data for the assessment of interspecific relationships (Hill *et al.* 1996, Kardolus *et al.* 1998, Aggarwal *et al.* 1999, Caicedo *et al.* 1999, Giannasi *et al.* 2001). Here I present a phylogeny based on AFLP data for perennial members of *Cryptantha* in the Section Oreocarya. I then combined this phylogeny with species specific substrate preferences

Figure 12. Preliminary analyses of *trnL* and ETS data. Note the low resolution within the Oreocarya in both trees. The only bootstrap support is for a monomophyletic Oreocarya.

A. Maximum Likelihood analysis of the *trn*L data. A Maximum Parsimony analysis produced an identical tree.



B. Maximum Likelihood analysis of the ETS data. This tree is identical to one of the 5 most parsimonious trees. Bootstrap values greater than 50 are shown.

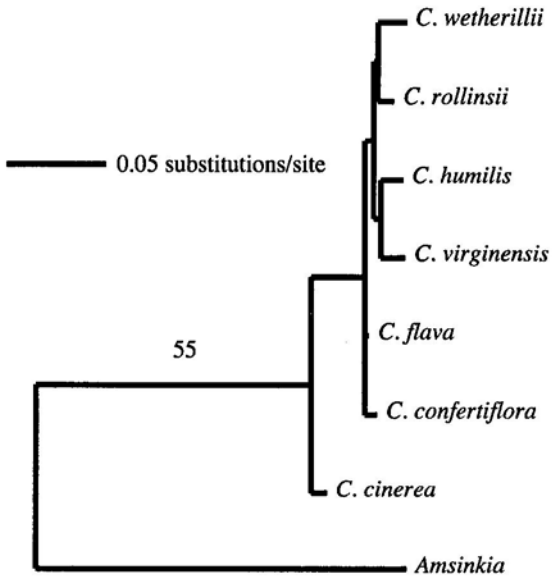
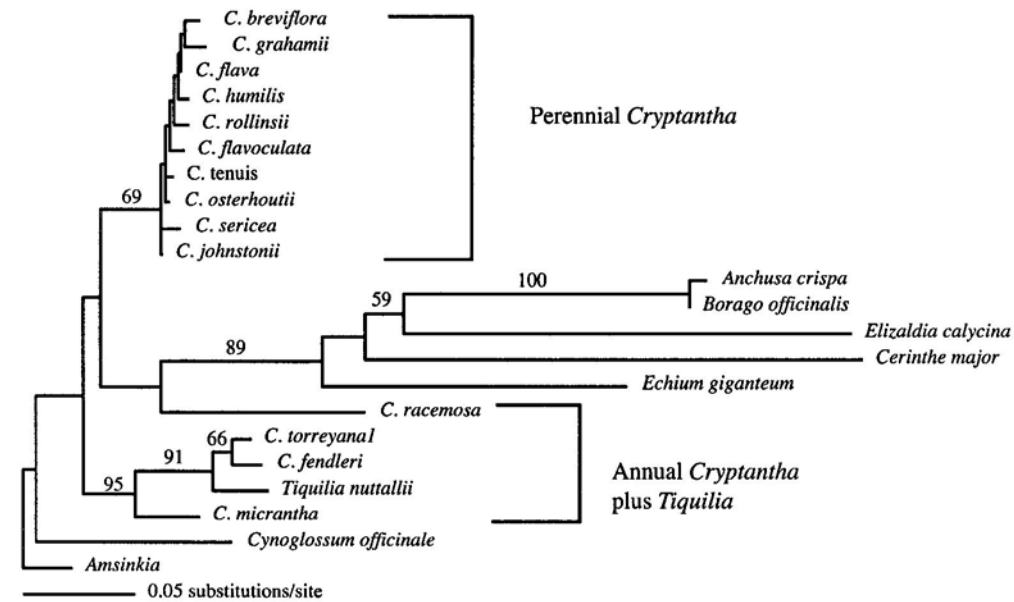


Figure 13. ML bootstrap analyses of ITS sequence data. Note the shorter branch lengths within the perennial *Cryptantha* and the lack of bootstrap support within the perennials.

A. entire data set



B. Data set reduced to exclude species with partial sequences

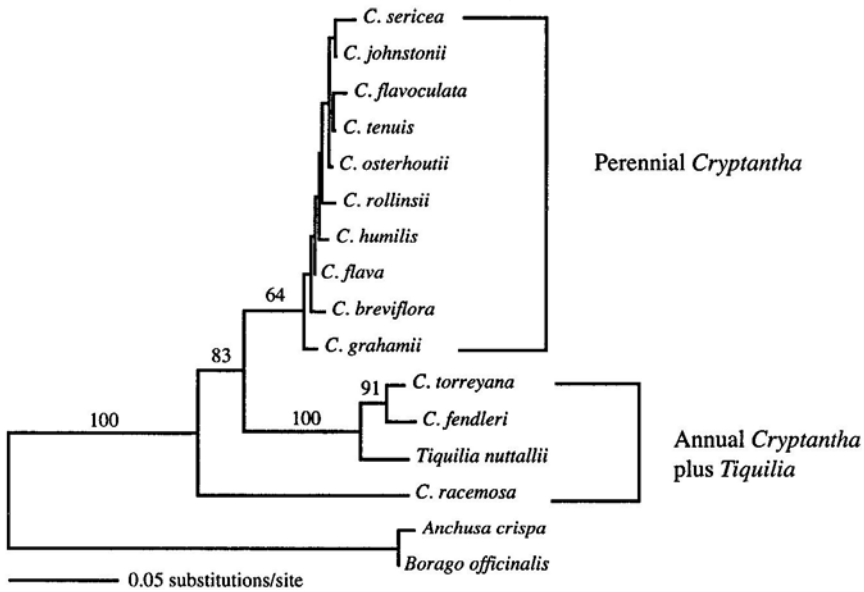


Table 6. Survey of *trnL* variation among species of *Cryptantha* with *Amsinkia*. Sequences are almost identical, even between *Cryptantha* and *Amsinkia*. P-distances ranged from 0.00704 (same value between all ingroup taxa) to 0.007129 (between *Amsinkia* and all ingroup taxa).

```
C. humilis    GAGCCTTGGT ATGGAAACCT ACTAAGTGAC AACTTTCAAA
C. flava      GAGCCTTGGT ATGGAAACCT ACTAAGTGAC AACTTTCAAA
C. sericea    GAGCCTTGGT ATGGAAACCT ACTAAGTGAC AACTTTCAAA
C. rollinsii GAGCCTTGGT ATGGAAACCT ACTAAGTGAC AACTTTCAAA
Amsinkia      GAGCCTTGGT ATGGAAACCT ACTAAGTGAC AACTTTCAAA


C. humilis    TTCAGAGAAA CCCCGGAATT AATCAAAATG GGCAATCCTG
C. flava      TTCAGAGAAA CCCCGGAATT AATCAAAATG GGCAATCCTG
C. sericea    TTCAGAGAAA CCCCGGAATT AATCAAAATG GGCAATCCTG
C. rollinsii TTCAGAGAAA CCCCGGAATT AATCAAAATG GGCAATCCTG
Amsinkia      TTCAGAGAAA CCCCGGAATT AATCAAAATG GGCAATCCTG

C. humilis    AGCCAAATCC GGTTTTCCGA AAACAAAAGT TGAAAAAGAA
C. flava      AGCCAAATCC GGTTTTCCGA AAACAAAAGT TGAAAAAGAA
C. sericea    AGCCAAATCC GGTTTTCCGA AAACAAAAGT TGAAAAAGAA
C. rollinsii AGCCAAATCC GGTTTTCCGA AAACAAAAGT TGAAAAAGAA
Amsinkia      AGCCAAATCC GGTTTTCCGA AAACAAAAGT TGAAAAAGAA

C. humilis    AAAAAAGGAT AGGTGCAGAG ACTCAATGGA AGCTGTTCTA
C. flava      AAAAAAGGAT AGGTGCAGAG ACTCAATGGA AGCTGTTCTA
C. sericea    AAAAA:GGAT AGGTGCAGAG ACTCAATGGA AGCTGTTCTA
C. rollinsii AAAAA:GGAT AGGTGCAGAG ACTCAATGGA AGCTGTTCTA
Amsinkia      AAAA::GGAT AGGTGCAGAG ACTCAATGGA AGCTGTTCTA

C. humilis    ACAAATGGAG TTGACTGGAA GAATCTTTCT CATAAAGTGT
C. flava      ACAAATGGAG TTGACTGGAA GAATCTTTCT CATAAAGTGT
C. sericea    ACAAATGGAG TTGACTGGAA GAATCTTTCT CATAAAGTGT
C. rollinsii ACAAATGGAG TTGACTGGAA GAATCTTTCT CATAAAGTGT
Amsinkia      ACAAATGGAG TTGACTGGAA GAATCTTTCT CATAAAGTGT


C. humilis    GAAAGTATAT ACATTTGTAT TGAATACTTT ATCAAATGAA
C. flava      GAAAGTATAT ACATTTGTAT TGAATACTTT ATCAAATGAA
C. sericea    GAAAGTATAT ACATTTGTAT TGAATACTTT ATCAAATGAA
C. rollinsii GAAAGTATAT ACATTTGTAT TGAATACTTT ATCAAATGAA
Amsinkia      GAAAGTATAT ACATTTGTAT TGAATACTTT ATCAAATGA:


C. humilis    ATGATTAATG AAATATAGTT TTTATGAAAA AAA:GAGTTG
C. flava      ATGATTAATG AAATATAGTT TTTATGAAAA AAA:GAGTTG
C. sericea    ATGATTAATG AAATATAGTT TTTATGAAAA AAA:GAGTTG
C. rollinsii ATGATTAATG AAATATAGTT TTTATGAAAA AAA:GAGTTG
Amsinkia      ::::TTAATG A::TATAGTT TTTATGAAAA AAAAGAGTTG


C. humilis    GTGTTAATCG ATTCCACATA GAAGAAAGAA TCGAATATTC
C. flava      GTGTTAATCG ATTCCACATA GAAGAAAGAA TCGAATATTC
C. sericea    GTGTTAATCG ATTCCACATA GAAGAAAGAA TCGAATATTC
C. rollinsii GTGTTAATCG ATTCCACATA GAAGAAAGAA TCGAATATTC
Amsinkia      GTGTTAATCG ATTCCACATA GAAGAAAGAA TCGAATATTC
```

```
C. humilis   ATTGATCAAA GATCAAATCA TTCACTCCAT AGTCTGATAG
C. flava     ATTGATCAAA GATCAAATCA TTCACTCCAT AGTCTGATAG
C. sericea   ATTGATCAAA GATCAAATCA TTCACTCCAT AGTCTGATAG
C. rollinsii ATTGATCAAA GATCAAATCA TTCACTCCAT AGTCTGATAG
Amsinkia     ATTGATCAAA GATCAAATCA TTCACTCCAC AGTCTGATAG

C. humilis   ATCTTTTGAA GAATTGATTT ATCGGACGAG AATAAAGATA
C. flava     ATCTTTTGAA GAATTGATTT ATCGGACGAG AATAAAGATA
C. sericea   ATCTTTTGAA GAATTGATTT ATCGGACGAG AATAAAGATA
C. rollinsii ATCTTTTGAA GAATTGATTT ATCGGACGAG AATAAAGATA
Amsinkia     ATCTTTTGAA GAATTGATTT CTCGGACGAG AATAAAGATA

C. humilis   GAGTCCCGTT CTACATGTCA ATACCGGCAA CAATGAAATT
C. flava     GAGTCCCGTT CTACATGTCA ATACCGGCAA CAATGAAATT
C. sericea   GAGTCCCGTT CTACATGTCA ATACCGGCAA CAATGAAATT
C. rollinsii GAGTCCCGTT CTACATGTCA ATACCGGCAA CAATGAAATT
Amsinkia     GAGTCCCGTT CTACATGTCA ATACCGGCAA CAATGAAATT

C. humilis   TATAGTAAGA GGAAAATCCG TCGACTTTAA AAATCGTGAG
C. flava     TATAGTAAGA GGAAAATCCG TCGACTTTAA AAATCGTGAG
C. sericea   TATAGTAAGA GGAAAATCCG TCGACTTTAA AAATCGTGAG
C. rollinsii TATAGTAAGA GGAAAATCCG TCGACTTTAA AAATCGTGAG
Amsinkia     TATAGTAAGA GGAAAATCCG TCGACTTTAA AAATCGTGAG

C. humilis   GGTTCAAGTC CCTCTATCCC CAAAAGCCTA CATCCCAACG
C. flava     GGTTCAAGTC CCTCTATCCC CAAAAGCCTA CATCCCAACG
C. sericea   GGTTCAAGTC CCTCTATCCC CAAAAGCCTA CATCCCAACG
C. rollinsii GGTTCAAGTC CCTCTATCCC CAAAAGCCTA CATCCCAACG
Amsinkia     GGTTCAAGTC CCTCTATCCC CAAAAGCCTA CATACCAACG

C. humilis   ATTTCTCCTA TATCTATTTT AGTTAGTGGT TCCAAATCCC
C. flava     ATTTCTCCTA TATCTATTTT AGTTAGTGGT TCCAAATCCC
C. sericea   ATTTCTCCTA TATCTATTTT AGTTAGTGGT TCCAAATCCC
C. rollinsii ATTTCTCCTA TATCTATTTT AGTTAGTGGT TCCAAATCCC
Amsinkia     ATTTCTCCTA TATCTATTTT AGATAGTGGT TCCAAATCCC

C. humilis   TCATCTTTAT CATTCACTCT ATTATTGTTT TACAAACGGA
C. flava     TCATCTTTAT CATTCACTCT ATTATTGTTT TACAAACGGA
C. sericea   TCATCTTTAT CATTCACTCT ATTATTGTTT TACAAACGGA
C. rollinsii TCATCTTTAT C
Amsinkia     TCATCTTTAT CATTCACTCT AT

C. humilis   TCTGACTGGA AACGCCTTTC ATCTTACACA AGTCTTGTGA
C. flava     TCTGACTGGA AACGCCTTTC ATCTTACACA AGTCTTGTGA
C. sericea   TCTGACTGGA AACGCCTTTC ATCTTACACA AGTCTTGTGA
C. humilis   GATACGTATG ATACACATAC AAATGCACAG GGAATCCCCT
C. flava     GATACGTATG ATACACATAC AAATGCACAG GGAATCCCCT
C. sericea   GATACGTATG ATACACATAC AAATGCACAG GGAATCCCCT
```

C. humilis TTTGAATTAT TTAC
C. flava TTTGAATTAT TTAC
C. sericea TTTGAATTAT TTAC

Table 7. Survey of variation in the ETS among species of *Cryptantha* with *Amsinkia*. Sequences are slightly more variable than the *trnL* sequences, but there remain few informative characters, even between *Cryptantha* and *Amsinkia*. The p-distances for the ETS data set ranged from 0.00787567 (between *C. flava* and *C. confertiflora*), and 0.148747758 (between *C. virginensis* and *Amsinkia*).

```
C. cinerea          GTAGCATTCC TCAACGAAGC GATCACCGCA CTCGCCATAA
C. virginensis      GTAGCATTCC TCAACGAAGC CATCACCGTA CTTGCCAAAA
C. rollinsii        GTAGCATTCC TCAACGAAGC CATCACCGTA CTTGCCAAAA
C. flava            GTAGCATTCC TCAACGAAGC CATCACCGTA CTTGCCAAAA
C. humilis          GTAGCATTCC TCAACGAAGC CATCACCGTA CTTGCCAAAA
C. confertiflora    GTAGCATTCC TCAACGAAGC CATCACCGTA CTTGCCAAAA
C. wetherillii      GTAGCATTCC TCAACGAAGC CATCACCGTA CTTGCCAAAA
Amsinkia                       AACGAAGC GAGCACCGCA CTTGCCCGAA

C. cinerea          GGCTCGAAAC GGGGATCGCT ATCGTTCGCA ACTAAGCGAT
C. virginensis      GGCTCGAAAC GGGGATCGCA ATCGTTCGCA ACTAAGCGAT
C. rollinsii        GGCTCGAAAC GGGGATCGCA ATCGTTCGCA ACTAAGCGAT
C. flava            GGCTCGAAAC GGGGATCGCA ATCGTTCGCA ACTAAGCGAT
C. humilis          GGCTCGAAAC GGGGATCGCA ATCGTTCGCA ACTAAGCGGT
C. confertiflora    GGCTCGAAAC GGGGATCGCA ATCGTTCGCA ACTAAGCGAT
C. wetherillii      GGCTCGAAAC GGGGATCGCA ATCGTTCGCA ACTAAGCGAT
Amsinkia            GGCTAGAAAC GGAGTTCGCA ATCGTTCGCA ACTAAGCGAT

C. cinerea          GAAAGCTTTT GTTCGGGCAA AAAGAGGACG TGAGACCTCA
C. virginensis      GAAAGCTTTT GTTCGGGCAG AAAGAGGACG TGAGACCTCA
C. rollinsii        GAAAGCTTTT GTTCGGGCAG AAAGAGGACG TGAGACCTCA
C. flava            GAAAGCTTTT GTTCGGGCAA AAAGAGGACG TGAGACCTCA
C. humilis          GAAAGCTTTT GTTCGGGCAG AAAGAGGACG TGAGACCTCA
C. confertiflora    GAAAGCTTTT GTTCGGGCAA AAAGAGGACG TGAGACCTCA
C. wetherillii      GAAAGCTTTT GTTCGGGCAG AAAGAGGACG TGAGACCTCA
Amsinkia            GAAAGCTTTT GTTAGGGCAA AAAGAGGACG TGAGCCCTCG

C. cinerea          TGCCCATATA TAATGCACCG CATCCAAGAG ATCAAGCAAA
C. virginensis      TGCCCATATA TAATGCACCG CATCCAAGAG ATCAAGCAAA
C. rollinsii        TGCCCATATA TAATGCACCG CATCCAGGAG ATCAAGCAAA
C. flava            TGCCCATATA TAATGCACCG CATCCAAGAG ATCAAGCAAA
C. humilis          TGCCCATATA TAATGCACCG CATCCAAGAG ATCAAGCAAA
C. confertiflora    TGCCCATATA TAATGCACCG CATCCAAGAG ATCAAGCAAA
C. wetherillii      TGCCCATATA TAATGCACCG CATCCAAGAG ATCAAGCAAA
Amsinkia            TGCCCATATA ACATGCACCG CATCCAAGAG CCCAAGCAAA

C. cinerea          TGCCCTATGC ACCACWCTGC AAGCACAAAT CAATGTGAGT
C. virginensis      TGCCCTATGC ACCACTCTAC AAGCACAAAT CAATGTGAGA
C. rollinsii        TGCCCTATGC ACCACACTAC AAGCACAAAT CAATGTGAGA
C. flava            TGCCCTATGC ACCACACTGC AAGCACAAAT CAATGTGAGA
C. humilis          TGCCCTATGC ACCACACTAC AAGCACAAAT CAATGTGAGA
C. confertiflora    TGCCCTATGC ACCACACTGC AAGCACAAAT CAATGTGAGA
C. wetherillii      TGCCCTATGC ACCACACTAC AAGCACAAAT CAATGTGAGA
Amsinkia            TACCTCGTGG ACCAATCCAC AAGCACAAAC GAATGTGAGT

C. cinerea          AGAGTGGGAC ACAGAGAYTG CTTCGTGGTT CACCTTACAT
C. virginensis      AGAGTGGGAC ACAGAGATTA CTTCGTGGTT CACCTTACAT
C. rollinsii        AGAGTGGGAC ACAGAGATTA CTTCGTGGTT CACCTTACAT
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C. flava           AGAGTGGGAC ACAGAGATTA CTTCGTGGTT CACCTTACAT
C. humilis         AGAGTGGGAC ACAGAGATTA CTTCATGGTT CACCTTACAT
C. confertiflora   AGAGTGGGAC ACAGAGATTA CTTCGTGGTT CACCTTACAT
C. wetherillii     AGAGTGGGAC ACAGAGATTA CTTCGTGGTT CACCTTGCAT
Amsinkia           GGAGTGGGAC ACAAAGATTG CTTCATGGTT CACCCTACST

C. cinerea         CAACCCAARA AGGGACGAGG AAAGGCGAAA :TGTAACATT
C. virginensis     CAACCCAAAA AGGGACGAGT AAAGGCGAAA :TGTAACATT
C. rollinsii       CAACCCAAAA AGGGACGAGG GAAGGCGAAA :TGTAACATT
C. flava           CAACCCAAAA AGGGACGAGG AAAGGCGAAA :TGTAACATT
C. humilis         CAACCCAAAA AGGGACGAGT AAAGGCGAAA :TGTAACATT
C. confertiflora   CAACCCAAAA AGGGACGAGG AAAGGCGAAA ATGTAACATT
C. wetherillii     CAACCCAAAA AGGGACGACG AAAGGCGAAA :TGTAACATT
Amsinkia           GGCCCCAATA AGGGACAACG GAAGGCGAAA :TGGGACGTT

C. cinerea         TAGACTATCG ATTGCCATCG CATAAGGTAC ACAACACAAG
C. virginensis     TAGACTATCG ATTGCCATCG CATAAGGTAC ACAACACAAG
C. rollinsii       TAGACTACCG ATTGCCATCG CATAAGGTAC ACAACACAAG
C. flava           TAGACTACCG ATTGCCATCG CATAAGGTAC ACAACACAAG
C. humilis         TAGACTAACG ATTGCCATTG CATAAGGTAC ACAACACAAG
C. confertiflora   TAGACTACCG ATTGCCATTG CATAAGGTAC ACAACACAAG
C. wetherillii     TAGACTACCG ATTGCCATTG CATAAGGTAC ACAACACAAG
Amsinkia           TAGACTACCG ATTGYCATCG TATAAGGTAC GCAACACAAG

C. cinerea         AAACCAATAC AGAACTCGAG ATAGTATGTA TCTAGAGACA
C. virginensis     AAACCAATAC AGAACTCCAG ATAGTACGTA TCTAGAGACA
C. rollinsii       AAACCAATAC AGAACTCGAG ATAGTATGTA TCTAGAGACA
C. flava           AAACCAATAC AGAACTCGAG ATAGTATGTA TCTAGAGACA
C. humilis         AAACCAATAC AGAACTCGAG ATAGTATGTA TCTAGAGACA
C. confertiflora   AAACCAATAC AGAACTCGAG ATAGTATGTA TCTAGAGACA
C. wetherillii     AAACCAATAC AGAACTCGAG ATAGTATGTA TCTAGAGACA
Amsinkia           AAACCAAGGC AACACTCTAG ATAATCTACA TCACGAGACC

C. cinerea         TGACTGAAGA TGCCTGTGAG AATGGACGTC GTTGCCAGAG
C. virginensis     TGACTGAAGA TGCATGTGAG AATGGACGTC GTTGCCAGAG
C. rollinsii       TGACTGAAGA TGCTTGTGAG AATGGACGTC GTTGCCAGAG
C. flava           TGACTGAAGA TGCCTGTGAG AATGGACGTC GTTGCCAGAG
C. humilis         TGACTGAAGA TGCATGTGAG AATGGACGTC GTTGCCAGAG
C. confertiflora   TGACTGAAGA TGCCTGTGAG AATGGACGTC GTTGCCAGAG
C. wetherillii     TGACTGAAGA TGCTTGTGAG AATGGACGTC GTTGCCAGAG
Amsinkia           CGACTGAGGA TGCATGTGAG GATGGACGTC GTTGCCAGAG

C. cinerea         CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
C. virginensis     CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
C. rollinsii       CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
C. flava           CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
C. humilis         CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
C. confertiflora   CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
C. wetherillii     CAAGGATCCA ACCAACCAAC ACAAGCAAAA CACCACTCAT
Amsinkia           CAAGGATCCA ACCAACCAAC ACAAGCAAAT CACCACTCAT

C. cinerea         GCGCCTACAC GTATCGCATT ATCAACCCTC AAATCAACAA
C. virginensis     GCGCCTACAC GTATTGCATT GTCAACCCTC AAATCAACAA
C. rollinsii       GCGCCTACAC GTATTGCATT ATCAACCCTC AAATCAACAA
C. flava           GCGCCTACAC GTATTGCATT ATCAACCCTC ACATCAACAA
C. humilis         GCGCCTACAC GTATTGCATT ATCAACCCTC TAATCAACAA
C. confertiflora   GCGCCTACAC GTATTGCATT ATCAACCCTC AAATCAACAA
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C. wetherillii    GCGCCTGCAC GTATTGCAAT ATCAACCCTC AAATCAACAA
Amsinkia          GCGCCTGCAC GTACAGCATT AACAACCC

C. cinerea        :TGGACCACC CCACAGAGAG AAAATCACTC TATGAAGCAA
C. virginensis    :TAGACCACC CCACAGAGAG AAAATCACTC TATGAAGCAA
C. rollinsii      ATAGACCACC CCACAGAGAG AAAATCACTC TATGAAGCAA
C. flava          :TAGACCACC CCACAGAGAS AAAATCACTC TATGAAGCAA
C. humilis        :TAGACCACC CCACAGAGAG AAAATCACTC TATGAAGCAA
C. confertiflora  :TAGACCACC CCACAGAGAG AAAATCACTC TATGAAGCAA
C. wetherillii    ATAGACCACC CCACAGAGAG AAAATCACTC TATGAAGCAA

C. cinerea        TCCAAGCCAA CGAGAGAATC GACAAAGCAC CGCTTGGCGC
C. virginensis    TCAAAGCCAA CGAGAGAACC GACAAAGCAC CGCTTGGCGC
C. rollinsii      CCCAAGCCAA CGAGAGAATC GACAAAGCAC CGCTTGGCGC
C. flava          TCCAAGCCAA CGAGAGAATC GACAAAGCAC CGCTTGGCGC
C. humilis        TCCAAGCCAA CGAGAGAATC GACAAAGCAC CGCTTGGCGC
C. confertiflora  TCCAAGCCAA CGAGAGAATC GACAAAGCAC CGCTTGGCGC
C. wetherillii    TCCAAGCCAA CGAGAGAATG GACAAAGCAC CGCTTGGCGC

C. cinerea        GAACGACCCC ACACCCCACC TCTGATTTT: ::::::::::
C. virginensis    GAAAGACCCC AACACACCAC CTCTGACCCA ACACACCACC
C. rollinsii      GAAAGGACCC AACACACCAC ACCACCTNTG ATGATCGATA
C. flava          GAAAGACCC: :::::::::: ::::::ACCA ACACACCACC
C. humilis        GAAGGACCC: :::::::::: :::::::::A ACCCACCACC
C. confertiflora  GAAAGAACCA CCAACACACC AC::::CCCA ACACACCACC
C. wetherillii    GAAAGGACCC AACACA:::: :CCACCTCTG ATGATCGATA

C. cinerea        :::::::::C GAAATGTGCA TCGACTAGCA AGTACGAGCT
C. virginensis    TCTGGTGATC GATATGTGCT TCGACTAGCA TGTACGAGCC
C. rollinsii      TGTGC::::: :::::::::C CCGACTAGCA TGTACGAGCC
C. flava          TCTGGTGATC GATATGTGCT TCGACTAGCA TGTACGAGCC
C. humilis        TCTGGTGATC GATATGTGTC TCGACTAGCA TGTACGAGCC
C. confertiflora  TCTGGAGATC GATATGTGTC TCGACTAGCA TGTACGAGCC
C. wetherillii    TGTGC::::: :::::::::T TCGACTAGCA AGTACGAGCC

C. cinerea        TTTCAAGACA CGATTCTACT GGTCAA
C. virginensis    TTTCAAGACA CGATTCTACT GGTCAA
C. rollinsii      TTTCAAGACA CGATTCTACT GGTCAA
C. flava          TTTCAAGACA CGATTCTACT GGTCAA
C. humilis        TTTCAAGACA CGATTCTACT GGTCAA
C. confertiflora  TTTCAAGACA CGATTCTACT GGTCAA
C. wetherillii    TTTCAGGACA CGATTCTACT GGTCAA
```

to make inferences about the environment as a selective force for speciation patterns in this group.

Methods

Plant Material: Leaf tissue was collected from 37 perennial Oreocarya species, 3 annual Krynitzkia species, and one species of *Amsinkia* (for use as out group taxa) during the Spring/Summers of 1998-2001 (Table 8). Samples were collected in the field and sent to UMCP on ice, where they were stored at -80^0 C, until DNA could be extracted. Tissue was collected from multiple (2-7) individuals for each species. Because populations of *C. oblata* could not be located in the field, leaf tissue for this species was collected from Brigham Young University herbarium specimens (Table 8).

DNA isolation: Total genomic DNA was extracted with the Nucleon Phytopure resin extraction kit following the manufacturer's protocol (Amersham International, Buckinghamshire, England) with an additional chloroform extraction performed before the chloroform/resin step. This additional step helps to remove cellular debris that would otherwise inhibit the removal of polysaccharides, abundant in *Cryptantha* leaf tissue, by the resin. DNA pellets were air dried and suspended in 100 μL of ultrapure water.

ITS amplification: PCR was conducted using primers 'ITS-4' (5'-TCCTCCGCTTATTGATATGC-3') and 'ITS-5' (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.* 1990) under standard conditions (50 υL reactions at the following final concentration: 2mM $MgCl_2$, 0.7 mg/mL BSA, 0.25 mM primers, 0.1mM dNTPS, 0.5 units taq polymerase) with the following reaction conditions: an initial 94 ° C denaturation for 4 minutes followed by 30 cycles of

Table 8. Location data for species used in this study. No GPS data were available for 4 taxa.

Species	Location
C. abata	N 37° 42.031' W 112° 16.715'
C. bakeri	N 39° 04.092' W 109° 41.941'
C. barnebyi	N 39° 43.452' W 109° 25.691'
C. breviflora	N 40° 35.716' W 109° 26.194'
C. caespitosa	N 41° 04.957' W 110° 10.100'
C. cana	N 41° 09.294' W 103° 05.520'
C. capitata	N 37° 05.212' W 111° 59.067'
C. celosoides	N 41° 09.294' W 103° 05.520'
C. cinerea var. cinerea	N 36° 51.157' W 111° 59.565'
C. cinerea var. cinerea	100 m S of Red Canyon Campground, Garfield county
C. compacta	N 38° 37.216' W 113° 50.768'
C. compacta	N 38° 37.216' W 113° 50.768'
C. confertiflora	N 37° 05.250' W 111° 59.052'
C. creuzfeldtii	N 39° 36.140' W 110° 49.981'
C. elata	N 39° 17.287' W 108° 49.233'
C. flava	N 40° 35.716' W 109° 26.194'
C. flavoculata	N 40° 35.716' W 109° 26.194'
C. fulvocanescens	N 38° 55.949' W 110° 36.107'
C. grahamii	N 39° 50.832' W 109° 37.147'
C. humilis	behind Univ. of Utah hospital center
C. johnstonii	N 39° 10.620' W 110° 29.901'
C. jonesiana	N 38° 53.032' W 110° 39.389'
C. longiflora	N 38° 37.818' W 109° 47.971'
C. mensana	N 39° 10.240' W 110° 27.450'
C. oblata	herbarium specimen # LCHiggins 3001 BYU
C. ochroleuca	2.75 mi. from jct. of Bryce Canyon rd.
C. osterhoutii	N 38° 49.531' W 109° 17.058'
C. paradoxa	N 38° 53.139' W 110° 39.469'
C. rollinsii	N 39° 45.630' W 109° 36.641'
C. rugulosa	N 39° 05.067' W 113° 34.292'
C. sericea	N 39° 44.912' W 109° 32.189'
C. semiglabra	N 36° 51.462' W 112° 43.816'
C. setosissima	North Rim of Grand Canyon, Arizona
C. stricta	N 40° 37.200' W 109° 34.279'
C. tenuis	N 38° 43.678' W 109° 21.016'
C. virginenesis	N 37° 15.335' W 113° 37.656'
C. wetherillii	N 39° 17.496' W 110° 52.401'
C. torreyana	N 36° 51.456' W 112° 43.905'
C. racemosa	N 37° 15.335' W 113° 37.656'
C. fendleri	N 37° 15.335' W 113° 37.656'
Amsinkia douglasiana	N 36° 33.787' W 119° 07.912'

94° C for 3 seconds, 55° C for 5 seconds, and 72° C for 45 seconds, with one additional extension at 72° C for 4 min. Additional ITS sequences were gathered from 9 perennial *Cryptantha*, 3 annual *Cryptantha*, and one *Amsinkia* species to determine which group represented an appropriate outgroup for the AFLP analyses. All remaining ITS sequences presented here were obtained from GenBank (http:// www.ncbi.nlm.nih.gov).

ETS amplification: To obtain ETS sequences, the intergenic spacer (IGS) was amplified using long-distance PCR with primers CNS1R (5'-GAGACAAGCATATGACTAC-3') (Eckenrode *et al.* 1985) and DN26s3331F (5'-CTGCCACGATCCACTGAGAT-3'; kindly provided by E. Zimmer). Standard PCR (see above) was conducted identical to the amplification for ITS except with 35 cycles of 94 ° C for 10 seconds, 60 ° C for 10 seconds, and 72 ° C for 2 minutes. Preliminary IGS sequence data were obtained using primer CNS1R in sequencing reactions and resolved using an ABI 3100. From these single-stranded reads a *Cryptantha/Amsinkia*-specific IGS primer (5'-CCTTGACCAGTAGAATCGTG-3', CrypETS) was designed and used with CNS1R to obtain ETS amplicons across a broad range of *Cryptantha* species. A second PCR (identical to the amplification used for IGS with 30 cycles, and an annealing temperature of 54 ° C) was performed with CNS1R and CrypETS to obtain the ETS product.

***trnL* amplification:** To obtain *trnL* products, a standard PCR (identical to ITS amplification except with 30 cycles of 94 ° C for 10 seconds, 55 ° C for 10 seconds, and 72 ° C for one minute) was performed using the primers *trnL*-c (5'-CGAAATCGGTAGACGCTACG-3') and *trnL*-f (5'-ATTTGAACTGGTGACACGAG-3') (Taberlet *et al.* 1991).

Purification of PCR product/Sequencing: All PCR products were visualized on a 1% agarose gel stained with ethidium bromide (0.1 μg/mL). An equal amount of 20% polyethylene glycol (PEG): 0.5M NaCl was added to the PCR products, and the solution was heated for 15 minutes at 37° C on a heat block. The tubes were centrifuged at 14000 rpm for 15 minutes, and the supernatant was removed by pipetting. To remove excess salts the pellet was covered with 100 μL of cold 80% ethanol, and the tubes were centrifuged for 10 minutes at 14,000 rpm. The alcohol was removed, and the pellet was allowed to air dry. Pellets were suspended in 20 μL ultrapure water, visualized and quantified on a 1% agarose gel stained with ethidium bromide, and stored at -20° C. The PEG-purified PCR product was sequenced on an ABI 3100 with the dye-terminator cycle-sequencing protocol following the manufacturer's protocol. The sequences were edited and assembled using Sequencher version 3.1.1, and subsequent alignment was done manually.

ETS/*trnL* analyses: The *trnL* and ETS sequence data were analyzed with PAUP* version 4.0b 10 (Swofford 2001). For each data set, phylogenetic reconstruction under maximum parsimony (MP) was conducted using the heuristic search option with TBR branch-swapping, MULPARS, and ACCTRAN options active. Characters were assigned equal weights at all nucleotide positions. Robustness of cladistic lineages were evaluated with 1000 bootstrap replicates. A maximum likelihood analysis using the K2P model and parameters that were estimated iteratively using the set of trees generated by the MP analysis was conducted on both data sets. Bootstrap methods with 1000 replicates were performed to estimate the robustness of nodal support. The 'show pairwise distances'

option in PAUP* was used to evaluate the largest and smallest distances between taxa in each data set to evaluate the variability in each of the data sets.

ITS analyses: A ML analysis was performed on the ITS sequence data using PAUP* version 4.0b 10 (Swofford 2001). This data set included 10 perennial *Cryptantha*, 4 annual *Cryptantha*, one *Amsinkia* species, and 8 other genera from the Boraginaceae. These include *C. sericea*, *C. humilis*, *C. flavoculata*, *C. johnstonii*, *C. breviflora*, *C. rollinsii*, *C. flava*, *C. grahamii*, *C. tenuis*, *C. osterhoutii*, *Amsinkia*, *C. torreyana*, *C. fendleri*, and *C. racemosa* (Table 1). Sequences for *C. micrantha* (AF402581), *C. flavoculata* (AF091154), *Anchusa crispa* (AY071853), *Borago officinalis*(AY092898), *Cynoglossum officinale* (AF402582), *Cerinthe major* (L43200), *Echium giganteum* (L43224), *Elizaldia calycina* (AF402583), and *Tiquilia nuttallii* (AF091207) were obtained from GenBank. Modeltest version 3.06 for Macintosh (Posada and Crandall 1998) was used to select the best-fit model of DNA substitution, among 56 possible models. Modeltest implements a series of hierarchical likelihood ratio tests and employs the AIC criterion to assess significant differences between the likelihood scores for alternative models (Posada and Crandall 1998). The best-fitting model was the GTR+I+Γ ($p \leq 0.01$). Analyses were performed on the full data set (22 taxa), and a smaller data set (16 taxa) that excluded taxa with incomplete ITS 1 or ITS 2 sequences (*Amsinkia*, *Cryptantha micrantha*, *Cynoglossum officinale*, *Elizaldia calycina*, *Echium giganteum*, and *Cerinthe major*) to determine if the topology was influenced by missing data. The GTR + I + Γ model was also used for the analysis of the reduced data set.

AFLP Construction: The AFLP technique (Vos *et al.* 1995) uses the principles of RFLP (restriction fragment length polymorphism) combined with polymerase chain reaction

(PCR) to magnify the isolated fragments. The method involves a double digestion of genomic DNA with restriction endonucleases, and ligation of double-stranded, short adapter sequences to the resulting fragments. The DNA sequence of the adapter and restriction site serve as primer binding sites for PCR amplification. These primers allow for selective amplification of only the fragments in which the sequence of the nucleotides flanking the restriction site is complementary. Six primers that differed at their 3' end (Table 9) were used for the final amplification (Vos *et al.* 1995, Hawthorne 2001).

A modified version of the AFLP method from Vos *et al.* (1995) was used to allow non-radioactive analysis, and detection of fragments with an automated sequencer (PE Applied Biosystems, Foster City, CA). This method favors the restriction of non-methylated DNA by using methylation-sensitive enzymes, *PstI* and *EcoRI*, and reduces the overall number of restriction fragments since *PstI* and *EcoRI* have a six base pair recognition sequence (Hawthorne 2001). AFLP constructs were assembled in a single step by mixing 2.5 μg of genomic DNA, New England Biolabs #4 restriction enzyme buffer [20 mM tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol (DTT) (Beverly, MA)], 1.8 mM DTT, 2 mM ATP, 100 ng/ml bovine serum albumen, 20 units PstI, 20 units EcoRI, 6 units T4 DNA ligase, and 5 pmoles of each double-stranded adapter (Table 9). The reactions were incubated for 14 h at 37° C. The restriction enzymes create "sticky ends" to which the adapters ligate, thereby changing the sequence of the restriction site and preventing subsequent cleavage (Hawthorne 2000).

Amplification: A two-step amplification strategy was used following Vos et al. (1995). First a PCR was performed with core primers complementary only to the adapter

Table 9. Adapter sequences, primer sequences, and primer combinations. Adapter sequences for each enzyme are listed (A.), followed by the core primer sequence (A.), and the selective extensions (B.). Primer combinations are listed in (C.), such that each number/letter represents a primer specific extension. Adapted from Hawthorne (2000).

Adapter and Primer sequences and primer combinations
A. The sequences of EcoRI and PstI adapters
EcoRI adapters 5'-AATTGGTACGCAGTC-3' 5'-CTCGTAGACTGCGTACC-3'
PstI adapters 5'-TGTACGCAGTCTTAC-3' 5'-CTCGTAGACTGCGTACATGCA-3'
The sequence of primers for *EcoRI* and *PstI* - the enzyme specific portion is underlined *EcoRI*: 5'-GACTGCGTACCAATTC-3' *PstI*: 5'-GACTGCGTACATGCAG-3'
B. Primer overhangs for selective amplification
Primer overhangs added to the *EcoRI* primer 1= AA 2= ACC 3= ACT 4= ACG Primer overhangs added to the *PstI* primer A=ACC B=AGG C=ACT
C. Primer combinations used
A1 A2 C2 B2 A4 A3

sequence (preamplification) (Table 9). The second amplification, selective amplification, uses primers with two or three overhanging nucleotides at the 3' end (Table 9). A standard PCR was performed for the preamplification round- [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit Taq DNApolymerase] that included2 ml of the AFLP construction as template and 5 pmoles of each primer (Table 9). This reaction was cycled at 94° C for 1 min, 56° C for 1 min and at 72° C for 1 min for 20 cycles. The preamplification products were diluted 1:1 with 10 mM Tris (pH 8.0) and stored at -20°. Six primer combinations were used for the selective amplifications (Table 9c). A standard PCR was performed for the second amplification (see reagents above) that included 1 ml of the diluted preamplification product as template, and one of the *EcoRI* and *PstI* selective primers (Hawthorne 2000) that were 5'-labeled with either *Fam* or *Hex* fluorescent dye (PE Applied Biosystems, Foster City, CA) (Table 9). Only one primer per reaction was dye-labeled. A total of 27 selective amplification cycles were performed. The reaction profile was a "touchdown-PCR" where a relatively high annealing temperature of 65° C was used for the first round and subsequently reduced by 0.7° for each of the next 12 cycles. The denaturing and extension step for each of these 12 cycles were 94° C for 10 s and 72° C for 90 s, respectively. After 12 touchdown cycles, 25 additional cycles were performed at 94° C for 10 sec, 56° C for 40 + 1 sec. per cycle, and 72° C for 90 sec. (Hawthorne 2000). These products were diluted 1:10 with a mixture of formamide and GeneScan™-500 *Rox* fluorescent size standard (50-500 bp – PE Applied Biosystems, Foster City, CA) (1 mL formamide to 37.2 mL *Rox* standard) and run on ABI PRISM® 3100 following the manufacturer's protocols (PE Applied Biosystems, Foster City, CA). Each sample had an internal size standard reaction labeled

with *Rox* (PE Applied Biosystems, Foster City, CA), and a second reaction labeled with either *Hex* or *Fam* (PE Applied Biosystems, Foster City, CA).

Data Analysis: Fragment data were analyzed with GeneScan® Analysis Software to identify and calculate the predicted size for each peak relative to the *Rox*-labeled internal size standard. All standard peaks were used (with the exception of the 35 and 250 bp) to calculate the size curve based on recommendation from the manufacturer (ABI product guide). Excluding the 35 and 250 bp fragments increases the accuracy of the size calculation (ABI product guide). GeneScan® fragment data were imported into Genotyper version 2.5 (PE Applied Biosystems, Foster City, CA) for scoring characters. The software provides a scaling factor that allows one to evaluate presence or absence of peaks while taking into account the strength of the reaction. An arbitrarily chosen reaction was used to normalize differences in the peak heights among samples. The sum of the total signal in each of the other lanes was divided by the sum of the total signal for the arbitrarily chosen reaction to yield a separate scaling factor for each lane. Scaling factors were also calculated for each size standard and sample reaction. Characters (called "categories" in Genotyper) were chosen by scanning the data by eye at low resolution for peaks that were not present in all individuals. Once the categories were assigned, the chromatograms for each individual were evaluated manually for the presence of a peak in each assigned category. Because the size standard ranged between 50 and 500 bp,only peaks in that range were scored. Bands outside the 50 to 500 bp range could not be accurately sized, and consequently were excluded from all analyses. The resulting data were proofread by repeating the entire process to re-evaluate the original chromatograms and ascertain the presence/absence of peaks.

Data were analyzed with PAUP* version 4.0b 10 (Swofford 2001). To determine the relationships among species of Oreocarya, Maximum Parsimony (MP) and Minimum Evolution (ME) analyses were conducted. The ME tree was constructed using Nei-Li distances (Nei and Li 1979). The MP tree was constructed with characters that were unordered and of equal weights. For both the MP and ME analyses, 100 bootstrap replicates were performed to assess the internal consistency of the data. Each bootstrap replicate consisted of 1000 replicates of random taxon addition and used the heuristic search procedure with TBR branch swapping.

A permutation test was done to assess phylogenetic signal in the data (Swofford 1996). A heuristic search with TBR branch swapping was performed on starting trees generated from 100 random taxon addition replicates for each of the 100 permutations of the data set. The permutation test was conducted on the entire data set and on a modified data set. The modified data set included only one individual per species to determine if phylogenetic signal was present throughout the data set, or only between individuals from like species.

Homology assesment: The PCR products from the selective amplification were resolved using SDS-PAGE (4.25% acrylamide gels for 4 hours at a constant 80 W) and silver stained (Promega, Madison WI) for the *EcoRI*acg and *PstI*acc primer pairs. The amplified fragments were visualized on a light box and compared with the electropherograms from the ABI 3100 analysis. The gels were scanned on a flatbed scanner in order to archive the image (Hawthorne 2000). Bands from the gels were hydrated with 0.5 mL of ultrapure water, removed with the aid of a small pipette tip, and placed in a 0.7 mL eppendorf tube containing 10 μL of ultrapure water. After 10

minutes, 1 μL of this solution was used as template in a standard PCR (see recipe under Amplification section) with the same primers used in the original selective amplification (both unlabeled). These PCR products were PEG purified and sequenced (see description above in Methods: Purification of PCR product/Sequencing).

Results

ETS/*trnL* analyses: Analyses of both preliminary data sets showed that the relationships among species of the Oreocarya could not be resolved using these markers (phylogenetic trees not shown). The p-distances for the *trnL* sequences ranged from 0.00704 (same value between all ingroup taxa) to 0.007129 (between *Amsinkia* and all ingroup taxa). The MP and ML analyses produced identical trees for the *trnL* data set; there was no bootstrap support in either analysis. For the ETS data, ML analysis resulted in a tree identical to one of the 5 most parsimonious trees (Figure 12), both with weak bootstrap support only for the monophyly of the Oreocarya, and not for the relationships within the group (Figure 12). The p-distances for the ETS data set ranged from 0.00787567 (between *C. flava* and *C. confertiflora*), and 0.148747758 (between *C. virginensis* and *Amsinkia*).

ITS analyses: Preliminary analysis of the entire ITS data set resulted in a clade of the perennial species, a clade of outgroup genera plus *C. racemosa*, a clade of annual *Cryptantha* plus *Tiquilia nuttallii*, and basal individual branches of *Cynoglossum officinale* and *Amsinkia* (Figure 13a). There was weak bootstrap support for monophyly of the perennial *Cryptantha* and strong bootstrap support for a polyphyletic annual clade (Figure 3a). The reduced data set, where all of the species with partial sequences were excluded, showed similar patterns (Figure 13b). There remained no bootstrap support for

any of the relationships among the ingroup taxa; however there was weak bootstrap support for the monophyly of the Oreocarya (Figure 13b). The clade containing the annual *Cryptantha* plus *Tiquilia nuttallii* also has high bootstrap support in the analysis of the reduced dataset, as does the branch leading to the polyphyletic lineage of annual *Cryptantha* (Figure 13b). The p-distances for the ITS sequence data ranged from 0.00469 (between *C. osterhoutii* and *C. johnstonii*), and 0.30262 (between *Cerinthe major* and *Cryptantha fendleri*).

AFLP analyses: A total of 177 bands were scored from 6 primer pairs (Table 9) for 83 individuals (38 species). The average number of bands per primer pair was 29.5, and the number of bands scored per primer pair ranged from 20-42. All individuals from like species grouped together, regardless of the analytical method (Figures 4 and 5). However, ME and MP analyses produced different tree topologies.

ME: The ME analysis resulted in one tree with a length of 0.77621 that differs from the MP tree in the branching order of the internal nodes. Many of the relationships among the peripheral branches are maintained between trees (Figure 14, Figure 15). Bootstrap analyses support clades of individuals from the same species, and for grouping *C. abata* with *C. capitata*, and *C. tenuis* with *C. osterhoutii* as sister taxa.

MP: The MP analysis yielded two equally parsimonious 1321-step trees that differed only in the placement of individuals within one of the outgroup species. Bootstrap support for the internal nodes was weak, but the terminal branches that group individuals from like species had strong bootstrap support (Figure 15). There were 174 parsimony informative characters.

Figure 14. Phylogeny of *Cryptantha* section Oreocarya based on AFLP data. Minimum evolution (Nei-Li distances) tree generated with 1000 random taxon addition sequences. The numbers above branches are bootstrap values generated with 100 replicates. Values less than 50% are not shown. The taxa limited to unique substrates are highlighted in specific colors defined in the key.

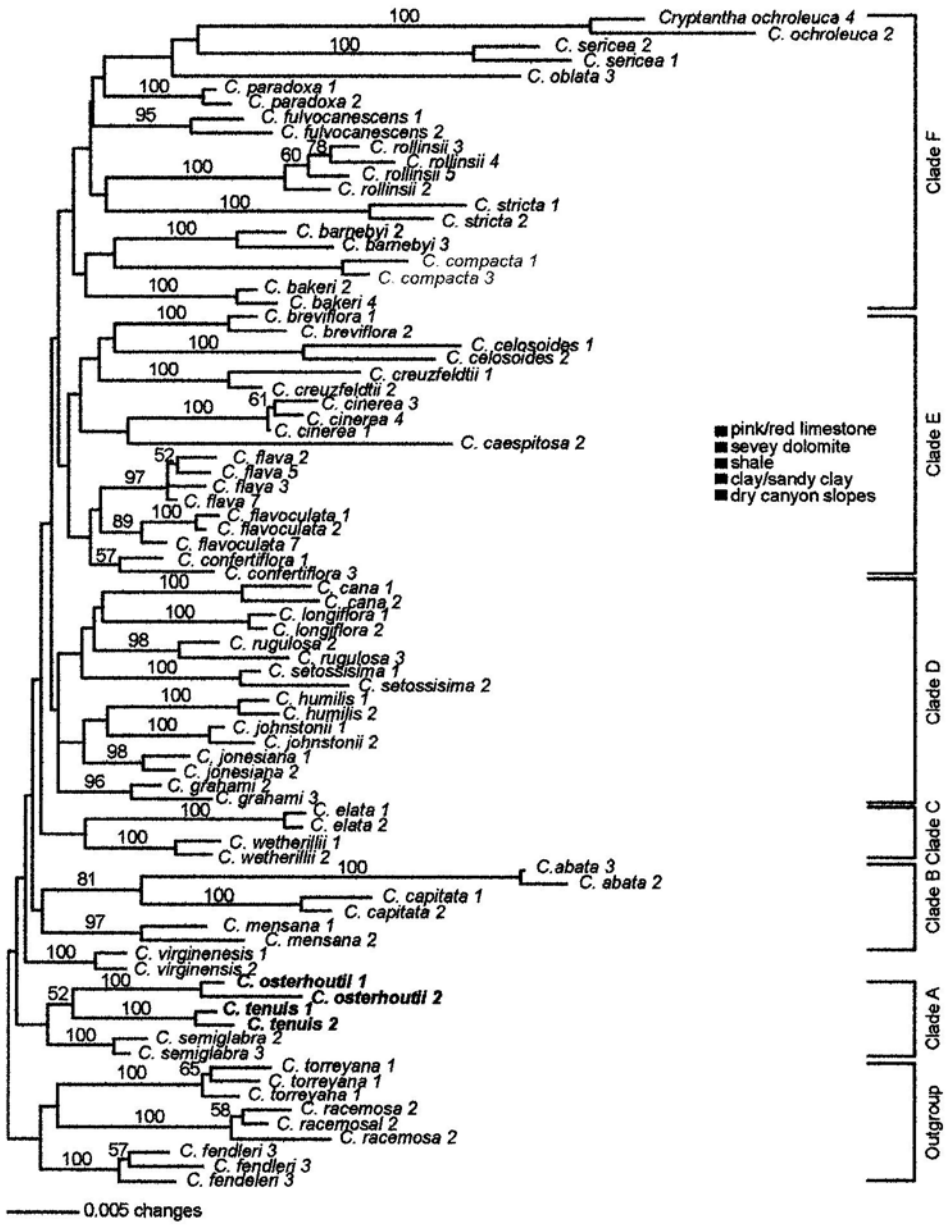
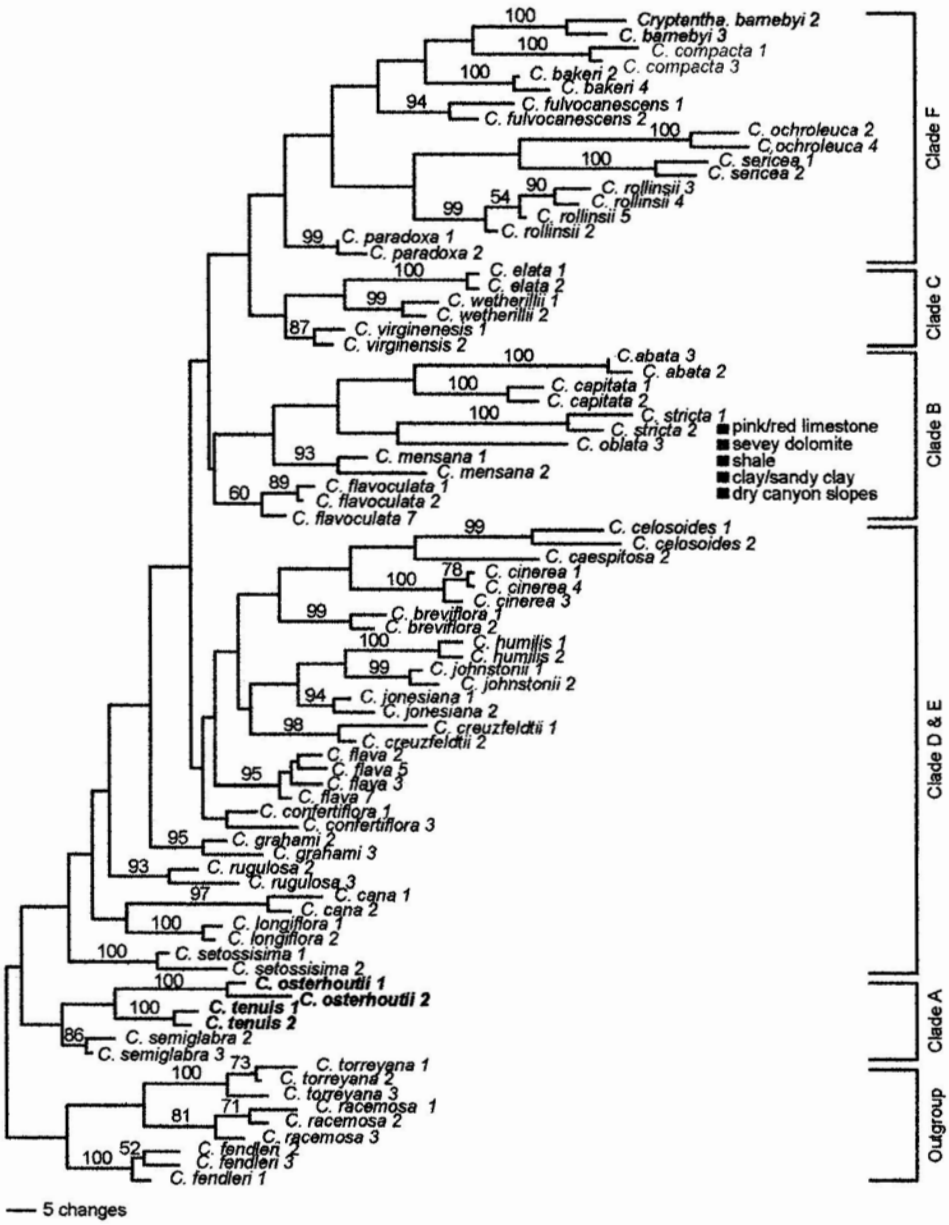


Figure 15. Phylogeny of *Cryptantha* section Oreocarya based on AFLP data. Maximum parsimony tree generated with 1000 random taxon addition sequences. The numbers above branches are bootstrap values generated with 100 replicates. Values less than 50% are not shown. The taxa limited to specific substrates are highlighted in specific colors defined in the key.



Comparison of the MP and ME tree topology: Six distinct clades were apparent in the ME tree toplogy, and clades in the MP tree were labeled relative to the ME clades (Figure 14, Figure 15). Many of the clades remain consistent between tree topologies. Clade A is identical in both trees, and the sister relationship of *Cryptantha tenuis* and *C. osterhoutii* has bootstrap support of 52% in the ME tree (Figure 14). *C. virginenesis* which branches between Clade A and Clade B in the ME tree shifts to become part of Clade C, sister to *C. wetherillii* and *C. elata*. The location of Clade C shifts so that it is sister to Clade F. Clades D and E merge in the MP tree. *Cryptantha jonesiana, C, johnstonii*, and *C. humilis* shift to become sister to *C. creuzfeldtii* in the MP tree. The relative branching order of many of the species in Clades D and E changes in the MP tree (see Figures 14 and 15 - i.e. *C. rugulosa, C. setossisima, C. flava, C. flavoculata*, etc.). *Cryptantha flavoculata* and *C. setossissima* are the only species that change clades completely (Figures 14 and 15). Clade F remains unchanged with the exception of *C. oblata* and *C. stricta* that shift into Clade B in the MP tree topology.

Permutation tests: The permutation test showed that the MP tree differed significantly from a distribution of trees that were built from 100 random permutations of the original data set ($p \leq 0.01$), for both the entire data set, and a smaller data set where only one individual per species was included.

Homology assessment: Bands extracted and sequenced from the silver-stained polyacylamide gel were readily aligned. Some of the bands were difficult to sequence, resulting in variability in the length of the sequences presented (Table 10).

Table 10. Alignment of a 275 bp band excised from the *EcoRI*acg and *PstI*acc primer pair acrylamide gel. Amplicons were readily aligned across taxa, supporting the homology of the band from different taxa. Bands from two separate accessions of *C. flava* were sequenced.

```
C. elata          TGTCTGGAAC TCTAATCGAT ATGGGATATC ANATTCTCTA ATACCATCTT
C. creuzfeldtii   TGTCTGGAAC TCTAATCGAT ATGGGATATC ATATTCTCTA ATACCATCTG
C. elata          :GTCTGGAAC TCTAATCGAT ATGGGATATC ATATTCTCTA ATACCATCTN
C. confertiflora  :::::::::: :::::::::: :TGGGATATC ATATTCTCTA ATACCATCTG
C. flava 2        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
C. paradoxa       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
C. flava 7        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

C. elata          GCAATGAAAC CTATTTTTTG TGAACACATG AATGCACATA ACAAAACCAA
C. creuzfeldtii   GCAATGAAAC CTATTTTTTG TGAACACATG AATGCACATA ACAA::::::
C. elata          GCAATGAAAC CTATTTTT:: :::::::::: :::::::::: ::::::::::
C. confertiflora  ACAATGAAAC CTATTTTTTG TTAACACATG AATGCACATA ACAAAACCAA
C. flava 2        :::::::::: :::::::::: :::::::::: ::TGCACATA ACAAAACCAA
C. paradoxa       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
C. flava 7        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

C. elata          GACTGATGGA TCTGTCAAAA TGTTTGAATC TTTGAATGAC ATCAGTGCAT
C. creuzfeldtii   GACTGATGGA TATGTCAAAA TGTTTGAATC TTTGAATGAC ATNAGTGCAT
C. elata          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
C. confertiflora  GACTGTTGGA TCTGTCAAAA TGTTTGAATC TTTGAATGAC ATCAGTGCAT
C. flava 2        GACTGTTGGA TCTGTCAAAA TGTTTGAATC TTTGAATGAC ATCAGTGCAT
C. paradoxa       :::::::::: :::::::::: :::::::::: :::::::::: ::::::GCAT
C. flava 7        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

C. elata          AATGTAAAAA ATTATTTAGT TTTTGAATAT GAAGTTCTTC AATTTAATGT
C. creuzfeldtii   AATGTAAAAA ATTATTTAGT TTTTGAATAT GATGTTCTTC AATTTAATGC
C. elata          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
C. confertiflora  AATGTACAAA ATTATTTAGT TTTTGAATAT GAAGTTCTTC AATTTAATGT
C. flava 2        AATGTACAAA ATTATTTAGT TTTTGAATAT GAAGTTCTTC AATTTAATGT
C. paradoxa       AATGTAAAAA ATTATTTAGT TTTTGAATAT GAAGTTCTTC AGTTTAATGT
C. flava 7        ::::::CAAA ATTATTTAGT TTTTGAATAT GAAGTTCTTC AATTTAATGT


C. elata          TCCGACAAAA CTATGAGCTG GTCTGCAT
C. creuzfeldtii   TTCGACAAAA CTATGAGCTG GTCTGCAT
C. elata          :::::::::: :::::::::: ::::::::
C. confertiflora  TTCGACAAAA CTATGAGCTG GTCTGCAT
C. flava 2        TTCGACAAAA CTATGAGCTG GTCTGCAT
C. paradoxa       TTCGACAAAA CTATGAGCTG GTCTGCAT
C. flava 7        TTCGACAAAA CTATGAGCTG GTCTGCAT
```

Discussion

Phylogenetic analyses:

ITS: ML analyses of both the entire data set and a data set that excluded species with partial ITS-1 or ITS-2 sequences support monophyly of the Oreocarya, indicate that the annual *Cryptantha* may be polyphyletic, and show that the annuals are a suitable outgroup for use in the AFLP analyses.

AFLP: Many species shifted or changed positions depending upon the type of phylogenetic analysis, MP or ME. The branch lengths of some taxa were notably longer than others (eg. *C. stricta* compared to *C. rollinsii*), and parsimony can be an inconsistent estimator under these conditions (Swofford *et al.* 1996). Parsimony tends to underestimate the true amount of change between two individuals unless the actual rate of change is small (Felsenstein 1978, Swofford *et al.* 1996). ME was a more appropriate analytical method for this data set since the rates of change between species were quite variable, based on differences in branch lengths (Figures 14 and 15). The permutation test demonstrated that there was phylogenetic signal in the data, and that signal was both intra- and interspecific, since tests on both the entire data set, and one where all but one individual per species was excluded yielded a significant p-value ($p \leq 0.01$). The relative branching order for many species changed between tree topologies; likely as a function of overall low phylogenetic signal for the deeper nodes of the trees.

Higgins (1971) proposed a qualitative phylogenetic arrangement of groups of species in the Oreocarya based upon morphological characters. None of the groups that he designated were recovered entirely in these analyses; however, some of the species

that he placed into a group are members of the same clade in the MP and ME analyses (Figures 16 and 17).

Environmental influence on species distributions: There are some interesting patterns that emerge when the soil type and habitat range occupied by individual species are considered in light of the section phylogeny. Clades D and E have several species (*C. celosoides*, *C. cinerea*, *C. confertiflora*, *C. flava* and *C. flavoculata*) that are found in a wide variety of soil types, and have extensive ranges. *C. celosoides* is found in the northern portion of the western United States and southern Canada including eight states and two provinces. *C. confertiflora* can be found in Utah, California, and northwest Arizona. *C. flava* is common in eastern Utah and western Colorado into Arizona and New Mexico. *C. cinerea* has a wide range including South Dakota, Nebraska, Kansas, Oklahoma, Texas, New Mexico, Arizona, Wyoming, Nevada, California, and into Mexico. *C. flavoculata*, can be found in almost any soil type in Colorado, Nevada, Wyoming, Arizona, and all of Utah. These species group together regardless of the analytical method (*C. flavoculata* shifts in MP). Empirical evidence overwhelmingly supports a strong correlation between environmental heterogeneity (both biotic and abiotic) and genetic heterogeneity (Clausen, 1951, Antonovics 1971, Hedrick *et al.* 1976, Linhart and Grant 1996). Soltis and Soltis (1996), in a phylogenetic analysis of *Schidea*, showed that species found on more than two of the Hawaiian islands clustered at the base of the tree. Similarly, the generalist species of *Cryptantha* may share high levels of genetic variation and thus cluster in the analyses presented here (Figure 14, Figure 15). An alternative explanation for the close phylogenetic relationships among the wide-

Figure 16. Comparison of Higgins proposed relationships within the Oreocarya and the ME tree. The numbers above branches are bootstrap values generated with 100 replicates. Values less than 50% are not shown. Numbers and brackets on the Higgins tree represent groups that he designated, while the letters and brackets on the ME tree represent arbitrarily defined clades discussed in the text of the paper.

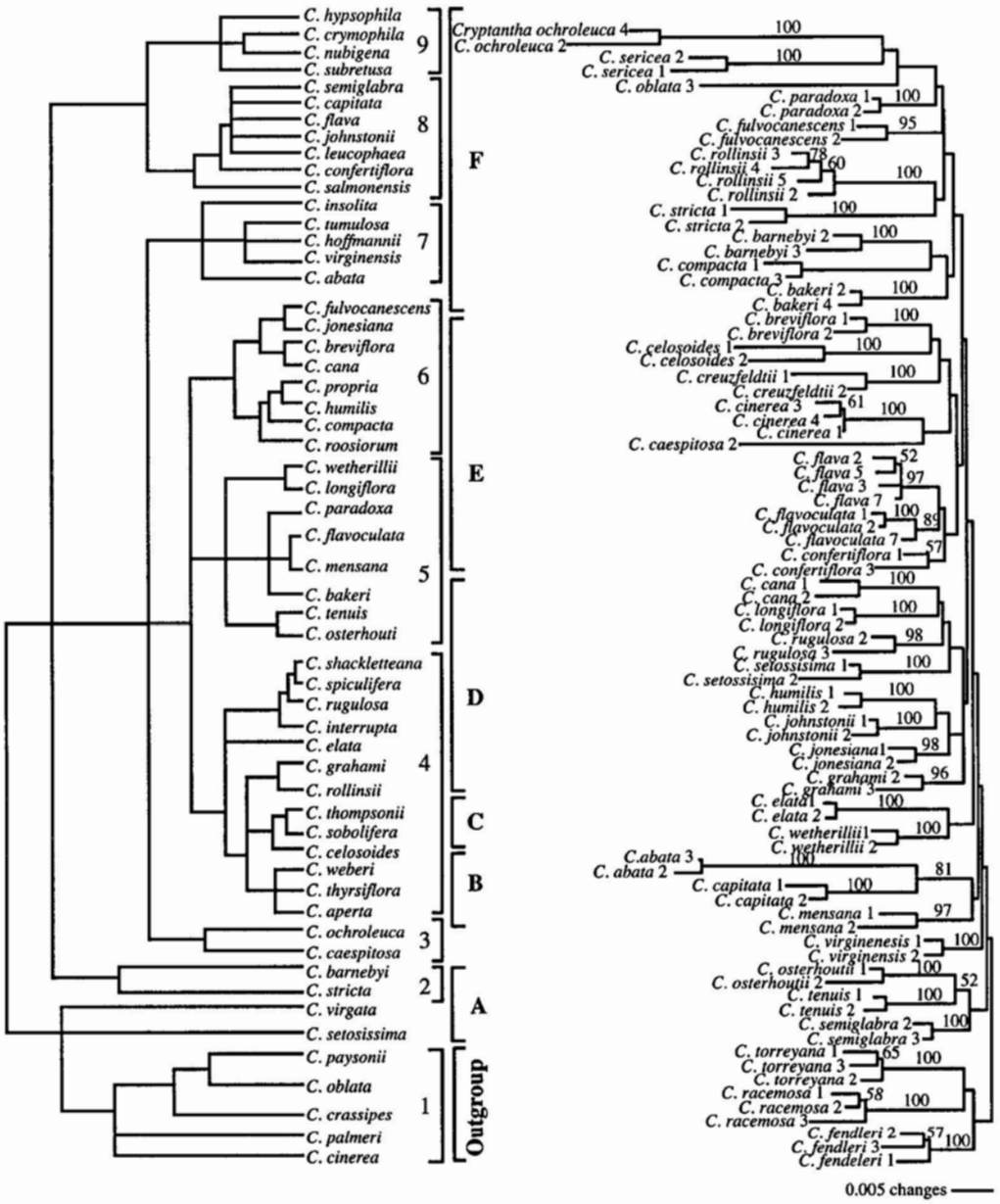
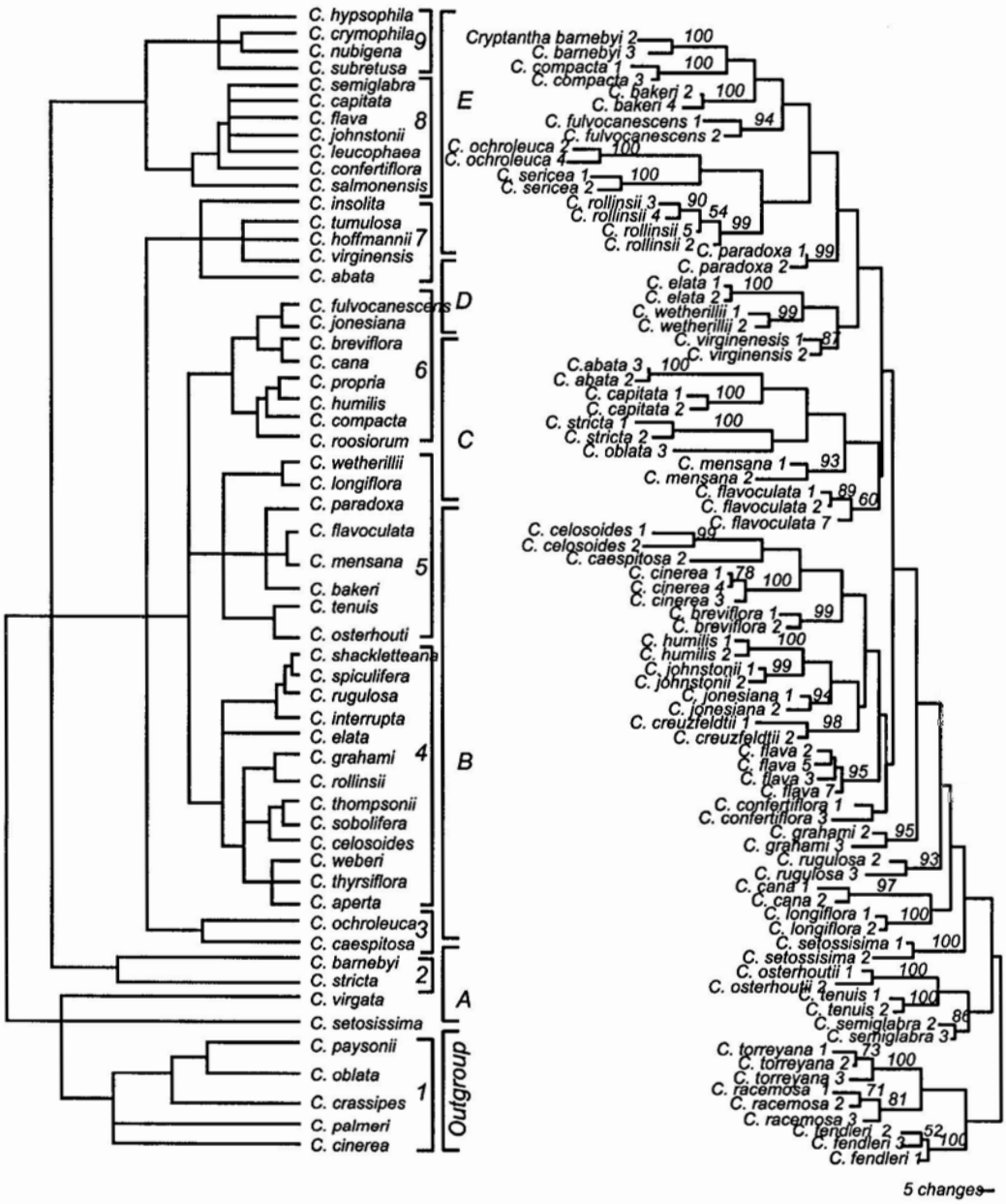


Figure 17. Comparison of Higgins proposed relationships within the Oreocarya and the MP tree.The numbers above branches are bootstrap values generated with 100 replicates. Values less than 50% are not shown. Numbers and brackets on the Higgins tree represent groups that he designated, while the letters and brackets on the ME tree represent arbitrarily defined clades discussed in the text of the paper.



ranging taxa in *Cryptantha* is that they share a generic, plastic phenotype. Higgins (1971) and Cronquist *et al.* (1984) have emphasized the high degree of phenotypic variation present in *C. celosoides*. Substantial morphological and ecological divergence may be associated with high levels of genetic identity, as was found in allozyme comparisons in *Tetramolopium* (Lowrey and Crawford 1985), and *Bidens* (Helenurm and Ganders 1985) from Hawaii. Plants typically respond to local conditions with plastic changes in morphology or physiology (Bradshaw 1965, Schlicting 1986, Sultan 1987). Plastic responses to environmental conditions allow organisms to express selectively advantageous phenotypes in a broad range of environments (Donohue *et al.* 2001). The fact that little variation was found in sections of DNA that are typically classified as 'rapidly evolving' and are used in most species-level studies (e.g., ITS, ETS, *adh*, *rps*, and *waxy*) provides some support for plastic environmental responses in these widespread species. That is, there appears to be variation in *Cryptantha* for the ability to colonize different substrates, however there is low genetic variation in genomic regions that are under little selective pressure, indicating that the colonization ability may be a plastic environmental response not under genetic control.

Island-like habitat patches may be identified on several scales, including mountain ranges surrounded by deserts, forest patches surrounded by clearings, and restricted soil substrates surrounded by contrasting soils (Levin 2001). For example, the retreat of glaciers in the Andes provided new habitats open for invasion, as demonstrated by the numerous species of *Espeletia* that vary in habit, stem morphology, energy allocation, and leaf pubescence (Monasterio and Sarmiento 1991). Well-preserved glacial deposits and geomorphic features such as moraines and cirques show that glaciation was

extensive in parts of the intermountain west during the Pleistocene epoch (Tidwell *et al.* 1972). Fossils of *Cryptantha* have been identified that date to the Miocene (24 MYA) and Pliocene (5 MYA) epochs (Leonard 1958, Gabel *et al.* 1998), implying that some species of *Cryptantha* were established prior to the last ice age. However, many species are located in regions that were affected by the Pleistocene glaciations, and these species may be much younger than the fossils from Nebraska, South Dakota and Texas. Lindsay and Vickery (1967) demonstrated that some habitats in the Great Basin are less than four thousand years old using a natural time clock provided by the recession of glaciers and lakes in the Bonneville Basin of Utah. The overall low genetic diversity found in the Oreocarya is likely a function of recent speciation. Most phylogenetic studies of island species rely on genetic markers typically used for population-level studies (Francisco-Ortega *et al.* 1996, Soltis and Soltis 1996). Levels of variation in the Oreocarya are similar to those found in island species, based on the need for similar population-level approaches to delimit species relationships in *Cryptantha* and island endemics. The data presented here support the species status of the Oreocarya as evidenced by the consistently high bootstrap values grouping individuals of like species (Figure 14, Figure 15).

Clade F has three species that are narrow endemics. *C. ochroleuca* is found only in the pink/red limestone of Garfield county, Utah. *C. barnebyi* is an endemic local to Uinta county, Utah, and is found growing on white barren shale knolls. *C. compacta* is endemic to Millard, Tooele, and Beaver counties in Utah and is said to grow only on Sevey Dolomite (Higgins 1971, Welsh *et al.* 1993). These species have colonized harsh substrates where there is little competition from other species (Welsh *et al.* 1993). The

soils of the Bonneville Basin where *C. compacta* is found tend to be saline and alkaline (Higgins 1971, Cronquist *et al.* 1972). Limestone and dolomite both contain carbonate (Coch and Ludman 1991), and carbonate soils are characterized as having unfavorable water regimes (Linhart and Grant 1996). The clay-sized particles that comprise shale cause water to diffuse at a slow rate through the small soil pores (Coch and Ludman 1991). Water is therefore limited to some degree in all of these soil types, a condition that is exacerbated by the low annual rainfall for the area (Cayan et al. 1998). Though the soil types and geographic distributions of these specialist species do not overlap, one possible explanation for their grouping is that the species are recently derived from an older, wide-ranging species. Recently derived species should be more alike than those of greater age, because genetic identities decline over time (Nei 1987, Levin 2001). A possible corollary to this hypothesis is that the ancestor to *C. barnebyi*, *C. compacta*, and *C. ochroleuca* was adapted to a soil type where water was limited, and glaciation lead to disjunction of the ancestral population resulting in these 3 extant species.

Cryptantha creuzfeldtii, *C. jonesiana*, and *C. johnstonii* group in the MP tree while (only *C. jonesiana*, and *C. johnstonii* group in the ME tree). These species are endemic to the San Rafael Swell in central Utah, and grow on unique geologic substrates exposed in the Swell. *C. johnstonii* is found on sandy-clay substrates; *C. jonesiana* on the clay slopes of the Moenkopi and Summerville geologic formations. *C. creuzfeldtii* is morphologically similar to *C. jonesiana*, but is found growing in shale and clay in the Blue Gate member of the Mancos Shale Formation (Cronquist *et al.* 1984). The ranges of these species are sharply delimited by substrate (Higgins 1971 and pers obs), and there is potential that the environment was an integral factor in the speciation process for these

taxa. The most likely scenario would be for an ancestral species to have colonized one of the substrates, with subsequent spread to the other substrate patches. The hypothesized scenario for *Cryptantha* fits a model for adaptive radiation in oceanic archipelagos - one island habitat is colonized, and the ancestral species spreads to other islands (substrate patches) that are similar in substrate. From these habitats the species invades 'new habitats' resulting in the evolution of new species (Carlquist 1965, 1974). Recent molecular phylogenetic studies of island species have offered support for this model (Francisco-Ortega *et al.* 1996).

There are several main patterns for plant distribution in Utah correlated on some level with the 'lines of least resistance' for migration routes. Dry canyon slopes along the major river systems in Utah serve as routes for the movement of plant propagules (Welsh *et al.* 1993). *C. osterhoutii* and *C. tenuis* are found along the Colorado river drainage. These species are sister taxa in both the ME and the MP tree (Figures 14 and 15). One explanation is that the Colorado River served as a migration route for their common ancestor.

To gain a more thorough understanding of the relationships among these species, individuals from across the entire range, encompassing several different populations should be sampled. Comparing the phylogenetic relationships among individuals within this and other widespread Oreocarya species would provide a useful foundation to compare the remainder of the section, especially given the low levels of genetic variation in Oreocarya as a whole (Tables 6 and 7, Figures 12 and 13).

Conclusions: Genetic variation among perennial species of *Cryptantha* within the Oreocarya appears to be limited. Typical species-level molecular tools were not

sufficient to explore the phylogeny of the group. This may reflect the recent adaptive radiation that occurred following the last major glaciation period in the Intermountain West. More appropriate approaches to resolving phylogenetic relationships in this group include markers that are more often used at the population or sub-species level. AFLP data provided a first estimate of species relationships in the *Oreocraya*. The data showed that bands between species are homologous, supporting the use of AFLP data for interspecific studies on closely related, or recently derived taxa. Additionally, these data demonstrated that individual species were well-marked genetically, since phylogenetic analyses consistently grouped individuals from like species together (Figure 14 and Figure 15). Nevertheless, the low genetic variation in this group argues for data from additional primer pairs to resolve the relationships among Oreocaya species more completely.

In general, plant species distributions tend to be correlated with geology in the Intermountain West. This was also true for Oreocarya, where interesting interactions between species distributions and geology were evident. Additional sampling within species across the species range would address several untested hypotheses about the course of the adaptive radiation and the evolution of specific traits (e.g., water transport on substrates with low water potentials, floral morphology, and breeding system) within the group.

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Evolutionary status of heterostyly in *Cryptantha* Section Oreocarya- inferences from phylogenetic analysis of AFLP data

Introduction:

Historically, studies of mating systems in plants have been addressed at a population level, using microevolutionary approaches (Barrett *et al.* 1996, Weller and Sakai 1999). However, the evolutionary processes that lead to the diversification of plant lineages are intrinsically linked to changes in mating patterns that govern the genetic structure of populations. Interspecific comparisons of reproductive characters associated with specific mating systems represents a largely untapped source of insight into the ecology and evolution of plant reproduction (Barrett *et al.* 1996). With the development of molecular systematics, several recent studies examined the evolutionary hypotheses of plant reproduction in a comparative framework. Specifically, ecological and systematic methodology is combined to test explicit hypotheses about evolutionary transitions in reproductive characters that have led to present day plant breeding systems (Barrett *et al.* 1996, Weller and Sakai 1999).

Heterostyly is one example of a breeding system that has been studied in a phylogenetic framework (Eckenwalder *et al.* 1986, Graham and Barrett 1995, Kohn *et al.* 1996). Heterostyly comprises a suite of traits including reciprocal placement of stigmata and anthers in two floral morphs (pins and thrums), diallelic self-incompatibility, and assorted secondary pollen and stigma polymorphisms (Vuilleumier 1967, Ganders 1979, Barrett 1992). Two models have been proposed to describe the progression in the

evolution of the major components of heterostyly (Charlesworth and Charlesworth 1979, Lloyd and Webb 1992). In the Lloyd and Webb (1992) model, reciprocal herkogamy (spatial separation of the stigmata and anthers) evolves initially to promote more efficient outcrossing. This transition is then followed by self-incompatibility. In contrast, the Charlesworth and Charlesworth (1979) model posits that self-incompatibility evolved first, to reduce the deleterious effects of inbreeding depression, and approach herkogamy followed due to the advantages of reducing the loss of gametes in incompatible matings. The comparative studies of Eckenwalder *et al.* (1986), Graham and Barrett (1995), Kohn *et al.* (1996) and Barrett *et al.* (1996) all found systematic data supporting the Lloyd and Webb model (1992). The conclusions of these previous studies relied heavily on character-weighting assumptions (Barrett *et al.* 1996).

Few groups of heterostylous taxa contain individuals that represent intermediate forms of the breeding system (Barrett *et al.* 1996). The section Oreocarya within the genus *Cryptantha* (Boraginaceae) is a group of approximately 40 desert perennials in the Colorado Plateau region of eastern Utah, western Colorado, and northern Arizona. This group includes many heterostylous species (Cronquist 1984), some of which lack the self-incompatibility and/or heteromorphic characters typically associated with the fully developed heterostylous condition (Casper 1985, B.B. Casper, University of Pennsylvania, unpublished data, Chapter 1, Chapter 2). Due to this variability in breeding systems within closely related species, the section Oreocarya may represent a model system with which to study the evolution of heterostyly.

In this study, amplified fragment length polymorphism (AFLP) data were used to construct a phylogeny for 35 members of section Oreocarya. Hand pollination

experiments were performed previously (Chapter 2) to determine the presence/absence of self-compatibility in seven monomorphic and nine dimorphic Oreocarya species. These breeding system characters were mapped onto the molecular phylogeny for Oreocarya using both a maximum likelihood and a maximum parsimony approach to determine the order of evolution of reproductive characters associated with the breeding system for *Cryptantha*. These patterns were then used to make inferences about the proposed models (Charlesworth and Charlesworth 1979, Lloyd and Webb 1992) for the evolution of the heterostylous syndrome.

The specific questions that were addressed include: 1) Do alternative methods for mapping characters onto phylogenetic trees yield the same conclusion regarding the favored ancestral state reconstruction in *Cryptantha*? 2) What is the ancestral state for the breeding system in *Cryptantha*? 3) Which model, if either, is supported by the phylogenetic relationships in *Cryptantha*?

Methods:

Taxon Sampling: Location data for the species used in this study were reported in the previous chapter (Chapter 4, Table 6). Two individuals from each species were sampled, with the exception of *C. cinerea* (3 individuals), C. *flava* (4 individuals), and *C. rollinsii* (4 individuals). Three annual species (*C. fendleri*, *C. racemosa*, and *C. torreyana*) from *Cryptantha* section Krynitzkia were used as an outgroup.

Molecular methods: DNA extraction and AFLP reactions were performed as described previously (Chapter 4 Materials and Methods).

Phylogenetic analyses: Phylogenetic analyses were performed using PAUP*4.08b (Swofford, 2001). Taxa were coded 0 for absence and 1 for presence of AFLP bands.

Maximum Parsimony (MP) and Minimum Evolution (ME) were used as optimality criteria. The starting tree was generated using stepwise addition with 1000 random taxon addition replicates since an initial survey showed that islands of optimal trees were present.

Parsimony mapping Optimal trees were imported to MacClade version 4.0 (Maddison and Maddison 2000), and reproductive characters were mapped onto the tree using an unweighted approach, since the Maximum Likelihood analysis estimated the separate gain and loss rates of self-incompatibility and heterostyly, thereby eliminating the need to make assumptions about whether the loss or gain of a complex trait should be favored (Barrett *et al.* 1996, Kohn *et al.* 1997, Takebayashi and Morrell 2001). Tracing characters determined the minimum number of changes along the branches of a particular tree that were required to account for the distribution of character states found at the tips of the branches in the terminal taxa. This most parsimonious reconstruction (MPR) procedure resolves the states present at the internal nodes of the tree and along the branches where changes of character state are assumed to have occurred (Weller *et al.* 1995, Maddison and Maddison, 2000).

Maximum Likelihood mapping Characters were also mapped onto the phylogenetic trees (Minimum Evolution and Maimum Parsimony) using a Maximum Likelihhood approach. Maximum Likelihood solutions make the observed data most likely given a model of the process under investigation (Edwards, 1972). In the case of mapping breeding system characters, this means reconstructing the ancestral character states to make the character states observed among the extant species most probable, given some statistical model of the way evolution proceeds. The program Discrete (Pagel 1994, 1997, 1999a,b) was used

to map breeding system characters (self-compatibility and heterostyly) onto the Minimum Evolution and Maximum Parsimony trees. The program requires a bifurcating phylogeny (no polytomies), and data on all species. For this reason, two analyses were run: one including all taxa only investigating the evolution of the stylar morphs (because the phenotype of all taxa included in the study was known), and one including only those taxa for which the compatibility status was known. For the second analysis, all taxa with no data on compatibility were pruned from the original trees in Paup*4.08b.

The reproductive characters that were mapped include heterostyly (dimorphic=1 vs. monomorphic species=0) and self-incompatibility. The self-compatibility index (Chapter 2) was split into two categories (0.1 – 0.5 (labeled as 0 in the matrix) and 0.6-1.1 (labeled as 1 in the matrix)).

Three annual species (*C. torreyana*, *C. racemosa*, and *C. fendleri*) from section Krynitzkia (*Cryptantha*) were used as the outgroup. To date no annual species of *Cryptantha* have been described as heterostylous (Higgins 1971,Ganders 1979, Cronquist 1984); however, there are examples of other heterostylous annual taxa – including members of the closely related genus *Amsinkia* (Ganders 1979). The compatibility status of these taxa is unknown, and so these taxa were coded as unknown for compatibility in the Parsimony mapping, and as homostylous for all of the mapping analyses. It is extremely unlikely given the size of the flowers that there is any spatial separation between stigmata and anthers in the annual *Cryptantha*.

A permutation test was performed to determine if the most parsimonious distribution of heterostyly differed significantly from that of a random distribution on either the ME or MP tree (Faith 1991, Swofford *et al.* 1996). This test was repeated on a

data set that excluded all but one member of each species to test whether phylogenetic structure, when present, was due to intraspecific or interspecific factors.

Terminology The term heterostyly typically implies those species that have 2 or three morphs that differ in the reciprocal placement of stigmata and anthers, and a diallelic self-incompatibility system (Barrett 1992). Homostyly has been used to describe individuals with equal stigma and anther position in an otherwise heterostylous population (Ganders 1979), and those species that have only one morph within a species. I use monomorphic and homostylous interchangeably in this paper. The degree of self-compatibility varies in many dimorphic species of Oreocarya (Chapter 1), and here the terms dimorphic and heterostylous are used synonymously after Lloyd and Webb (1992).

Results

Mapping with Parsimony

Parsimony analyses: Treating heterostyly as unweighted and unordered, 10 steps for the tree length were required, and the basal condition was homostylous with at least 9 gains of heterostyly and 2 reversals to homostyly (Figure 18).

The basal condition for self-compatibility was self-incompatible; and there were at least 4 gains of self-compatibility, and 4 reversals to self-incompatibility. Unweighted self-incompatibility mapped with 7 steps (Figure 19).

Distance analyses When heterostyly was unweighted, it added 10 steps to the tree length, and there were at least 7 gains of dimorphism - the basal condition was monomorphic (Figure 20).

Figure 18. Mapping heterostyly onto the Maximum Parsimony (MP) tree using unweighted Parsimony. The key demonstrates the colors associated with homostyly and heterostyly on the branches. The ancestral condition is predicted to be homostylous based on this analysis.

Figure 19. Mapping the self-compatibility index onto the Maximum Parsimony (MP) tree using unweighted Parsimony. The key demonstrates the colors associated with self-compatibility and self-incompatibility on the branches. In some cases the mapping of an ancestor was equivocal, and an additional color was added for this condition. The ancestral condition is predicted to be self-incompatible based on this analysis.

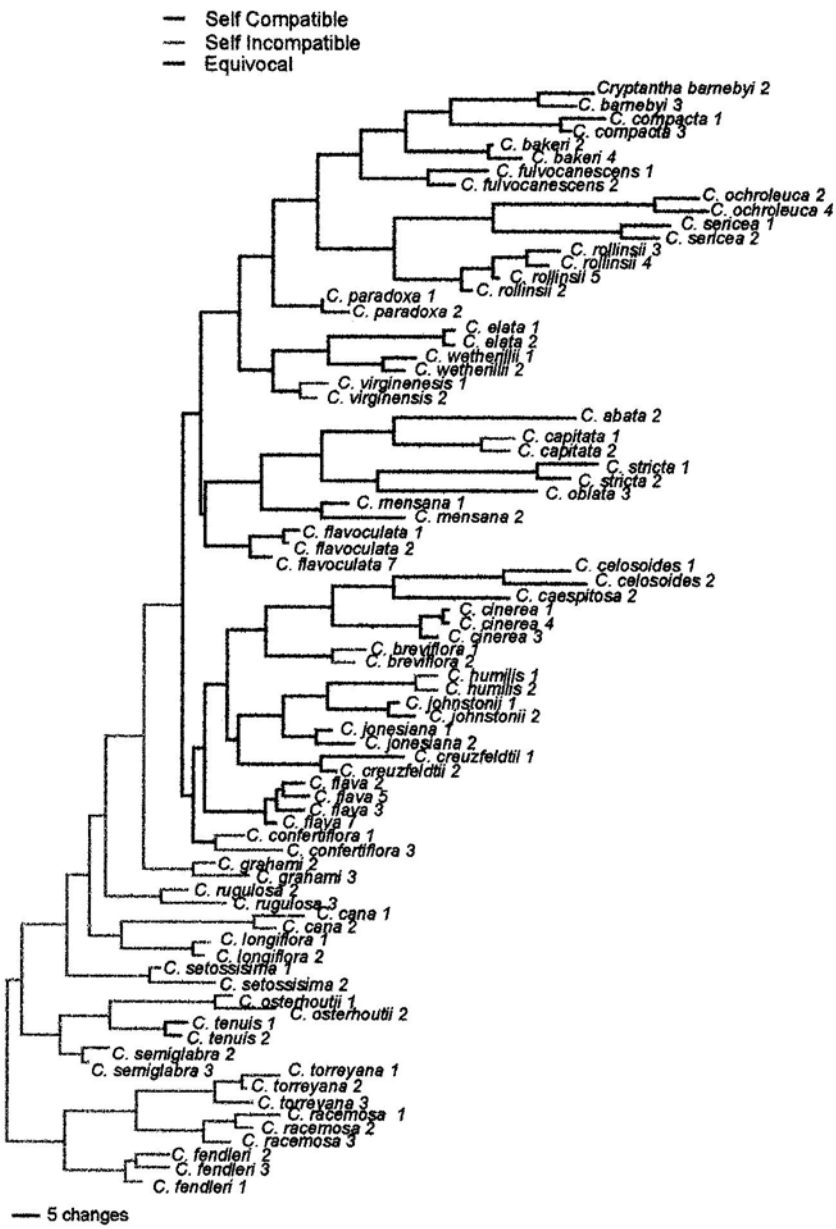


Figure 20. Mapping heterostyly onto the Minimum Evolution (ME) tree using unweighted Parsimony. The key demonstrates the colors associated with homostyly and heterostyly on the branches. In some cases the mapping of an ancestor was equivocal, and an additional color was added for this condition. The ancestral condition is predicted to be homostylous based on this analysis.

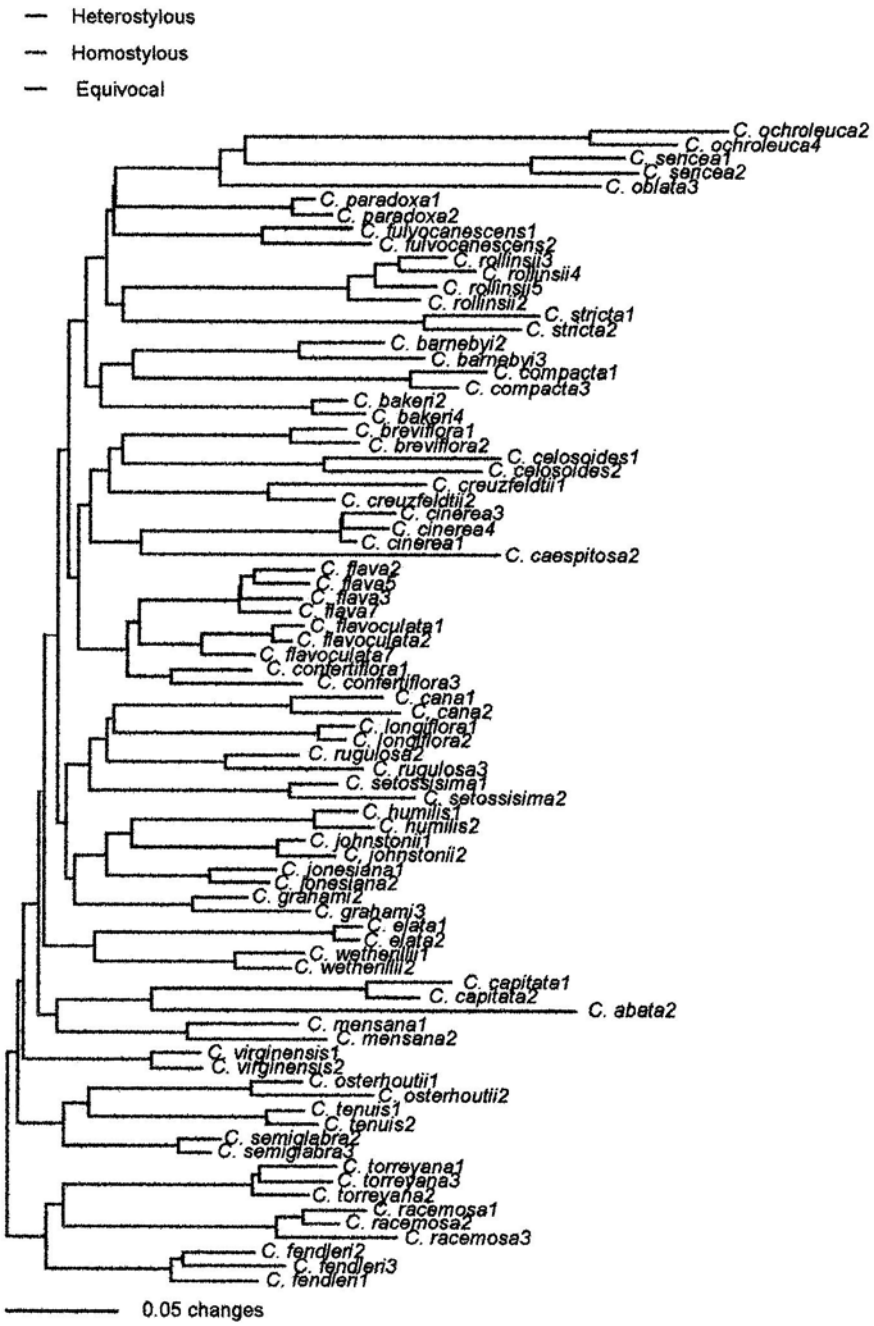
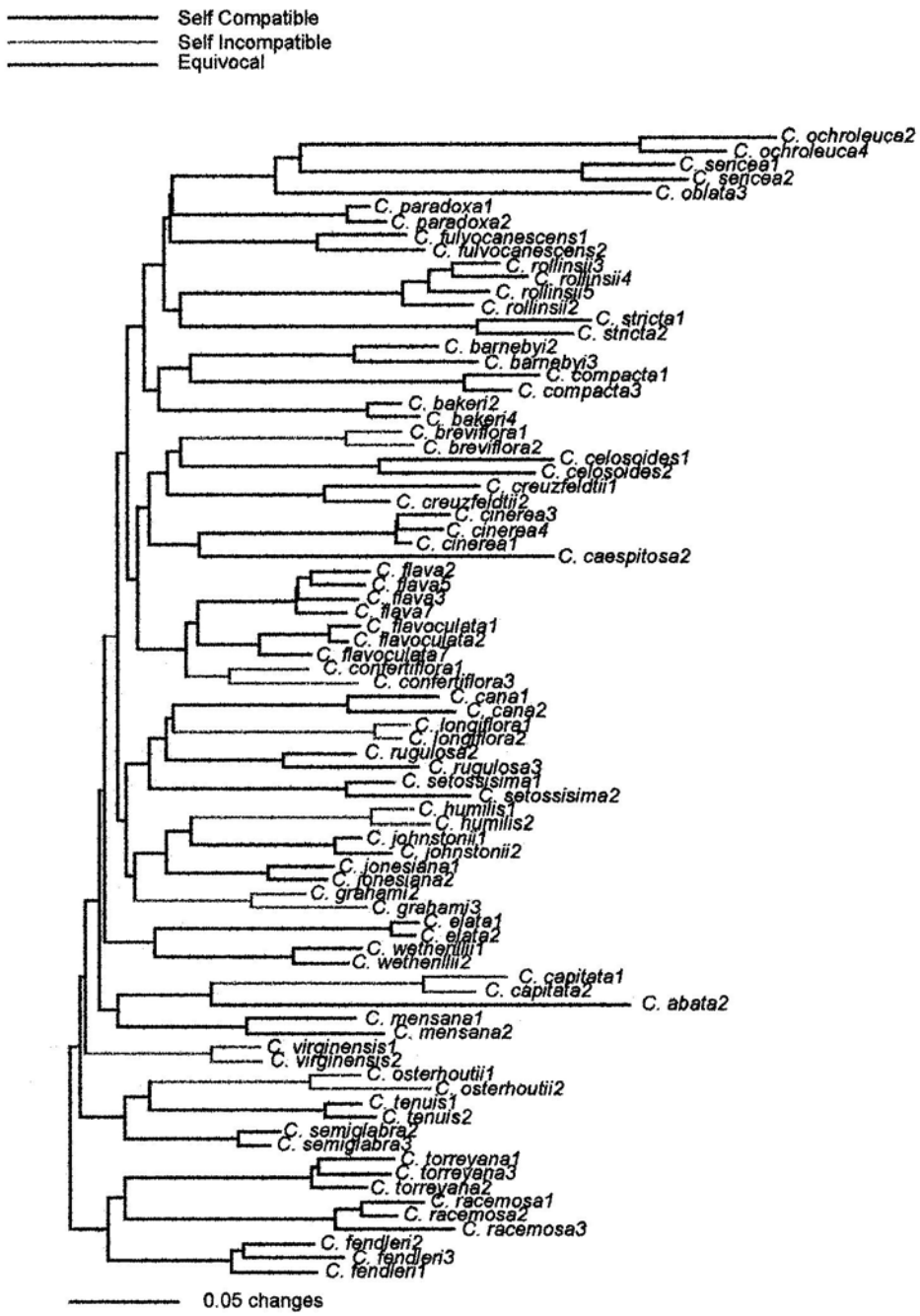


Figure 21. Mapping the self-compatibility index onto the Minimum Evolution (ME) tree using unweighted Parsimony. The key demonstrates the colors associated with self-compatibility and self-incompatibility on the branches. In some cases the mapping of an ancestor was equivocal, and an additional color was added for this condition. The ancestral condition is equivocal based on this analysis.



The ancestral condition for self-incompatibility was equivocal. The number of changes from one character state to another was unclear since many of the nodes were equivocal (Figure 21).

Mapping with Maximum Likelihood

The Discrete output yields both global and local estimates for ancestral states. The difference between these estimators is based upon whether the parameters of the Maximum Likelihood model of evolution are found separately for each combination of ancestral states (local) or the parameters are estimated only once (global) and not for any specific ancestral state, but as the single best set maximized over all possible states (Pagel 1999). The figures presented here report the character state predicted by both local and global likelihood scores in the table to the left of the phylogenetic tree for each species included in the analysis (Figure 22-25). The branches of the phylogenetic trees are coded to reflect the local estimates of ancestral states. It is important to note that the global and local estimates for the reproductive characters for the tips of the tree (the species) occasionally disagree with each other, or with the known phenotype for the analyses of the entire data set (Figure 22-25). When the data set is pruned to include only taxa for which the self-compatibility status is known, these inconsistencies are not found (Figure 23 and Figure 25).

Parsimony analyses For the analyses of the entire data set for the Maximum Parsimony tree (Figure 22), the predicted ancestral condition was heterostylous, and for the pruned data set (Figure 23), the predicted ancestral condition was homostylous and self-compatible. Both the global and local likelihood estimates for the basal ancestral condition support these conclusions for each data set (data not presented). The Maximum

Figure 22. Maximum Parsimony Tree with heterostyly mapped using Maximum Likelihood. The colors used in the phylogenetic tree are based upon the local estimators from the Discrete program output. The colors of the branches correspond to the styly condition as indicated in the legend. The ancestral condition for the stylar condition based on this analysis is heterostylous. The table to the right of the tree indicates the reproductive characters associated with the phenotype (P), the local estimate from the likelihood analysis (L), and the global estimate from the likelihood analysis (G).

Figure 23. Pruned Maximum Parsimony Tree with heterostyly and self-compatibility mapped using Maximum Likelihood. The tree was pruned to exclude all taxa for which the compatibility condition was unknown. Colors represent the possible character state combinations as indicated in the legend. The colors used in the phylogenetic tree are based upon the local estimators from the Discrete program output. The phenotype (P), the character state based on global estimators (G), and the character state based on local estimators (L) are included in the table to the right of the phylogenetic tree. Ho represents homostyly, and He represents heterostyly. The colored boxes around the abbreviations for the styly condition represent the compatibility and correspond to the legend. The ancestral condition for the breeding system characters based on this analysis is homostylous and self-compatible.

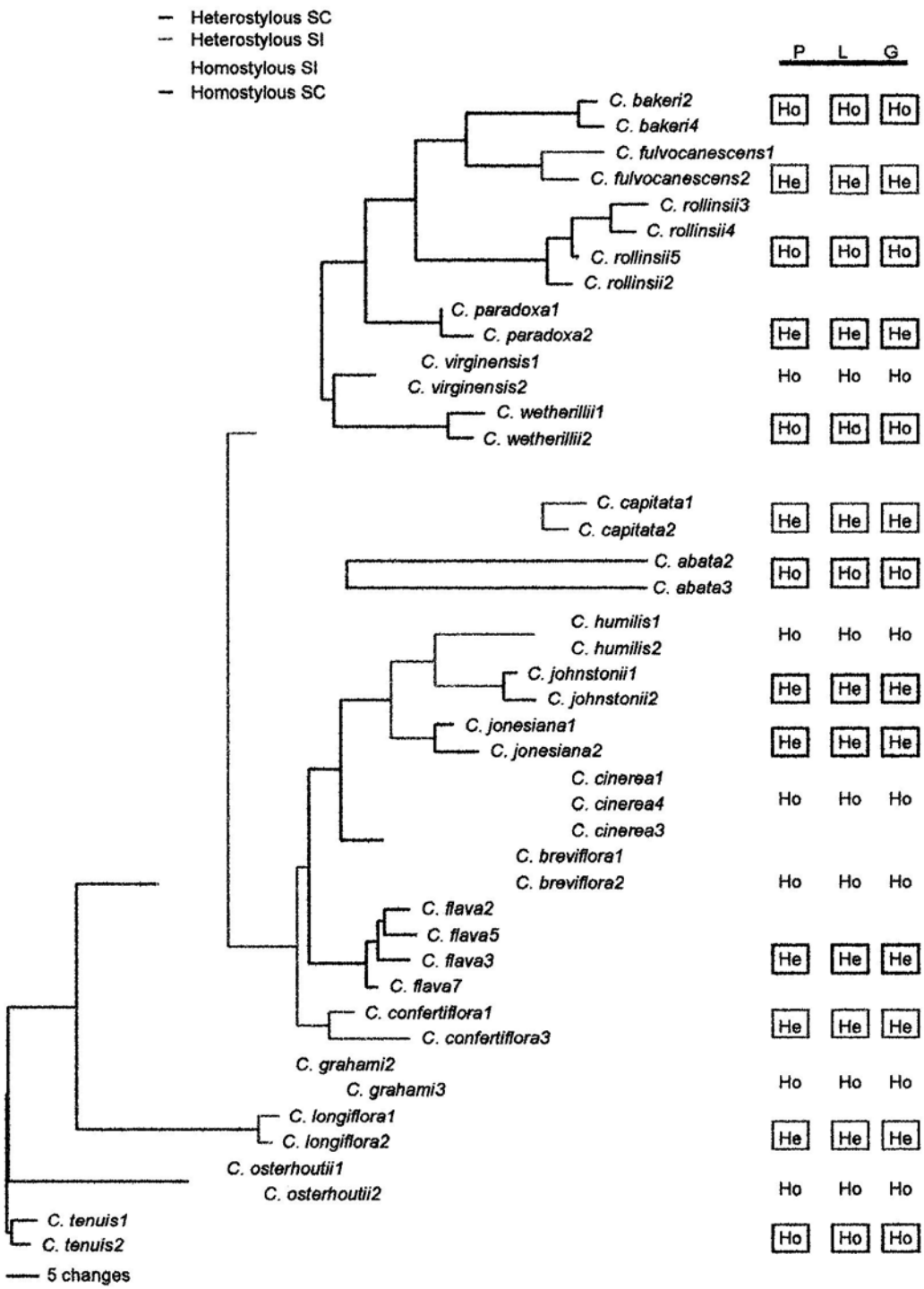


Figure 24. Minimum Evolution Tree with heterostyly mapped using Maximum Likelihood. The colors of the branches correspond to the styly condition as indicated in the legend. The colors used in the phylogenetic tree are based upon the local estimators from the Discrete program output. The ancestral condition for heterostyly based on this analysis is heterostylous. The table to the right of the tree indicates the reproductive characters associated with the phenotype (P), the local estimate from the likelihood analysis (L), and the global estimate from the likelihood analysis (G).

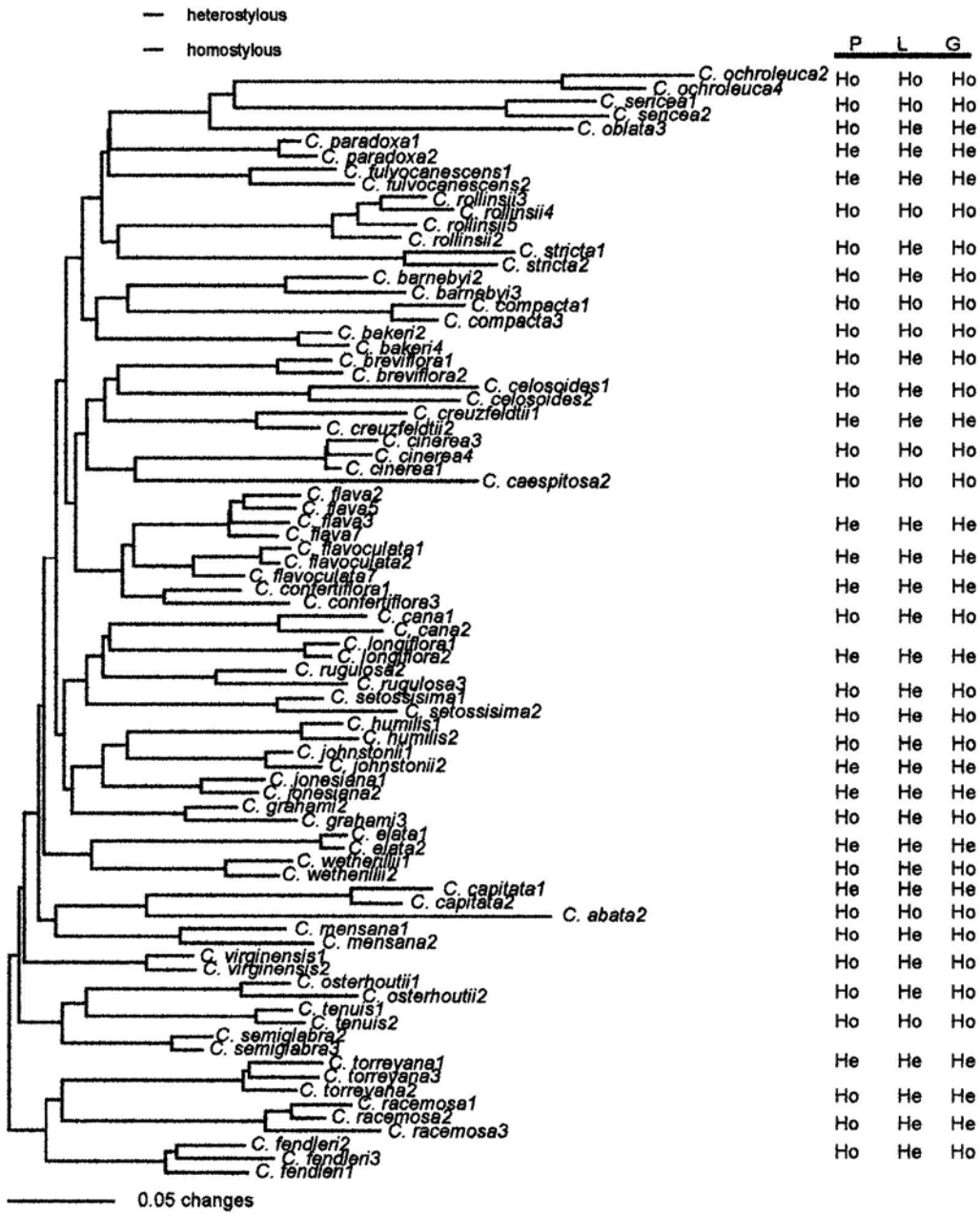
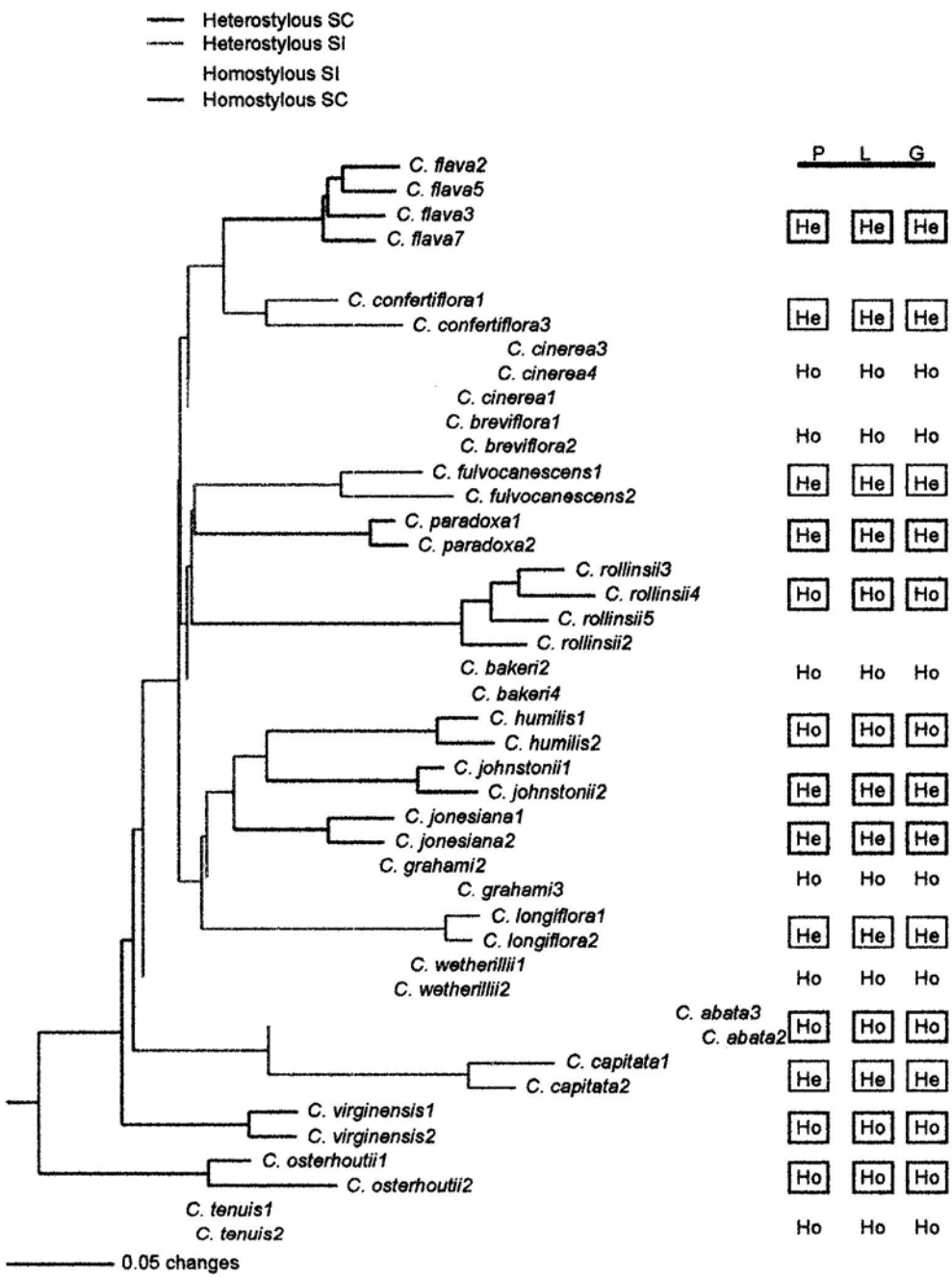


Figure 25. Pruned Minimum Evolution Tree with heterostyly and self-compatibility mapped using Maximum Likelihood. The tree was pruned to exclude all taxa for which the compatibility condition was unknown. Colors represent the possible character state combinations as indicated in the legend. The colors used in the phylogenetic tree are based upon the local estimators from the Discrete program output. The phenotype (P), the character state based on global estimators (G), and the character state based on local estimators (L) are included in the table to the left of the phylogenetic tree. Ho represents homostyly, and He represents heterostyly. The colored boxes around the abbreviations for the styly condition represent the compatibility and correspond the to legend. The ancestral condition for the breeding system characters based on this analysis is homostylous and self-incompatible.



Likelihood estimates for the loss of heterostyly (a transition from 1 to 0) were 0.02332 (about 2x the value for the gain of heterosyly) for the analysis based on the pruned data set and 0.03256 (about 100x the value for the gain of heterostyly) for the entire data set. The gain of self-incompatibility was favored slightly over the loss (0.02543 versus 0.01943) in the analysis of the entire data set.

Distance analyses The Maximum Likelihood mapping of the Minimum Evolution tree favored a heterostylous ancestor based on the entire data set (Figure 24), and a self-incompatible homostylous ancestor based on the pruned data set (Figure 25). Again, both the global and local estimates for likelihood favor these conclusions regarding the basal ancestral condition for the Oreocarya for both data sets (data not presented). The estimate for the transition from heterostyly to homostyly was 45.88789 (about 3.5x the value for the gain of heterostyly) for the pruned data set while the estimate based on the entire data set favored the gain of heterostyly with a value of 17.73769 (about 17x the value for the loss of heterostyly). The loss of self-incompatibility was favored by about 1.5x (36.6719 versus 21.58346).

Permutation tests

The distribution of heterostyly across the MP and ME tree differed from random when the complete data set was used for the trees ($p = 0.01$ when either tree was used as a constraint). When the data set was scaled down to include only one individual from each species, the distribution of heterostyly did not significantly differ from random on either the MP tree ($p= 0.71$) or the ME tree ($p=0.65$).

Discussion:

Permutation tests The permutation test indicated that the distribution of heterostyly across both the MP and the ME tree topologies was nonrandom when the entire data set was used in the analysis. However, when only one individual per species was used, the distribution of heterostyly across the tree did not differ significantly from random. This indicates that the phylogenetic signal underlying the structure of heterostyly across the tree was intraspecific, and not interspecific. With only one individual per species, the observed structure could be obtained by chance alone. The most likely explanation for this observation is linked to the ancestral state for the group – heterostyly was present in the ancestor of Oreocarya and has been randomly lost in some taxa. Thus the current distribution is random.

In contrast to the permutation test using heterostyly alone, a permutation test on all of the characters in the data set resulted in a signal that coded for a non-random tree topology for all of the taxa, and the scaled down data set (T. Marushak, Chapter 3). This means that there was significant signal for the relationships among the species in the ME and MP trees.

Mapping analyses Many authors have discussed the importance of character weighting in ancestral reconstructions (Barrett *et al.* 1996, Kohn *et al.* 1996, Omland 1999, Weiblen *et al.* 2000, Takebayashi and Morrell 2001). Complex character states (such as breeding system characters) are thought to be more easily lost than gained (Kohn *et al.* 1996, Hart *et al.* 1997, Omland 1997, Lee and Shine 1998, Omland 1999). Thus, the implicit assumption of simple parsimony that the probability of change from state A to state B for any character equals the probability of change from state B to state A can be invalid when

considering the evolution of complex characters (Omland 1999). Studies that address the gain or loss of complex characters using equally weighted parsimony can result in unlikely evolutionary scenarios (Kohn *et al.* 1996, Schoen *et al.* 1997, Bena *et al* 1998). More probable evolutionary histories can be recovered by estimating the separate gain and loss rates using Maximum Likelihood, thereby avoiding the need to make untested assumptions about rates of character state change. I will discuss the two selection models for heterostyly in light of an unweighted character scheme (Parsimony mapping analysis) and a Maximum Likelihood mapping analysis, where the rates of change for the reproductive characters are estimated, and incorporated into a model of evolution.

When heterostyly and self-compatibility were treated as unweighted characters in the Parsimony mapping analysis, the basal condition on the MP tree was homostylous and self-incompatible (Figure 18, Figure 19) and homostylous and equivocal (Figure 20, Figure 21) on the ME tree for compatibility. When the stylar condition alone is mapped onto the entire data set using Maximum Likelihood, heterostyly is the predicted ancestral state on both the ME and MP trees (Figure 22, Figure 24). However, when the data set is reduced to include only those taxa for which self-compatibility data are known, the predicted ancestral condition is homostylous and self-compatible for the MP tree (Figure 6) and homostylous and self-incompatible for the ME tree (Figure 25). Only two analyses are in agreement in favoring a homostylous ancestor that was self-incompatible (Figure 18, Figure 19, Figure 25). The fact that there is disagreement among the methods is likely linked to the fact that the reproductive states in *Cryptantha* appear to be relatively plastic and actively undergoing change, and alternate methodologies estimate the rates of change along the branches of a phylogenetic tree in different manners. Thus

if the rate of change for a particular character is high, alternative analytical methods will favor different conclusions based on the assumptions of the method.

The global and local estimates for the reproductive characters found at the tips of the tree (in the species) occasionally disagree with each other or the known phenotype when the stylar condition is mapped on the entire data set (Figure 21 and Figure 23). The subject of which estimator to use when modeling complex characters is a current subject of debate (Pagel 1999), and will not be addressed here. However, it is likely that the inconsistencies witnessed in these data are linked to the fact that the breeding system in the Oreocarya appears to be actively undergoing change, with species exhibiting a variety of partially heterostylous combinations (changes in style without reciprocal changes in anthers, variability in the degree of self-compatibility, etc. see Chapter 2 and 3). When there is a great deal of fluctuation between character states as is witnessed with these data, it becomes difficult to test between alternative hypotheses due to the loss and recovery of the complex character states of interest through time (Whiting *et al.* 2003).

Model interpretation

Mapping with Parsimony. These data lend some support to the Charlesworthian model, since the ancestral reconstructions indicate that the basal condition is homostylous, but in order to completely support their model the ancestral condition should be self-compatible (the MP tree maps a self-incompatible ancestor while the ME tree does not favor either state). There is one reversal to self-compatibility and back to self-incompatibility for *Cryptantha capitata* on the MP tree, and this specific case would fully support the Charlesworth model since *C. capitata* is heteromorphic, and there was a monomorphic self-compatible ancestor that leads to a self-incompatible, heteromorphic

species (*C. capitata).* The Lloyd and Webb model predicts self-compatible heteromorphic individuals as the ancestral condition for self-incompatible heteromorphic species. One would expect to see a slow transition from a monomorphic (but approach herkogamous- according to the model) state to a heteromorphic state with self-incompatibility developing at the tips of the tree, and this scenario is not evidenced with the Parsimony mapping analyses. Thus these data do not support the Lloyd and Webb model.

Mapping with Maximum Likelihood. When the entire taxon set is used in the Maximum Likelihood mapping analysis, the predicted ancestral condition on the MP tree is heterostylous (Figure 22), and one witnesses a number of reversals between heteromorphy and monomorphy throughout the tree. This scenario implies that there are random losses of heteromorphy in *Cryptantha*, and that the intermediate stages witnessed in the group are in the process of losing the heteromorphic state, thus not lending support to either model. The pruned data set reveals a different scenario (Figure 23), with a monomorphic self-compatible ancestor giving rise to monomorphic self-incompatible individuals, which then lead to heteromorphic self-incompatible species. This is the transition series that the Charlesworth model predicts, and is witnessed in the ancestral reconstructions that are located in the shallow part of the tree. There are additional reversals within the deeper parts of the tree, leading to 4 species that fit the classic definition of heterostyly. The transition series that leads to *C. confertiflora* favors the Charlesworth model, with a shift from monomorphic self-compatibility to monomorphic self-incompatibility to the heteromorphic self-incompatibility observed in *C. confertiflora.* The Lloyd and Webb model is supported by the transition from a

monomorphic self-compatible ancestor to a heteromorphic self-compatible individual, and finally to the heteromorphic self-incompatible condition in *C. fulvocanescens*. The other two cases of heteromorphic self-incompatible species (*C. capitata* and *C. longiflora*) do not support either model. The immediate ancestor to *C. capitata* appears to have lost heteromorphy and it was subsequently regained for *C. capitata*, while the immediate ancestor to *C. longiflora* was homostylous and self-compatible according to these data. The intermediate stages of the breeding system witnessed in the species at the tips of the tree then represent species in the process of losing some aspect of the breeding system, either self-incompatibility or heteromorphy.

Mapping ancestral states onto the ME tree for the entire taxon set yields results similar to those found on the Parsimony tree; that is, the predicted ancestral condition for the entire data set is heteromorphic (Figure 24). There are fewer shifts between heteromorphy and monomorphy throughout the tree than with the Maximum Likelihood mapping of the Parsimony tree. This is likely due to the differences in the estimates for gain and loss of heterostyly between the two data sets, the estimate for the rate of transition from heterostyly to monomorphy on the ME favored the gain of heteromorphy over the loss, which would explain the differences witnessed between the MP and the ME trees with regard to the mapping of heteromorphy. The analysis of the pruned data set yields little support for either model since all of the transitions to the classically defined heterostylous species (*C. capitata*, *C. longiflora*, *C. confertiflora*, and *C. fulvocanescens*) are from a homostylous self-compatible ancestor directly to the heteromorphic self-incompatible state associated with heterostyly (Figure 25). Again, these data support the

idea that breeding system is randomly changing in *Cryptantha*, and the intermediate stages observed in many of the extant taxa may represent the breakdown of heterostyly.

The fact that the species that show intermediate stages of heterostyly do not form a monophyletic group helps to demonstrate a random distribution for the loss of heterostyly. It appears that heterostyly is in the process of breaking down in several taxa that are scattered throughout the tree. It is possible that heterostyly in these species is breaking down by following an alternate path, since either the anther height or stigma height remains constant between morphs. Barrett *et al.* (2000) report a stable stigma-height dimorphism in *Narcissus*, and conclude that the polymorphism is maintained due to an increase in cross-pollination by reducing self-pollination and interference. Whether the dimorphisms found in *Cryptantha* are stable floral strategies is unknown. Additional populations of the species exhibiting these unique floral patterns need to be investigated to gain a better perspective on the frequency with which they occur.

There were at least 4 transitions from outcrossing (self-incompatible) species to selfing (self-compatible) species when the self-compatibility index was treated as a binary unweighted character (MP), 5 transitions when the self-compatibility index was mapped on the pruned MP tree, and 7 transitions when the self-compatibility index was mapped on the pruned ME tree using Maximum Likelihood. The evolution of selfing lineages from primarily outcrossing ancestors is one of the most common evolutionary transitions in the plant kingdom (Stebbins 1950, Grant 1981). However, it is unusual for dimorphic species to be self-compatible, since heterostyly is typically invoked as a mechanism to increase outcrossing, but there are other examples of dimorphic self-compatible taxa (Ganders 1979, Lloyd and Webb 1992, Barrett *et al.* 1996). The

ancestral reconstructions for the dimorphic, self-compatible state in Oreocarya imply that these species are the product of a breakdown in heterostyly (Fig 18-25).

Several other genera in the Boraginaceae show patterns that are suggestive of the breakdown of heterostyly. In *Anchusa*, there are reports of 2 species with multiallelic self-incompatibility (Dulberger 1970a, Phillip and Schou 1981), and the absence of stamen polymorphisms (*Anchusa officinalis*). Self-incompatibility typical of distylous species is absent in several *Amsinckia* species (Ray and Chisaki 1957, Ganders 1975c, 1976, Ornduff 1976, Weller and Ornduff 1977), and cryptic incompatibility has been demonstrated (Casper *et al.* 1988). Morphologically heterostylous and functionally dioecious species have been reported in *Cordia*, indicating a trend from heterostyly to dioecy (Opler *et al.* 1975). Opler *et al.* 1975 conclude that the primitive member of *Cordia* was likely heterostylous, as some of the data presented here indicate. *Cryptantha* species show similar trends to other genera in the Boraginaceae including the loss of self-incompatibility and the absence of stamen polymorphisms or stigma polymorphisms (Chapter 2 and 3).

It is possible that the intrasectional level is inappropriate for the study of the evolutionary steps involved in heterostyly, at least in *Cryptantha*. The perennial Oreocarya section may have undergone a recent adaptive radiation (Chapter 4) resulting in several closely allied species. Therefore, a study of the entire genus, including members of other sections, and species in geographically separated locales, such as South America may yield data with greater resolution. Alternatively, a study including representatives of the entire family Boraginaceae may be more informative, since many boraginaceous genera include heterostylous species (Ganders 1979), and several of these

have what appear to be intermediate stages or unusual variants of heterostyly – e.g. *Amsinckia*, *Anchusa*, *Cordia*, *Lithospermum*, and *Pulmonaria* (Vuilleumier 1967, Ganders 1979, Duhlberger 1992).

Conclusions There is wide interspecific variation in breeding system traits within the Oreocarya. Heterostyly in Oreocarya appears to be a basal condition (or at least somewhat ancestral) that is in the process of breaking down. Many of the heterostylous species demonstrate some level of self-compatibility, definitive clusters of pins and thrums were absent in some species, and no change in anther height between morphs was witnessed for 3 species (Chapter 3). There are two comparative approaches that should be taken to resolve these issues. First, more populations of those species that exhibit reproductive characters not usually associated with heterostyly need to be examined to gain a better understanding of how widespread these anomalies are in the perennial section of the genus. Second, an inter-sectional and/or inter-generic comparison should be combined with the results in this study. Many heterostylous genera in the Boraginaceae show patterns that indicate a breakdown in heterostyly. Phylogenetic relationships within the family are not well understood. Given the diverse breeding systems found throughout the Boraginaceae, a robust phylogeny for the group will yield better insight into patterns in mating system evolution.

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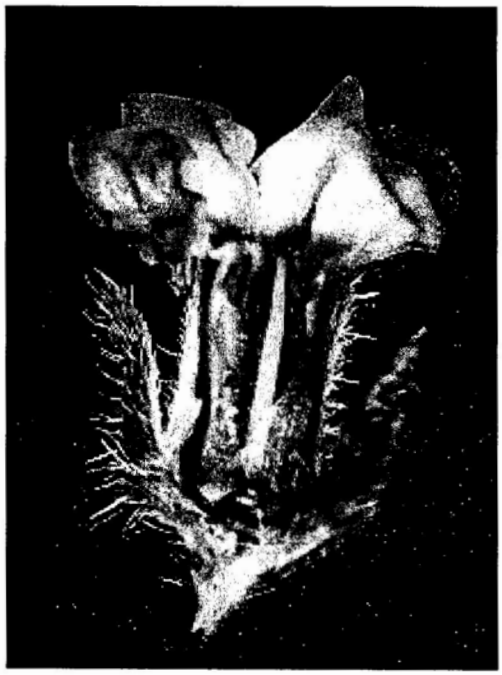
Conclusions

- Species of the Oreocarya have self-compatibility indices that range from 0.1 to 1.1; the entire range of compatibility is found in the group.
- All of the heteromorphic species in the Oreocarya studied here are at least partially self-compatible.
- The homomorphic species are more self-incompatible than the heteromorphic species.
- Homomorphic species may be less self-compatible due to the limited spatial separation between stigmata and anthers.
- The actual frequency of selfing in self-compatible, heteromorphic species may be relatively low, that is; other factors (herkogamy, delayed selfing) may promote outcrossing in these species.
- The ancestral character state for heterostyly and self-incompatibility in the Section Oreocarya, varied according to the analytical method used to analyze the data. It appears that these character states are actively undergoing change, and that there are many shifts between self-compatibility and self-incompatibility, and between heteromorphy and monomorphy.
- Not all of the partially or fully heteromorphic species showed differences among pins and thrums that designated them as statistically significant groups. *Cryptantha barnebyi*, *C. rollinsii*, *C. wetherillii* and *C. tenuis* show patterns that are representative of monomorphic species where anther and stigma height did not change in a reciprocal fashion between morphs. C. *jonesiana* and *C. creutzfeldtii* show a stylar morph type of variation where there are significant differences between stigma height but not anther height between morphs.

- Corolla height was significantly different between clusters for *C. barnebyi*, *C. creutzfeldtii*, *C. johnstonii*, C. *jonesiana*, *C. rollinsii*, *C. tenuis*, C. *semiglabra*, and *C. wetherillii*.
- Differences in corolla size in Oreocarya may be related to pollen flow, such that the morph with the larger corolla preferentially attracts pollinators.
- There was low overall genetic variation among species of the perennial section of the *Cryptantha*.
- AFLP bands were homologous within and between species, and the AFLP technique represents an informative source for genetic markers in species-level phylogenetic analyses.
- There was phylogenetic signal both within and between species in the AFLP data set used in the ME and MP analyses.
- Speciation patterns in Section Oreocarya were likely influenced by the unique geologic substrates present in the Intermountain West.
- The speciation patterns in some *Cryptantha* fit a model developed for adaptive radiation in oceanic archipelagos- one substrate was colonized, and the ancestral species spread to other substrates, finally invading new substrates where the evolution of a new species results.
- Individual species in Section Oreocarya are well-marked genetically (i.e. it does not appear that there was recent introgression among species), though the variation between species is low.
- The distribution of heterostyly on both the ME and MP trees did not differ significantly from random when only one individual per species was considered.

- The random distribution of heterostyly on the phylogenetic trees supported the concept that heterostyly in the genus is in the process of breaking down.
- It was unclear which, if either, model for the evolution of heterostyly was supported by the Parsimony mapping data since the most parsimonious reconstruction for the ancestor was homostylous and self-incompatible or equivocal for compatibility, depending upon which tree the character states were mapped onto.
- The Maximum Likelihood mapping data support a heterostylous basal condition for the group when the entire data set is used to map characters. When the data set is reduced to include only those members for which data on self-compatibility exists, a monomorphic self-compatible ancestor is predicted based upon the MP tree, and a monomorphic, self-incompatible ancestor is predicted based upon the ME tree.
- The fact there is disagreement among analytical methods regarding the determination of an ancestral state for the breeding system in *Cryptantha* Section Oreocraya implies that these characters are actively undergoing change, with many gains and losses of heteromorphy and self-incompatibility.
- Additional studies at the intergeneric level in the Boraginaceae will provide useful insight to the evolution of heterostyly, especially since many genera in the family show patterns that indicate a breakdown of heterostyly.

Appendix I. Photographs of examples of the floral morphology in *Cryptantha*. *C. tenuis* homostyle variant (A.), reverse herkogamous *C. tenuis* (B.), *C. compacta* demonstrating a monomorphic approach herkogamous morphology (C.), *C. creutzfeldtii* pin morphology (D.), *C. creutzfeldtii* thrum morphology (E.), and an example of an undissected *C. creutzfeldtii* flower (F.). Note the close proximity of the anthers and stigma in *C. compacta* (C.), and the spatial separation of the stigma and anthers in *C. tenuis* (B.). The thrum morphology (E.) resembles reverse herkogamy (B.).



A.

B.

C.

D.

E.

F.

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