# Utility of RAPD Markers in Evaluating the Status of the Hawaiian Tree Fern *Cibotium* ×*heleniae*[1]

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**Abstract:** Randomly amplified polymorphic DNA (RAPD) markers provide data consistent with the conclusion based on morphological characters that the recently named taxon *Cibotium* ×*heleniae* is indeed of hybrid origin. This assertion is supported by (1) placement of *C.* ×*heleniae* intermediate to the parent taxa, as determined by genetic similarity data; (2) location of *C.* ×*heleniae* individuals on a clade intermediate to the parent species in the cladistic analysis; and (3) clustering of the *C.* ×*heleniae* individuals between clusters of parental individuals in principal components analysis. Additivity of parental genetic markers in the putative hybrid ranged from 54 to 64%, providing additional though modest support for the hypothesized origin of *C.* ×*heleniae*. Our results indicate that RAPD data can be of considerable value in assessing potential hybridity of individuals in naturally occurring populations.

INVESTIGATIONS OF THE Hawaiian pteridophytes over the past decade have revealed over two dozen previously unrecognized putative interspecific hybrids. They are often easily recognized by the combination of their coexistence with the hypothesized parent species, morphological intermediacy, sporadic occurrence, and their irregular and abortive spores (Palmer 1998). Several of these putative hybrids have already been documented, vouchered, and named (e.g., Palmer 1998, Wagner et al. 1999). Of these, our study focuses on the putative hybrid individuals among species of *Cibotium* tree ferns on the island of O'ahu (Palmer 1998).

Four *Cibotium* species are currently recognized in Hawai'i (Palmer 1994). Two of these, *C. chamissoi* Kaulf. and *C. menziesii* Hook., are common in the lower montane forests of several islands and frequently grow sympatrically. These species are easily distinguishable; the diagnostic characters do not overlap (Palmer 1994). *Cibotium chamissoi* has matted, woolly, golden yellow trichomes formed of flattened cells found only at the base of the stipe, whereas *C. menziesii* has unmatted, stiff, straight, reddish brown trichomes composed of tubular cells located over the entire stipe and on the rachis (Palmer 1994). Leaf segments in these species are also distinct, with *C. chamissoi* having acuminate pinnae with deeply cut sinuses and long abaxial arachnoid trichomes, whereas *C. menziesii* has oblong pinnae with shallowly cut sinuses and punctate trichomes (Palmer 1994).

Palmer (1998) examined morphological variation within this group and identified putatively natural hybrids between these two species in several locations of the central Ko'olau Mountains on the island of O'ahu. These plants have been formally named *C.* ×*heleniae* and are recognized by stipes entirely covered with matted golden yellow hair composed of both tubular and flattened cells, intermediacy of pinna shape and sinus depth, as well as the presence of both punctate and arachnoid trichomes on the abaxial leaf sur-

TABLE 1

Comparison of Diagnostic Morphological Characters among the Three *Cibotium* Taxa
(Revised from Palmer 1998)

| Characters | *C. chamissoi* | *C. ×heleniae* | *C. menziesii* |
|---|---|---|---|
| Stipe trichomes | | | |
| Distribution | Basal | Entire stipe | Entire stipe |
| Color | Golden to mustard· | Reddish brown | Reddish brown to dark brown |
| Type | Woolly | Woolly, straight, or mixed | Straight |
| Cell shape | Flattened | Flattened, tubular, or mixed | Tubular |
| Frequency of short, thick, dark waxy trichomes | Absent | Scarce | Uncommon to common |
| Pinnule segments | | | |
| Width | 4–5 mm | 5.5–8 mm | 5–9 mm |
| Abaxial trichomes | Arachnoid | Arachnoid, punctate, and short arachnoid trichomes on punctae | Punctate |
| Depth of sinus between segments | 7/8 to pinna costa | 2/3 to 7/8 | 1/3 to 2/3 |

face. Other characters identified by Palmer (1998) are presented in Table 1.

Endemic island species, such as Hawaiian *Cibotium*, frequently exhibit large amounts of morphological variation, but appear to lack a correspondingly high amount of genetic variation that can be detected by enzyme electrophoresis (Rick and Fobes 1975, Helenurm and Ganders 1985, Lowrey and Crawford 1985, Crawford et al. 1987, 1988, 1990, Witter and Carr 1988, Wendel and Percy 1990, Aradhya et al. 1991, Lebot et al. 1991, DeJoode and Wendel 1992, Elisens 1992). More recently, molecular tools have been developed that have greater powers to resolve levels of genetic variation in populations of closely related island species (Williams et al. 1990, Vos et al. 1995, Blouin et al. 1996). Among these tools, randomly amplified polymorphic DNA (RAPD) markers have been repeatedly demonstrated to identify numerous loci from throughout the plant genome and identify variation among different individuals or populations based upon the presence or absence of amplified products (for example see Rieseberg et al. 1988, Arnold et al. 1990, 1991, Crowhurst et al. 1991, Rieseberg and Brunsfeld 1992, Morden and Loeffler 1999).

Although the RAPD markers are useful in revealing variation among closely related species, there are limitations to the technique. Because the DNA fragments are dominantly inherited (Williams et al. 1990, Hadrys et al. 1992), levels of heterozygosity cannot be accessed for individuals without knowledge of the parental banding patterns. Moreover, two nonhomologous bands that are of equal size can be mistakenly scored as homologous. There are also questions about repeatability (Lynch and Milligan 1994). However, many of the limitations of the technique can be avoided with proper sampling of populations, standardized methods, and the use of unambiguous, repeatable loci (Lynch and Milligan 1994). This method has been used successfully to determine hybrids in several flowering plant families including the Asteraceae (Caraway 1997), Cyperaceae (DeGreef and Triest 1999), Fabaceae (McCoy and Echt 1993), Gesneriaceae (Smith et al. 1996), Iridaceae (Arnold et al. 1991, Arnold 1993, Cruzan and Arnold 1993), Lactoridaceae (Brauner et al. 1992), Oleaceae (Marsolais et al. 1993), Poaceae (Welsh et al. 1991, Heun and Helentjaris 1993, Ayres et al. 1999), Ranunculaceae (Van Buren et al. 1994), Rosaceae (Crawford et al. 1993, Rieseberg and Gerber 1995), Solanaceae (Waugh et al. 1992), Typhaceae (Marcinko Kuehn et al. 1999), and Violaceae (Neuffer et al. 1999).

RAPD analyses were performed to evalu-

ate putative natural hybridization between *Cibotium* species. This study supports the interpretation that individuals of *C.* ×*heleniae* are indeed of hybrid origin and that RAPD markers are useful for verifying hybrid individuals within a natural population.

## MATERIALS AND METHODS

Nine *Cibotium* individuals (three from each of the parental species and three of the putative hybrid) were sampled and vouchered by Palmer (1998) from the 'Aiea Ridge hybrid population. These collections represented approximately one-fourth of the population at this site. Individuals of *C. chamissoi* and *C. menziesii* were chosen to best represent the diagnostic characteristics of each species. Individuals were also collected that displayed those morphological characters described above (see also Table 1) suggestive of a putative hybrid origin. Neither of the other two *Cibotium* species were found in the vicinity of the study.

Total DNA was extracted from 1 to 1.5 g of fresh leaf material using a modified CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1987, Stewart and Via 1993) and purified using cesium chloride equilibrium centrifugation (Sambrook et al. 1989). The DNA samples were vouchered and accessioned into the Hawaiian Plant DNA Library (HPDL) (Morden et al. 1996) as HPDL 614–616 (*C. menziesii*), HPDL 617–619 (*C.* ×*heleniae*), and HPDL 620–622 (*C. chamissoi*). DNA amplifications were performed in 25-μl reaction volumes consisting of 4 mM random 10-mer primer (Operon Technologies, Inc., Alameda, California), 0.2 mM dNTP, 1x polymerase buffer, 25 mM $MgCl_2$, 1 unit Taq Polymerase (Promega), and approximately 25 ng of isolated DNA overlaid with 2 drops of mineral oil. The samples were exposed to the following conditions on a temperature cycler (Hybaid OmniGene): one cycle at 94°C for 3 min, 35°C for 30 sec, and 72°C for 2 min; 43 cycles at 95°C for 45 sec, 35°C for 30 sec, and 72°C for 2 min; and a final cycle at 94°C for 45 sec, 35°C for 30 sec, and 72°C for 6 min. The amplification products were assayed on

1.5% agarose gels in 0.5x TBE (tris-borate-EDTA) (Sambrook et al. 1989). Gels were stained with ethidium bromide and photographed under UV light. Loci were identified based on the size of the band relative to a known molecular marker. The genetic markers (bands) for each individual were scored for each locus as being either present or absent. Only markers that were unambiguous, well amplified, and reproducible in replicate tests were scored.

The data were analyzed using Nei and Li's (1979) genetic similarity coefficient. This statistical test allows comparisons of genetic similarity within and among populations correcting for variation in experimental conditions and amplification artifacts (Lamboy 1994*a,b*). The bands were scored as a positive match (present in both subjects), a negative match (absent in both subjects), or as a mismatch (present in one subject and absent in the other). Using the formula $2sp/[2sp + (1 - s)]$, where $s =$ the percentage of positive and negative matches, $p =$ the percentage of positive matches, and $1 - s =$ the percentage of mismatches, a genetic similarity was calculated between two subjects. The final value is on a scale of 0 to 1, in which a value of 1 equals genetically identical individuals. We further investigated genetic relationships among the species and putative hybrids by principal components analysis (PCA) using the software program MINITAB (Minitab 1996).

The additivity of RAPD markers in the hybrid individuals was determined following the methods of Smith et al. (1996). Bands found in the hybrid were scored as common to both parent populations, shared with one of the parental populations, or unique to the hybrid. Markers that were common to both parents and those that were polymorphic in the parent populations were ignored in the analysis. Percentage additivity was calculated by adding the number of bands shared by the hybrid and the first parent to bands shared by the hybrid and the second parent, and dividing this sum by the total number of bands. Additivity was expected to be near 100% for an $F_1$ hybrid (Smith et al. 1996). As a test to determine if the putative hybrids were indeed

of hybrid origin, the data were reanalyzed as if each of the parents were the hybrid and the hybrid was one of the parents. A sharp decrease in additivity would be expected in these test scenarios if the original individuals were hybrids and not merely the result of common ancestry (Smith et al. 1996).

Cladistic analyses of the data were undertaken, and characters (fragment presence [1] or absence [0]) were analyzed using the nonadditive parsimony criterion (Fitch 1971) using PAUP 3.1.1 software (Swofford 1993). Heuristic tree searches of 1000 random replicates were executed using TBR branch swapping with the MULPARS option in effect (ACCTRAN optimization with equal weights). Parsimony jackknife (1000 replicates, SPR branch swapping, and five random entry orders per replicate [Xac program by J. S. Farris, unpublished]) analyses were applied to the matrix as an evaluation of topological support.

### RESULTS

Sixteen of the 17 primers produced good amplifications. Primer OPD-10 (GGTCTA-CACC) did not amplify the samples and was discarded from the analysis. The numbers of bands produced per primer ranged from one to nine with a mean of approximately 4.5 bands per primer. DNA amplification with 16 primers produced 163 scorable markers (Table 2), 89 of which were present in all individuals sampled. Of the remaining 74 variable markers, 13 were unique to *C. chamissoi*, seven were unique to *C. menziesii*, and one marker was unique to *C. ×heleniae* (Table 3). *Cibotium chamissoi* and *C. ×heleniae* shared 12 DNA markers that were not in *C. menziesii*. Similarly, *C. menziesii* shared 10 markers with *C. ×heleniae* that were not expressed in *C. chamissoi*. Of these 22 markers shared between a putative parent and *C. ×heleniae*, 13 were in all individuals of the hybrid and nine were polymorphic. Three markers were found to be present in both parental species but absent in *C. ×heleniae;* two of these were polymorphic in the parents. Although polymorphic, the remaining 28 markers were

TABLE 2

RAPD Primers Utilized and a Summary of Banding Patterns Produced by Each

| Primer | Primer Sequence | Loci | Variable Loci[a] |
|--------|-----------------|------|-------------|
| OPA-1  | CAGGCCCTTC      | 7    | 5           |
| OPA-13 | CAGCACCCAC      | 4    | 3           |
| OPB-17 | AGGGAACGAG      | 17   | 9           |
| OPB-18 | CCACAGCAGT      | 12   | 6           |
| OPB-20 | GGACCCTTAC      | 14   | 7           |
| OPC-7  | GTCCCGACGA      | 13   | 6           |
| OPC-8  | TGGACCGGTG      | 14   | 5           |
| OPC-12 | TGTCATCCCC      | 11   | 6           |
| OPD-3  | GTCGCCGTCA      | 15   | 8           |
| OPD-4  | TCTGGTGAGG      | 7    | 1           |
| OPD-5  | TGAGCGGACA      | 11   | 3           |
| OPD-7  | TTGGCACGGG      | 11   | 4           |
| OPD-8  | GTGTGCCCCA      | 8    | 2           |
| OPD-11 | AGCGCCATTG      | 1    | 0           |
| OPD-13 | GGGGTGACGA      | 10   | 6           |
| OPD-15 | CATCCGTGCT      | 8    | 3           |

[a] Includes those loci with markers variable within and among the species examined.

present in both species and the putative hybrids.

Genetic similarities were determined among individuals within and between the parent populations. The mean similarity coefficients within populations of *C. chamissoi* and *C. menziesii* were 0.937 and 0.955, respectively, and the mean similarity coefficient between these two species was 0.743. The mean genetic similarity between *C. chamissoi* and *C. ×heleniae* was 0.868 and between *C. menziesii* and *C. ×heleniae* 0.845 (Table 4). These data indicate that *C. ×heleniae* is genetically more similar to each parent than the parents are to each other.

Parsimony analysis of the data yielded a single shortest tree of 96 steps. The consistency and retention indices (CI and RI) were relatively high (0.771 and 0.805, respectively), indicating low levels of homoplasy among the characters in the data set. The tree was rooted with each of the putative parent taxa (Figure 1*A* and *B*). In both cases, *C. ×heleniae* individuals were basal in topological position to the putative parent taxa, which is the expected position for taxa of hybrid origin (McDade

## TABLE 3

Summary of RAPD Data Showing the Distribution among Primers of the Shared and Unique Markers within and among the Populations of *C. menziesii*, *C. chamissoi*, and *C. ×heleniae*

| Primer | Markers Shared Between *C. menziesii* and *C. ×heleniae* | Markers Shared Between *C. chamissoi* and *C. ×heleniae* | Markers Unique to *C. menziesii* | Markers Unique to *C. chamissoi* | Markers Unique to *C. ×heleniae* |
|---|---|---|---|---|---|
| OPA-1 | 1 | 1 | 0 | 1 | 1 |
| OPA-13 | 0 | 1 | 0 | 0 | 0 |
| OPB-17 | 1 | 1 | 2 | 1 | 0 |
| OPB-18 | 2 | 1 | 1 | 0 | 0 |
| OPB-20 | 0 | 0 | 1 | 2 | 0 |
| OPC-7 | 1 | 0 | 1 | 2 | 0 |
| OPC-8 | 0 | 1 | 1 | 2 | 0 |
| OPC-12 | 1 | 1 | 0 | 3 | 0 |
| OPD-3 | 0 | 1 | 0 | 1 | 0 |
| OPD-4 | 0 | 1 | 0 | 0 | 0 |
| OPD-5 | 1 | 0 | 0 | 0 | 0 |
| OPD-7 | 1 | 1 | 0 | 0 | 0 |
| OPD-8 | 1 | 0 | 0 | 0 | 0 |
| OPD-13 | 0 | 2 | 0 | 1 | 0 |
| OPD-15 | 1 | 1 | 1 | 0 | 0 |
| Total | 10 | 12 | 7 | 13 | 1 |

## TABLE 4

Genetic Similarity Coefficients within and among Populations of *C. menziesii* (M), *C. chamissoi* (C), and *C. ×heleniae* (H)

| Population or Individuals[a] | All Markers | | Fixed Markers[b] | |
|---|---|---|---|---|
| | Genetic Similarity Coefficient | Population Mean Values | Genetic Similarity Coefficient | Population Mean Values |
| M × C | — | 0.743 | — | 0.176 |
| M614 × M615 | 0.960 | | 0.852 | |
| M614 × M166 | 9.953 | | 0.828 | |
| M615 × M616 | 0.953 | 0.955 | 0.862 | 0.847 |
| C620 × C621 | 0.910 | | 0.754 | |
| C620 × C622 | 0.932 | | 0.769 | |
| C621 × C622 | 0.968 | 0.937 | 0.902 | 0.808 |
| M × H617 | 0.858 | | 0.494 | |
| M × H618 | 0.838 | | 0.421 | |
| M × H619 | 0.838 | 0.845 | 0.411 | 0.457 |
| C × H617 | 0.834 | | 0.455 | |
| C × H618 | 0.858 | | 0.503 | |
| C × H619 | 0.911 | 0.868 | 0.547 | 0.502 |

[a] Numbers indicate the HPDL accession number (Morden et al. 1996) of the individual within the population; lack of reference to an accession number identifies instances in which similarity was calculated based on population averages.

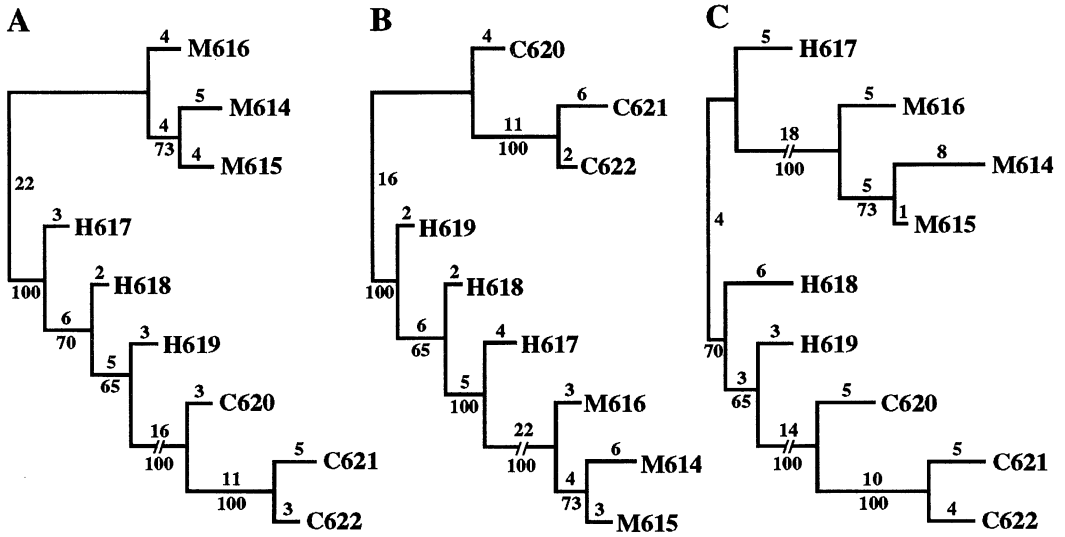[b] Those markers that are nonvarying within a taxon.

FIGURE 1. Parsimony cluster analysis of *Cibotium* individuals based on RAPD data. The analysis produced a single most parsimonious tree of 96 steps with a consistency index of 0.771 and a retention index of 0.805. The trees were rooted with (A) *C. menziesii* individuals, (B) *C. chamissoi* individuals, and (C) midpoint rooting. OTUs are identified by their HPDL accession (Morden et al. 1996).

1990, 1992, 1997). In addition, the phylogeny was analyzed using midpoint rooting (Figure 1*C*). This tree consisted of two major clades with one *C.* ×*heleniae* individual (HPDL 617) basal to the *C. menziesii* individuals in one clade and the other two individuals (HPDL 618 and 619) basal to the *C. chamissoi* individuals in the other.

The relationship among the individuals and populations was also depicted by principal components analysis (Figure 2). The first two principal components account for 65.8% of the variation in the data (48.3 and 17.5%, respectively). The first principal component identified genetic markers that clearly separate the two parental species (with *C.* ×*heleniae* occupying an intermediate position), whereas the second principal component identified those markers that separate *C.* ×*heleniae* individuals from each of the parents. One individual of *C. chamissoi* (HPDL 620) was intermediate between the other two and the *C.* ×*heleniae* cluster, and may represent a hybrid backcross progeny.

Percentage additivity was calculated for the hybrid individuals and for the putative

parent species treated as hybrids (Smith et al. 1996). Based on these data, putative hybrids were classified as hybrids if the DNA markers were additive, or partially additive, from both of the putative parents. The additivity values obtained ranged from 54 to 64% (Table 5).
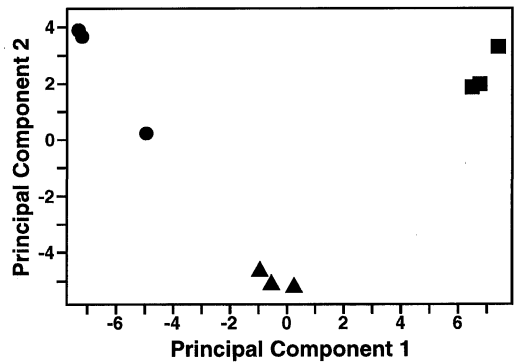


FIGURE 2. Principal components analysis of *Cibotium* individuals based on RAPD analysis. The first two components accounted for 65.8% of the variation. Circles denote *C. chamissoi*; squares denote *C. menziesii*; triangles denote *C.* ×*heleniae*.

TABLE 5

Additivity among RAPD Markers Scored for *C.* ×*heleniae* and for Both Parents Used as a Putative Hybrid (Smith et al. 1996) (Markers Were Present in All Individuals, and Markers Polymorphic in the Parent Populations Were Excluded)

| Test Hybrids[a] | Test Parents of Hybrid Cross[b] | Shared Bands/Unique Bands, Parent 1[c] | Shared Bands/Unique Bands, Parent 2[d] | % Additivity[e] | % Additivity Species Mean Values |
|---|---|---|---|---|---|
| *C.* ×*heleniae* 617 | *C. chamissoi* × *C. menziesii* | 11/17 | 10/16 | 64 | |
| *C.* ×*heleniae* 618 | *C. chamissoi* × *C. menziesii* | 10/16 | 6/12 | 57 | |
| *C.* ×*heleniae* 619 | *C. chamissoi* × *C. menziesii* | 10/16 | 4/10 | 54 | 58 |
| *C. chamissoi* 620 | *C. menziesii* × *C.* ×*heleniae* | 1/8 | 9/10 | 56 | |
| *C. chamissoi* 621 | *C. menziesii* × *C.* ×*heleniae* | 1/12 | 9/10 | 45 | |
| *C. chamissoi* 622 | *C. menziesii* × *C.* ×*heleniae* | 1/11 | 9/10 | 48 | 50 |
| *C. menziesii* 614 | *C. chamissoi* × *C.* ×*heleniae* | 1/9 | 3/4 | 38 | |
| *C. menziesii* 615 | *C. chamissoi* × *C.* ×*heleniae* | 1/8 | 3/4 | 33 | |
| *C. menziesii* 616 | *C. chamissoi* × *C.* ×*heleniae* | 1/7 | 4/5 | 42 | 38 |

[a] The individual treated as a hybrid in the analysis. Numbers indicate the HPDL accession number (Morden et al. 1996).
[b] The taxa treated as parents in the analysis.
[c] The number of markers shared between the putative hybrid ([a]) and the first parent/the number of markers found in parent 1.
[d] The number of markers shared between the putative hybrid ([a]) and the second parent/the number of markers found in parent 2.
[e] The sum of the numerators divided by the sum of the denominators of ([c]) and ([d]), expressed as a percentage.

When the data were examined with *C. menziesii* individuals as the putative hybrids and *C.* ×*heleniae* as putative parents, the additivity values were lower (33 to 42%) as is expected if *C.* ×*heleniae* is of hybrid origin from this cross (Smith et al. 1996). However, the additivity values obtained from the analysis of *C. chamissoi* as the putative hybrid were much higher (45 to 56%) and overlapped with those values obtained originally with *C.* ×*heleniae* (Table 5). Although the mean additivity values for *C.* ×*heleniae* were 10–20% higher than the mean values for the parent populations used as hybrids, there was a broad range of values and the differences were not significant.

## DISCUSSION

Analysis of the RAPD data suggested that natural hybridization has occurred among *Cibotium* species as was originally hypothesized by Palmer (1998) based on morphological examination. Field observations by Palmer and one of us (T.J.M.) suggested that hybrid individuals of *Cibotium* are well established in rain forest ecosystems and are found in several other localities in addition to the

'Aiea Ridge population on the island of O'ahu (Palmer 1998). Several individuals of *C.* ×*heleniae* examined appear to be later-generation hybrids or backcross progeny because they exhibit a broad range of variation in the morphological characters usually considered diagnostic of the parent species.

Based on morphological data, it appears that *C.* ×*heleniae* shares characteristics of *C. chamissoi* and *C. menziesii*. As with characters observed in *Drosophila* (Kaneshiro 1990), it was expected that morphological traits in *C.* ×*heleniae* would be under genetic control and that individuals would share genetic markers that are unique to the parent populations, as has been observed in *Dubautia* hybrids (Caraway 1997). There has been controversy over the most statistically accurate method for interpreting RAPD data (Lamboy 1994*a,b*, Lynch and Milligan 1994). We chose to use the greatest rigor to examine the data in separate analyses to test the genetic relationship of *C.* ×*heleniae* to the parental species. These results show that (1) genetic similarities place *C.* ×*heleniae* intermediate to the parent species; (2) cladistic analysis places *C.* ×*heleniae* individuals on a clade intermediate to the parent taxa; and (3) PCA analysis clusters the

*C.* ×*heleniae* individuals between the clusters of parent species. Each of these results is consistent with the hypothesis that the individuals of *C.* ×*heleniae* are indeed of hybrid origin.

In contrast, the results from the analysis of additivity were ambiguous. Additivity of loci was expected to be nearly 100% in $F_1$ individuals (Smith et al. 1996). The additivity of RAPD markers for known hybrid individuals has been shown to be a reliable criterion in *Nuphar* (Padgett et al. 1998; Padgett, pers. comm.) and Hawaiian *Cyrtandra* (50–100% [Smith et al. 1996]). In each case, the levels of additivity dropped when either of the parent populations was treated as being a putative hybrid. This trend was also found to be the case for *C.* ×*heleniae* with the exception of a high level of additivity for a single *C. chamissoi* individual. Low levels of additivity (<100%) in the hybrid are likely due to the high level of polymorphism identified at each locus. For example, markers present in all individuals of a parental species may not necessarily indicate that the individuals are homozygous for that marker. Because both heterozygotes and homozygotes for expression of the allele share the same phenotype (presence of a band), the two forms may not be differentiated. If the hybrid is derived from a cross involving a locus that is heterozygous in one individual and homozygous for the null allele (i.e., no band) in the other, it is expected that half the hybrid progeny will also be homozygous for the null, thus lowering the additivity. Alternatively, the absence of expression of a band in the hybrids may be a consequence of the individuals being $F_2$ or later generations. Similarly, the presence of the marker in *C.* ×*heleniae* that was not found in either of the parents is likely also the consequence of polymorphism at the locus within one of the parental species.

We expect that other hybrids among the Hawaiian species of *Cibotium* will be found though some combinations will be harder to detect by morphological assessment because the parents may be less distinct than in the example given here. In such cases, molecular approaches may be of particular value. As we have shown in this study, genetic analyses

using molecular markers were valuable for confirming the hybrid origin of *C.* ×*heleniae*. RAPD markers have also proved to be useful in evaluating hybrid swarms among other morphologically similar species (Caraway 1997), and we anticipate that they would similarly be useful in further understanding the biology of hybrid populations in other groups of ferns.

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