Aliso: A Journal of Systematic and Evolutionary Botany

Volume 13 | Issue 2 Article 10

1992

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ALISO
13(2), 1992, pp. 395–409

PLANT SPECIES DISJUNCTIONS: PERSPECTIVES FROM MOLECULAR DATA

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ABSTRACT

The use of molecular data in the study of plant species disjunctions is reviewed and evaluated. The major reason for employing molecular information is to estimate genetic divergence between morphologically similar disjunct species. Flavonoid chemistry offers few advantages over morphology because it is difficult, if not impossible, to infer genetic divergence from the arrays of flavonoid compounds sequestered by two species. Also, flavonoids can, like morphological characters, undergo stasis. Rather direct evidence for this comes from the fact that extant and fossil species may have identical or nearly identical flavonoids. Enzyme electrophoresis is useful for estimating divergence between disjunct species at gene loci encoding soluble enzymes. Disjunct species pairs in several genera are highly divergent at isozyme loci despite their morphological similarity. Restriction site analysis of chloroplast DNA (cpDNA) has proven useful for measuring divergence between disjunct species. The conservative rate of nucleotide substitutions in cpDNA allows one to estimate (albeit with several assumptions) sequence divergence between the DNAs. Whether isozyme and cpDNA data can be used to estimate divergence times with reasonable confidence remains an open question. In two studies employing both methods, similar divergence times were calculated with each. As two species become more divergent at isozyme loci, the variance in estimates of divergence times becomes larger, and the calculated times become less certain. Despite limitations, enzyme electrophoresis and cpDNA restriction site data are valuable for estimating genetic divergence between disjunct species. Future studies of plant species disjunctions will likely include nucleic acid sequence data. The molecular information should always be part of a broader study of species disjunctions, including detailed investigations of morphological features, chromosome numbers, ecology, and the geological histories of the species.

Key words: species disjunctions, flavonoid chemistry, enzyme electrophoresis, chloroplast DNA.

INTRODUCTION

Plant species disjunctions have always been recognized on the basis of morphology, that is, disjunct species are morphologically very similar but occur in widely separated geographic areas. As emphasized by Wood (1972), comparative morphology is the basis for the study of all disjunctions. In the present paper we will focus on the use of molecular data in the study of disjunctions; our primary purpose is to evaluate the advantages and limitations of molecular data for addressing various questions about plant species disjunctions.

In one sense, most plant species have disjunctions because they occur in discrete populations rather than being distributed continuously. While the level of gene exchange between plant populations is a matter of continuing discussion and research, in the present paper we will focus on those situations where populations occur in geographical areas sufficiently distant from each other that there can be little question that gene exchange no longer occurs.

As mentioned earlier, the recognition of species disjunctions is based on the morphological similarity of the plants in the two or more areas. A much-cited and remarkable example is the morphological similarity of plants from many different groups in eastern Asia and eastern North America (Li 1952). Depending on the taxonomist and/or the group, the plants have been treated as members of the same taxon, as distinct at the infraspecific level, or as distinct species. Regardless of the taxonomic treatment, the fact is that these plants are more similar to each other morphologically than either is to any other taxon.

Consider first the reasons for applying molecular data to studies of disjunct species. A basic question about these disjunct taxa concerns *why* they are so similar in morphology. The two possible explanations are recent divergence in the form of long distance dispersal *or* morphological stasis following an ancient separation of the two taxa. Also, ascertaining the genetic basis of morphological differences between plants is often difficult, and it is desirable to have other methods by which to measure genetic divergence. It may be, for example, that two species are judged to be genetically similar based on morphological similarity while in reality they are quite divergent when other data sets are considered. One advantage that certain molecular approaches have is the ability to quantify genetic differences in a rather precise manner as compared to morphology. It is then possible to compare genetic divergence in molecular features for disjunct, morphologically similar species. In simplest terms, if two disjunct taxa show little or no divergence in molecular features, one assumes that interruption of gene flow has been recent. By contrast, divergence in molecular features would be indicative of larger divergence time with the accumulation of differences via mutation.

Another question regarding molecular data concerns how reliable they are for providing estimates of times of divergence for taxa. This issue is concerned with whether there is a so-called molecular evolutionary clock or molecular clock. In simple terms, with the molecular clock hypothesis the degree of sequence divergence at the molecular level would be proportional to the amount of elapsed time since the copies shared a common ancestor (Clegg 1990). Suffice it to say that despite considerable discussion of the matter there is no consensus on whether anything approaching a molecular clock exists (see Thorpe 1982; Gillespie 1984, 1986; Hillis 1987; Clegg 1990; Melnick 1990 as examples). Critics of the molecular clock have emphasized the necessity of accurate paleontological data to calibrate it; if the calibration is wrong then estimates based on the molecular data will be wrong (Clegg 1990). Another critical issue is whether celestial or generation time is important. All of these caveats (plus others to be discussed later) must be kept in mind when calculated divergence times are discussed.

Three kinds of data will be considered, namely, flavonoid chemistry, enzyme electrophoresis (allozymes and isozymes), and restriction site analysis of chloroplast DNA (cpDNA). Flavonoid compounds (so-called secondary metabolites or micromolecules) differ from isozymes and cpDNA because they represent the end products of a biosynthetic pathway with several enzyme-mediated steps. It is not feasible in most cases to equate different flavonoid arrays in two species with genetic differences (Giannasi and Crawford 1986). Despite these limitations, flavonoids still offer additional characters for assessing whether species are the same or different. The rationale for using enzyme electrophoresis as a method for estimating genetic variation within and divergence among populations, subspecies

and species was presented by Gottlieb (1977, 1981) and Crawford (1983, 1989, 1990). The ability to equate electrophoretic variation with genetic variation represents a basic advantage of the method as compared to flavonoid compounds. The utility of restriction site analyses of cpDNA for constructing phylogenies and estimating divergence among species has been discussed in various reviews (Curtis and Clegg 1984; Palmer et al. 1988; Sytsma 1990, as examples). With various assumptions, it is possible to infer sequence divergence between two DNAs from the restriction site differences. The further assumption of a certain mutation rate then allows one to estimate divergence times for the DNAs (and thus the species).

DISCUSSION

Flavonoid Chemistry

Comparative flavonoid chemistry has been an integral part of many systematic studies of plants for the past three decades (see Giannasi and Crawford 1986 for a recent review). It achieved its greatest popularity from about 1960 until approximately 1980; during the past decade the use of various macromolecular approaches has resulted in a declining number of flavonoid studies. Flavonoids, being secondary metabolites or micromolecules, are the end products of a biosynthetic pathway consisting of several enzyme-mediated steps (see review by Stafford 1990).

Several workers have employed flavonoid chemistry to study species disjunctions between eastern Asia and eastern North America. Bohm et al. (1978) identified flavonoid compounds in four species of *Elliottia* (Ericaceae), two of which occur in Japan, one is from the southeastern United States and the fourth grows in the northwestern United States. *Elliottia pyroliflora* (Bong.) Brim & P. F. Stevens of the northwestern United States appears most distinct morphologically and it is likewise most divergent from other species in its flavonoid chemistry. The average flavonoid similarity between the western North American species and the two Japanese is lower than for species from other disjunct geographical areas. One of the Asian species, *E. bracteata,* (Maxim.) Bentham & Hooker f. has a very reduced flavonoid profile and this accounts for its lowered similarities with other species. Nearly all differences among the species result from sugar substituents on the same flavonoid nuclei.

Soltis and Bohm (1984) examined flavonoid compounds in the three disjunct species that comprise the genus *Tiarella* (Saxifragaceae). One species, *T. cordifolia* L., occurs widely in the eastern United States and Canada while *T. polyphylla* D. Don. is found in a wide area of eastern Asia, and *T. trifoliata* L. is widespread in the western United States and Canada. Twenty-four flavonoid compounds were isolated from the three species, with 22 of them partially or completely identified. The eastern Asian species and the two North American species share approximately 25% of the compounds while the two North American species have about half of their flavonoid components in common. The lower similarity of the Asian species was due almost totally to its reduced flavonoid profile, that is, it contains essentially a subset of the compounds detected in the two North American species. By contrast, the latter two species contain unique components. Soltis and Bohm (1984) interpreted the reduced profile of *T. polyphylla* as the specialized condition; but with regard to the disjunct species they suggested that the flavonoid chemistry

is concordant with morphology in documenting the equally distinct nature of each taxon. Differences among the taxa result from the kinds of glycosides of the flavonols (quercetin, kaempferol and myricetin) present in each.

Vogelmann (1984) studied flavonoids in species of the genus *Agastache* section *Agastache* (Labiatae) distributed in eastern and western North America, and in China. Three species occur in the eastern United States, four grow in the western United States, and one is native to China. On the basis of morphology, the Asian *A. rugosa* (Fisch. ex Myer) Kuntze is most similar to two species from the eastern United States, but the flavonoid chemistry of the Asian species is identical to the four species from western North America. The eastern United States species to which *A. rugosa* is most similar morphologically lacks several compounds characteristic of *A. rugosa* and the western North American taxa. Vogelmann (1984) suggested that the Asian and western North American species have been isolated for at least 12 million years and that during this time there has been no divergence in flavonoid components.

Rieseberg and Soltis (1987) examined flavonoid compounds in fossil (Miocene age) *Platanus* and compared them to extant species. The results indicate greater chemical similarity of the fossil plants to the Asiatic *P. orientalis* L. than to North American species despite the fossils being morphologically more similar to *P. occidentalis* L. of eastern North America. This situation is similar to that in *Agastache* described above and will be discussed later. Comparisons with other related taxa are necessary to determine whether the flavonoids or morphology represent the pleisiomorphic condition and thus which is informative with regard to relationships.

Wen and Crawford (1989) examined flavonoids in the morphologically very similar disjunct species pair *Aralia chinensis* L. and *A. spinosa* L. of eastern Asia and the eastern United States, respectively. A total of nine flavonoids was detected in the two species, with *A. chinensis* having a total of seven compounds, including both quercetin and kaempferol glycosides, while *A. spinosa* contains only two compounds and lacks kaempferol glycosides. Thus, these two species have very divergent flavonoid profiles despite their morphological similarity.

Bohm (1988) found that the two disjunct species of *Datisca, D. cannabina* L. (southwest and central Asia) and *D. glomerata* (Presl.) Baill. (California and Baja California) have identical leaf flavonoid profiles. The species are quite similar morphologically with the primary differences being that *D. cannabina* is always dioecious with pinnatisect mature leaves while *D. glomerata* is androdioecious with incised-serrate mature leaves (Liston, Rieseberg, and Elias 1989*b*).

Niklas and Giannasi (1978) used flavonoid chemistry to examine the biogeographical affinities of the fossil species *Quercus consimilis* Newberry from the Succor Creek Flora (15–20 million years old) of the northwestern United States. Morphology suggested that the closest relatives to the fossil species are two species from eastern Asia, *Q. stenophylla* Makino and *Q. myrsinaefolia* Blume. The two latter species have very similar flavonoid arrays but differ considerably from the fossil plant, including lacking a whole class of compounds, the C-glycosylflavones. A survey of 30 extant species of *Quercus* from Asia, North America and Europe revealed two species of *Quercus* (*Q. acutissima* Caruther from Japan and *Q. chenii* Nakai from Korea) that have flavonoid profiles essentially identical to the fossil plant, including the same two C-glycosylflavones. In this instance, the flavonoid chemistry altered previously suggested relationships between disjunct species.

Additional comparisons between the flavonoids of fossil and extant congeneric species have revealed little divergence in constituents. Giannasi and Niklas (1977) showed that flavonoid profiles between fossil (ca. 15–20 million years old) and extant *Celtis* are quite comparable, at least with regard to the class of compounds produced. Niklas, Giannasi, and Baghai (1985) examined the flavonoids of fossil *Liriodendron* (of Miocene age) and compared them to those found in the two extant disjunct species *L. tulipifera* L. of eastern North America and *L. chinense* Helmsley from eastern Asia. The profiles proved to be essentially identical in all three species.

Studies of flavonoids in disjunct species of plants in eastern and western North America and eastern Asia have revealed various levels and patterns of divergence for taxa from the different areas. In certain cases such as *Agastache, Datisca,* and *Liriodendron,* the flavonoid chemistry is identical for two disjunct species. In other instances such as *Aralia* and *Tiarella* there has been considerable flavonoid divergence between congeners on different continents. There are at least two possibilities for the different patterns and levels of flavonoid divergence observed. First, it may be that similar distribution patterns do not reflect similar biogeographic histories. Tiffney (1985*a*) has argued that this is likely the case for disjunct species of eastern North America and eastern Asia. Secondly, flavonoid divergence may not be a good indicator of genetic divergence between species and thus is of little value for inferring relative times of divergence for different taxa. Also, it is possible that a combination of these two factors is involved. While we cannot provide a critical evaluation of each of the genera above relative to their historical biogeography, we can make some comments on the tempo of flavonoid divergence. It is clear that considerable flavonoid divergence can occur between very closely related congeneric species, this having been documented in genera from a wide variety of families of flowering plants (see Crawford 1978 for discussion). By contrast, it is also apparent from comparisons of fossil and extant species that congeners may sequester identical flavonoids despite millions of years of divergence. These results show that stasis may occur for flavonoids as well as morphology.

It seems fair to conclude that flavonoid compounds have been of limited value for inferring genetic divergence between and the biogeographical history of disjunct species. The results for flavonoids will be compared to other data obtained for disjunct species.

Enzyme Electrophoresis

Enzyme electrophoresis, or the use of allozymes and isozymes, has been widely employed in plant systematics during the past two decades. The rationale for using the method for estimating genetic variation within and genetic divergence among populations, subspecies, and species was presented by Gottlieb (1977, 1981; Crawford 1983, 1989, 1990). The ability to equate electrophoretic variation with genetic variation represents a basic advantage of the method as compared to using flavonoid compounds. Thorpe (1982) and Nei (1987) have discussed the use of allozyme data to estimate divergence times, i.e., time since two populations ceased exchanging genes.

There are a number of potential limitations in using the electrophoretic mobility of enzymes in gels for inferring divergence between taxa and then in turn at-

tempting to place a time of divergence on the taxa. One problem is detecting all the different allozymes, that is, amino acid substitutions not affecting electrophoretic mobility with the methods employed would go undetected. Another problem with species that have diverged over longer time periods is the probability of back mutations. Also, the enzymes studied may affect results because of the different mutation rates of the genes encoding them. These problems were discussed by Thorpe (1982) and in considerable detail by Nei (1987). Nei (1987:230–240) considered the use of genetic distances derived from electrophoretic data for inferring evolutionary time. If one assumes that the rate of amino acid substitutions in proteins is approximately constant per year, and a distance measure is proportional to codon substitutions, then the measure should be proportional to evolutionary time. Depending on the level of divergence between species, certain assumptions are made with regard to whether each mutation creates a new allozyme (infinite allele model) or a change could represent a back mutation (stepwise mutation model). With back mutations, there would be no linear relationship between genetic distance and time. However, the proportionality between codon substitutions (genetic distance) and time presumably hold over shorter periods of time because of the low probability of back mutations. Taking these factors and others (no migration between populations, alleles at a locus selectively neutral, etc.) into account, Nei (1987) derived several formulas for estimating time of evolutionary divergence. An in-depth discussion of the formulas can be obtained by consulting Nei (1987:230–240).

Wendel and Percival (1990) examined electrophoretic divergence between *Gossypium klotzschianum* Anderss. and *G. davidsonii* Kell; the former species is endemic to the Galapagos Archipelago while the latter occurs in Baja California. The two species are very similar morphologically, are highly interfertile, and the totality of data suggests that the two species are more closely related to each other than either is to any other species (see discussions in Wendel and Percival 1990). Allozyme data were used to estimate the genetic divergence between the two species (and from this to estimate the time of divergence) and to compare the levels of variation in each. The second objective was of particular interest because the species from the Galapagos (*G. klotzschianum*) had been viewed as the derivative of *G. davidsonii* from Baja California. The Galapagos species was found to have a subset of the allozyme variation found in *G. davidsonii,* which would be expected if the former is derived from the latter (see Gottlieb 1977, 1981; Crawford 1989, 1990 for discussions).

A standard genetic distance of 0.142 (±0.061) was calculated for the two species with Nei's (1987) formula of time = $D/_{2a}$ (D is the genetic distance and a is the substitution rate per locus per year). The value of 10^{-7} was used for a (this is a commonly employed rate), which then allows one to calculate t as $(5 \times 10^6)D$. This formula, because of the low divergence between the species, is based on an infinite allele model. With these assumptions, the time of divergence was calculated to be 710,000 years (±305,000 years). The Galapagos Islands are estimated to be three to four million years old, and thus the calculated time for the separation of the two species of *Gossypium* is well within the time of origin of the archipelago. As will be discussed below, the divergence time estimated from chloroplast DNA restriction site differences is comparable to the allozyme data for these two species. The electrophoretic evidence provide strong confirmation that *Gossypium klotzschianum* of the Galapagos Islands is derived from *G. davidsonii* of Baja California.

Liston, Rieseberg, and Elias (1989*a*) examined allozymes in the two annual species *Senecio flavus* (Decne.) Schultz-Bip. and *S. mohavensis* Gray, with the former occurring in the Saharan and Namibian deserts of Africa and the Arabian desert of southwest Asia while the latter species grows in the Mojave and Sonoran deserts of western North America. Two subspecies of *S. flavus* are recognized; subsp. *flavus* occurs in Namibia (southwest Africa) and in northern Africa, while subsp. *breviflorus* occurs in southwest Asia. Allozymes were employed to measure the pattern and level of genetic divergence between plants in these four disjunct areas. Hopefully, these data would also provide insight into how the present distribution pattern was achieved. The two species are very similar morphologically; that is, they are considered to be more similar to each other than either is to any other species of *Senecio*. *Senecio flavus* is diploid ($2n = 20$) while *S. mohavensis* is tetraploid ($2n = 40$).

Genetic identities computed for plants from the different geographic areas are 0.857 for *S. flavus* subsp. *flavus* from Namibia and from North Africa; 0.805 and 0.857 for *S. flavus* subsp. *breviflorus* Kadereit compared to subsp. *flavus* from Namibia and North Africa, respectively, and 0.805, 0.857 and 0.952 when *S. mohavensis* is compared to subsp. *flavus* from Namibia, subsp. *flavus* from North Africa and subsp. *breviflorus*, respectively. Different numbers of isozymes were found among the taxa, with *S. flavus* subsp. *breviflorus* having two additional isozymes compared to subsp. *flavus* (both taxa are diploid). The tetraploid *S. mojavensis* has four isozymes in addition to the two it has in common with *S. flavus* subsp. *breviflorus*.

Based on the electrophoretic data, Liston, Rieseberg, and Elias (1989*a*) hypothesized that *Senecio flavus* originated in southern Africa, spread to northern Africa and then to southwest Asia where morphological differentiation occurred to produce subsp. *breviflorus*. Presumably, the tetraploid *S. mohavensis* of the Sonoran and Mojave deserts was derived from the diploid *S. flavus* subsp. *breviflorus*. The distribution of gene duplications is concordant with this biogeographical hypothesis because one would interpret the lack of duplications (above the "usual" minimal conserved number for diploid plants [Gottlieb 1982; Weeden and Wendel 1989]) as the ancestral condition with the two additional loci representing the derived condition in subsp. *breviflorus*. The additional four loci in the tetraploid *S. mohavensis* indicate a further derived condition. Mean genetic identities among plants from the different areas are generally concordant with this hypothesis, with the most noteworthy result being the very high identity between subsp. *breviflorus* and *S. mohavensis*.

Liston, Rieseberg, and Elias (1989*a*) discussed evolution and migration in *Senecio* relative to geological history. The Sahara first became a desert in the Pliocene but the present desert vegetation dates only from the Pleistocene. The origin and present vegetation of the northern part of the Sonoran desert likewise dates from the Pliocene-Pleistocene and Holocene, respectively. By contrast, the Namibian desert, the presumed place of origin of *S. flavus*, is considerably older (i.e., the early Oligocene). Liston, Rieseberg, and Elias (1989*a*) suggest that conditions were right for the spread of *S. flavus* to North Africa, followed by its movement to southwest Asia. The dispersal of the progenitor of *S. mohavensis* (presumably a plant similar to *S. flavus* subsp. *breviflorus*) remains more problematical.

If one calculates divergence times based on the electrophoretic data, then the Namibian to North African split of subsp. *flavus* would be placed at 770,000 years

which puts it within the Pleistocene. The allozyme data suggest that the divergence between plants in North Africa and southwest Asia (i.e., subsp. *breviflorus*) occurred at about the same time, which implies that the spread from North Africa to southwest Asia may have occurred shortly after the introduction of the species into the former area. The calculated divergence time for *S. mohavensis* is approximately 245,000 years. While the exact timing of events has not been elucidated, the electrophoretic data were useful for inferring the probable sequence of events leading to the present distribution of *Senecio flavus* and *S. mohavensis.* Also, the high genetic similarity between *S. flavus* subsp. *breviflorus* and *S. mohavensis* argues that long distance dispersal accounts for the present distribution of the latter species.

Liston, Rieseberg, and Elias (1989*b*) employed enzyme electrophoresis to measure genetic divergence between *Datisca cannabina* and *D. glomerata.* These two disjunct species are diploid ($n = 11$), long-lived, perennial herbs and are the only taxa recognized for the genus. As discussed earlier, *D. glomerata* occurs from northern California to northern Baja California, Mexico, while *D. cannabina* is found in southwest and central Asia from Crete in the east Mediterranean to northwestern India. Its range is disjunct because of its absence from an area of about 1000 km in the desert east of the Caspian Sea to northwestern Afghanistan. Geological history and paleobotanical data suggest that migration across the mid-Atlantic some 23 to 65 m.y.a. may account for the present distribution of these two species (see discussion in Liston, Rieseberg, and Elias, 1989*b*).

Enzyme electrophoresis revealed that the two species are highly divergent at isozyme loci, with a mean genetic identity of 0.142 and standard distance of 1.95 for all populations (Liston, Rieseberg, and Elias 1989*b*). The two populations of D. cannabina from southwest Asia have a higher mean identity (0.184) with *D. glomerata* than does the central Asian population (0.064) of *D. cannabina.* This provides support for the dispersal via islands across the Atlantic rather than over the Bering bridge (see later discussion of the two routes).

Depending on the method used to calculate divergence time from the electrophoretic data, the time of divergence for these two species is in the broad range of 10 to 40 million years ago. If one applies formula 9.62 in Nei (1987, p. 236) a divergence time in excess of 49 million years is obtained. Liston, Rieseberg, and Elias (1989*b*) emphasized the uncertainties associated with making such estimates when divergence is so high between species. Regardless of the problems with estimating actual divergence times, however, the electrophoretic data clearly show that these two species are highly differentiated at isozyme loci and are not an example of recent long distance dispersal. This is an insight not provided by secondary chemistry because the two species sequester an identical array of compounds (Bohm 1988). Although the two species differ in several respects, the most conspicuous being breeding systems, they are morphologically quite similar, and Liston, Rieseberg, and Elias (1989*b*) suggest that there has been morphological stasis in these two species; the allozyme data indicate this is a most reasonable hypothesis.

Eastern Asian-eastern North American plant species disjunctions have fascinated botanists for many years; both the large number of genera with species showing this pattern of disjunction and the distance involved probably account for this interest. The literature on this topic is voluminous; Boufford and Sponberg

(1983) provided an excellent review of this literature, while Tiffney (1985*a*, *b*) evaluated geological and paleobotanical aspects relative to the development of these disjunctions. As emphasized by Tiffney (1985*a*), it is important to consider all the possible ways by which this pattern could have arisen, and he is of the view that these disjunctions are neither the result of a single historical event nor are they just the product of chance long distance dispersal events. For further discussion of the historical patterns that could explain Asian–eastern North American species disjunctions see Tiffney (1985*a*).

A number of electrophoretic studies have been conducted on species disjuncts in eastern Asia and eastern North America. Vogelmann and Gastony (1987) examined genetic distances between the eastern Asian *Agastache rugosa* and North American populations of closely related species in sect. *Agastache*. These values range from 0.288 to 0.673 (identity values of 0.510 to 0.749). Depending on the method of calculation, the estimated divergence times would be 1.45 to 3.2 million years ago or 2.0 to 7.0 million years ago. While these estimates are on the low side, it is possible that divergence could have occurred in the late Miocene, which would place the oldest estimate of divergence within this time frame (Vogelmann and Gastony 1987). As discussed earlier, Vogelmann (1984) found that the flavonoids of *A. rugosa* and five species of *Agastache* from the western United States are identical despite *A. rugosa* being morphologically more similar to eastern United States species. This apparently represents a situation in which the flavonoids or morphology are plesiomorphic; thus it is not possible to determine from available data whether *A. rugosa* is more closely related to the eastern or western North American species of *Agastache*. A broader study of the genus could help to resolve the situation. The electrophoretic data are inconclusive in placing *A. rugosa* closer to the eastern versus western North American species, i.e., *A. rugosa* is distinct from all other taxa (Vogelmann and Gastony 1987).

Parks and Wendel (1990) carried out an extensive electrophoretic study of the well-known eastern Asia–eastern North American species pair *Liriodendron chinense* and *L. tulipifera*. The estimated divergence time from these data ranged from 10 to 16 million years, depending on the method employed. These authors feel that this a reasonable estimate given the fossil record. The estimate of divergence time based on cpDNA restriction site differences will be discussed later. It will be recalled that Niklas, Giannasi, and Baghai (1985) found no flavonoid differences between the two species of *Liriodendron*.

Hoey and Parks (1991) examined four disjunct species of *Liquidambar* electrophoretically. The two species from eastern Asia (*L. acalycina* H. T. Chang and *L. formosana* Hance) are highly divergent from the eastern North American species (*L. sturaciflua* L.), with mean genetic identities of 0.485 and 0.431 respectively. These values are very similar to those reported by Parks and Wendel (1990) for the two disjunct species of *Liriodendron*.

Preliminary electrophoretic data are available for disjunct taxon pairs of perennial herbs occurring in eastern North America and eastern Asia (Lee and Crawford, unpublished). These include *Phryma leptostachya* L. var. *leptostachya*–var. *asiatica* Hara, *Menispermum canadense* L.-*M. dauricum* DC., and *Saururus cernus* L.–*S. chinense* (Lour.) Baill.

Material was collected from natural populations in all instances, and the number of populations, sample sizes for each population, and number of loci, respectively,

are given after each taxon: *P. leptostachya* var. *leptostachya* (2; 28,27; 20); *P. leptostachya* var. *asiatica* (3; 34,15,14; 20); *M. canadense* (3; 22,18,19; 21); *M. dauricum* (2; 46,18; 21); *S. cernuus* (2; 46,21; 18); *S. chinense* (2; 21,9; 17).

The genetic identity values for these pairs of species are 0.291, 0.273 and 0.263, respectively. If one uses equation 9.62 of Nei (1987, p. 236), the estimated divergence times range from about 25 to 30 million years. Fruit and seed fossils of *Menispermum* and *Saururus* go back to the early Eocene, some 50 m.y.a. while the fossil record for *Phryma* is not known (Tiffney 1985*a*, pers. comm.). The divergence times estimated from the electrophoretic data, therefore, fall within the earliest known time in the fossil record for two of the genera (Tiffney, pers. comm.).

Tiffney (1985*a*) emphasized that genera of herbaceous angiosperms with disjunctions in eastern North America and eastern Asia may have achieved the distribution by various processes. Some genera may trace the origins of their species from the early Tertiary (as part of the boreotropical flora) whereas in other genera the disjunctions may be much younger with long distance dispersal a possibility in certain instances. Tiffney (1985*a*) indicated that the Miocene was the last time when temperate plants could have been exchanged between the two areas, either via the Bering bridge or possibly by island "stepping stones" across the North Atlantic. The calculated divergence times for the three genera mentioned above fall near the Oligocene-Miocene boundary, which is early enough for either of the two possible dispersal scenarios (Bering bridge or North Atlantic) put forth by Tiffney. Elisens (1990) examined allozyme divergence between *Alnus maritima* (Marsh.) Nutt. from North America and a population of the presumed sister species from Asia. Genetic identities between populations of the two species is 0.47 and 0.50, depending on whether one compares populations of *A. maritima* from Oklahoma or the Atlantic Coast plain. These identities are very similar to values calculated for *Liriodendron* and *Liquidambar*.

Enzyme electrophoresis has been valuable for the study of plant species disjunctions. Despite limitations, it can be used to assess genetic divergence at a number of loci (typically 15–30). These divergence values may then be employed to provide estimates, crude as they may be, of divergence times between species. For the majority of disjunct species (primarily between the Old and New Worlds) examined electrophoretically, the levels of isozyme similarity are lower than normally found among congeneric species of flowering plants (see Gottlieb 1977, 1981; Giannasi and Crawford 1986; Crawford 1989, 1990 for reviews). In most other electrophoretic studies of species as similar morphologically as the disjunct species the taxa often represent progenitor-derivative pairs that have presumably diverged recently and in contrast to the disjunct species, they have high genetic identities at isozyme loci. The electrophoretic data provide a compelling argument for morphological stasis in the disjunct species pairs. The only alternative explanation for the low similarity at isozyme loci would be that there has been extremely rapid divergence at these loci relative to other congeneric species, but there is no evidence to suggest that this is the case. Later discussions of studies of cpDNA divergence in some of the same species examined electrophoretically support the hypothesis of morphological stasis rather than rapid rates of isozyme divergence.

Whether allozymes can be used to estimate divergence times with any degree of confidence remains an open question. One problem with evaluating the utility of electrophoretic data for this purpose is the inability to date divergence times

by other means such as the fossil record or the known ages of the habitats where species occur. As indicated earlier, the allozyme data are, within broad limits, concordant with other information such as the fossil record for eastern Asian–eastern North American species disjuncts. It would be desirable to have a situation where evolutionary divergence has been more recent so there is less variance in the estimates from the electrophoretic data. The allozyme data of Witter and Carr (1988) for the genus *Dubautia* on the Hawaiian Islands may be used to compute divergence times for species restricted to islands of known ages. When this is done, the oldest calculated time for species on the island of Kauai (5.1 million years) is 4.2 million years, for Oahu (2.65–3.35 million years old) the time is 1.55 million years and for Hawaii (0.38 million years old) the value is 0.300 million years. In no case were the calculated times greater than the ages of the islands, which gives some confidence in the method for instances of more recent speciation.

Chloroplast DNA

The use of chloroplast DNA (cpDNA) for studying systematic and phylogenetic relationships in plants has increased greatly during the past five years (see Palmer 1986, 1987; Palmer et al. 1988; Crawford 1990 for reviews). Species are compared on the basis of the number of restriction sites by which they differ (or are the same) in their cpDNAs. The data may be used to construct phylogenetic hypotheses and to estimate sequence divergence between the species (see Nei 1987, chapt. 5 for discussion). In the present discussion, we will be concerned only with inferring sequence divergence from cpDNA restriction site differences and then estimating divergence times by assuming a certain mutation rate for the cpDNA genome.

The study of Wendel and Percival (1990) was discussed earlier with regard to isozyme divergence between two disjunct species of *Gossypium* in Baja California and the Galapagos Islands, and a divergence time of roughly 710,000 years was calculated for the two taxa. The authors also used cpDNA to estimate time of separation. With an estimated sequence divergence of 0.091% and assuming a mutation rate of 0.14% per million years, they calculated a time of 650,000 years for the split of *Gossypium davidsonii* and *G. klotzschianum.* Thus, the estimates from isozyme and cpDNA data are in close agreement.

Parks and Wendel (1990) used allozymes to calculate a divergence time of 10–16 million years for *Liriodendron chinense* and *L. tulipifera,* as was mentioned earlier. In the same study, they estimated a sequence divergence of 1.24% for the cpDNAs of the two species. Using a mutation rate of 10^{-9} nucleotide substitutions per site per year (or 0.1% sequence divergence per million years), the estimated divergence time for the two species is 12.4 million years. This value agrees rather well with the 10–16 million years calculated from isozyme data.

The study of Liston, Rieseberg, and Elias (1989*b*) discussed earlier demonstrated very low genetic identities at isozyme loci for the two disjunct species of *Datisca.* Therefore, it is of interest to note that a preliminary study of the cpDNAs of the two taxa indicate that they differ by many restriction site mutations in their chloroplast genomes (Rieseberg and Elias, pers. comm.).

Wendel (1989) found a low level of divergence between the chloroplast genomes of Old World diploid cottons (*Gossypium*) and the New World tetraploids. That is, the data indicated clearly that the latter group received the chloroplast genome from a species in the former group, yet divergence between the genomes was not

concordant with previous hypotheses of a Cretaceous origin for the tetraploids followed by separation via the drifting continents of Africa and South America. Assuming that the sequences diverged on an average of 0.14% per million years and estimating that the sequences of the diploids and tetraploids are 0.20% divergent, the tetraploid cotton would have evolved about 1.5 million years ago (Wendel 1989). While the calculated divergence time represents an approximation, the fact still remains that the molecular data provide strong evidence for a much more recent disjunction time for New World tetraploid and Old World diploid species of *Gossypium*.

Soreng (1990) surveyed cpDNA restriction site mutations in species representing 19 sections (or groups) and three subgenera of the genus *Poa* (Poaceae) and the phylogenetic hypotheses generated were interpreted from a biogeographical perspective. The data suggest a Eurasian origin for *Poa*, with six or more separate colonizations of North America, and with the subsequent dispersal of two of these groups to South America. Soreng (1990) also points out that the cpDNA provides a conservative estimate of the number of dispersal events from North to South America because of the number of distinct species present in the areas as compared to the number of distinct chloroplast genomes detected. The cpDNA provided very useful insights into disjunct distributions in *Poa*.

Jansen and Palmer (1988), in a study of cpDNA variation of the tribe Mutisieae (Asteraceae), found that the two genera *Gerbera* (South Africa) and *Leibnitzia* (Mexico) form a monophyletic group and they estimated their sequence divergence as 0.7%. This suggests (assuming sequence divergence of 0.14% per million years) that the two genera may have been separated for about five million years.

Additional cpDNA studies of disjunct species are to be desired to assess divergence between the taxa. The relatively conservative nature of the chloroplast genome (Palmer 1986, 1987; Palmer et al. 1988) makes it suitable for assessing differences between species that became isolated tens of millions of years ago, as may be the case with many disjunct species.

GENERAL CONCLUSIONS AND FUTURE PROSPECTS

Several general conclusions seem justified from the foregoing discussion of the application of molecular data to studies of plant species disjunctions. Flavonoid chemistry is less useful than allozymes or cpDNA restriction site mutations for inferring genetic divergence between disjunct species. In certain genera, *Datisca* and *Liriodendron* being good examples, allozymes and cpDNA data indicate extensive differences yet flavonoids of the disjunct species are identical. Also, in *Liriodendron* and other genera, fossil taxa are identical or nearly identical to extant species in flavonoid chemistry. It seems evident, therefore, that flavonoid chemistry can exhibit stasis in a manner similar to morphology. Yet, as indicated earlier in the discussion of flavonoid chemistry, there is ample evidence in the literature that recently diverged species can be very divergent in flavonoids.

Enzyme electrophoresis is preferable to flavonoid chemistry for assessing genetic divergence between disjunct species. Thus far there are no examples of disjunct species of suspected ancient divergence that are very similar at isozyme loci. It is also well known that recently diverged species typically show high similarity at loci encoding isozymes (see Gottlieb 1977, 1981; Crawford 1989, 1990 for reviews). The extent to which allozyme data may be used to provide estimates

of divergence times is an open question, but as the time since separation becomes greater, estimates become less certain.

Although restriction site analysis of cpDNA has not been widely used for inferring genetic divergence between disjunct species, the studies of *Gossypium* and *Liriodendron* discussed earlier attest to the potential of the approach. Because the data may be used to infer sequence divergence (however imperfectly), the method is preferable to allozymes. Also, because many disjunct species, particularly in eastern Asia and eastern North America, have probably been separated for millions of years the more conservative nature of the chloroplast genome makes it more appropriate than allozymes for such studies.

A recent study by Golenberg et al. (1990) in which a segment of the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) was sequenced from fossil *Magnolia* of Miocene age (15–20 million years) after amplification of the segment using the polymerase chain reaction (PCR) offers exciting possibilities for studies of disjunct species. Because the ages of the fossils are known, it is possible to calculate mutation rates by comparing sequence divergence between the fossil and extant species. Once this has been done for fossil and extant species in several different genera, it should be possible to gain much needed insights into variation in rates of mutation for the same genes in different organisms. Hopefully, eventually this will allow for more refined and informed uses of molecular data for inferring divergent times in disjunct taxa.

Molecular studies of species disjunctions should be done in collaboration with persons who know the morphology, ecology, and historical geology of the plants. Morphological studies using many characters, modern numerical methods, and population samples from throughout the ranges of the taxa are needed. These collaborative studies would allow the molecular data to be viewed within a morphological, ecological, and historical context. Lastly, whole clades and their appropriate outgroups should be considered when studying disjunct taxa, especially when the species occur in large genera.

ACKNOWLEDGMENTS

Research was supported in part by NSF grant BSR-8906988 to D.J.C. and T.F.S. An earlier version of this manuscript was presented at the Sixth Annual Southwestern Botanical Systematics and Evolution Symposium held at the Rancho Santa Ana Botanic Garden. One of us (D.J.C.) thanks S. Carlquist, T. Elias, T. Philbrick, L. Rieseberg, and R. Thorne for their hospitality during the symposium. Aaron Liston and Doug Soltis provided comments on the manuscript.

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