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SINGLE COPY DNA HOMOLOGIES IN *ATRIPLEX*. II. HYBRID THERMAL STABILITIES AND MOLECULAR PHYLOGENY*

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SUMMARY

Single copy DNA sequence homologies were measured by interspecific molecular hybridization and thermal denaturation techniques for eight species of the genus Atriplex and spinach (*Spinacia oleracea*). Thermal stability profiles for Atriplex interspecific hybrids indicated more base pair mismatch than has been observed in most previously reported intrageneric comparisons of animal DNA. On the assumption that sequence divergence by base substitution is proportional to the time which has elapsed subsequent to speciation, the data are interpreted as indicating that lines leading to many modern Atriplex species probably originated during a single period of rapid speciation. This group includes C_3 and C_4 photosynthetic types from both subgenera. Thus the molecular data do not support the classical subgeneric distinction, and it is no longer necessary to postulate a polyphyletic origin for C_4 photosynthesis within Atriplex.

1. INTRODUCTION

ATRIPLEX is a cosmopolitan angiosperm genus of the family *Chenopodiaceae*, comprised of halophytic species native to many varied marginal and/or extreme environments (Osmond *et al.*, 1980). The phylogenetic relationships within the genus have recently come under review with the discovery that it includes species with both C3 and C4 photosynthetic pathways (Björkman *et al.*, 1973). The most recent phylogenetic evaluation (Hall and Clements, 1923) was based solely on morphological characteristics and suggests that Atriplex should be split into two subgenera. Combination of this scheme with modern physiological data leads to the conclusion that C4 species have evolved on at least two occasions, once in each subgenus. While a polyphyletic origin for C4 photosynthesis is widely accepted for angiosperms in general it might be viewed as improbable within a group so closely related as a single genus.

In addition to the question of C4 species origin, there is also the question of how closely various Atriplex species are related. Hall and Clements (1923) describe an order of branching in their morphologically-based phylogenetic tree. The length of branches, or the interspecific divergence distances, are not specified although a gradually reticulating evolutionary pattern is implied. Experimental breeding programs were undertaken to

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clarify these relationships (Nobs, 1976). However, with a few exceptions, mating barriers were strong and sexual crosses failed to even produce seed. Thus a molecular approach to measuring evolutionary relationships in Atriplex seemed desirable.

The use of interspecific DNA sequence comparison in phylogenetic studies is based on the assumptions that (1) all sequences are subject to random point mutation, and (2) the fixation rate of random point mutations is constant with respect to time when averaged over the entire genome (for reviews see Kohne, 1970, and Wilson *et al.*, 1977). When a population becomes subdivided into genetically isolated groups, the DNA sequences of each group diverge from those of other subgroups at a constant rate. Thus interspecific sequence divergence should be directly proportional to time subsequent to genetic isolation, and estimates of sequence divergence may provide a kind of molecular clock, indicating (at least in relative terms) the time at which two lineages diverted from a common ancestor. Using molecular hybridization techniques, homologous DNA sequences from two species can be annealed *in vitro* and the hybrid duplexes subjected to thermal dissociation to reveal the extent to which the base sequences of each species are diverged from one another. For eukaryotes containing significant amounts of repetitive DNA sequences, such sequence divergence estimates should be restricted to single copy sequences. Point mutations can occur in all DNA sequences, including those which belong to repetitive families. Thus there will be sequence divergence among the members of repetitive families *within* ony one genome, obscuring any difference between different genomes. We have argued previously (Stein *et al.*, 1979) that interspecific differences in repeated DNA hybrids must arise from events *other than* random base substitution (*e.g.*, amplification or reamplification) and thus cannot be expected to reflect divergence time. However, as pointed out by Kohne (1970), hybrids formed from single copy DNA should be composed only of sequences descended from the same ancestral sequence; such hybrids should thus provide a valid measure of the extent to which base substitution has occurred since separation of any two lineages.

For the present study eight species were chosen to represent diverse placements in the morphologically-based phylogeny (see fig. 1 of Belford and Thompson, 1980). *A. hortensis* (C3, *A. triangularis* (C3), *A. rosea* (C4) and *A. sabulosa* (C4) were chosen to represent the Hall and Clements subgenus Euatriplex, while *A. phyllostegia* (C3), *A. truncata* (C4), *A. fruticulosa* (C4) and *A. serenana* (C4) represent the subgenus Obione. Spinach (*Spinacia oleracea*, which also belongs to the family *Chenopodiaceae*) was included to provide an extrageneric comparison. Sequence divergence was measured for all species with respect to each of two references, *A. serenana* and *A. rosea*, permitting construction of a partial matrix of relationships.

In addition to clarification of Atriplex evolutionary relationships, this work was also undertaken to gain information on the extent of sequence divergence among members of an angiosperm genus and between genera of a single angiosperm family. Previous divergence measurements have been made for various animal taxa (Harpold and Craig, 1978; Angerer *et al.*, 1976; Shields and Straus, 1975; Sohn *et al.*, 1975; Rice, 1972; Laird *et al.*, 1969) and among three species of the fern genus Osmunda (Stein *et al.*,

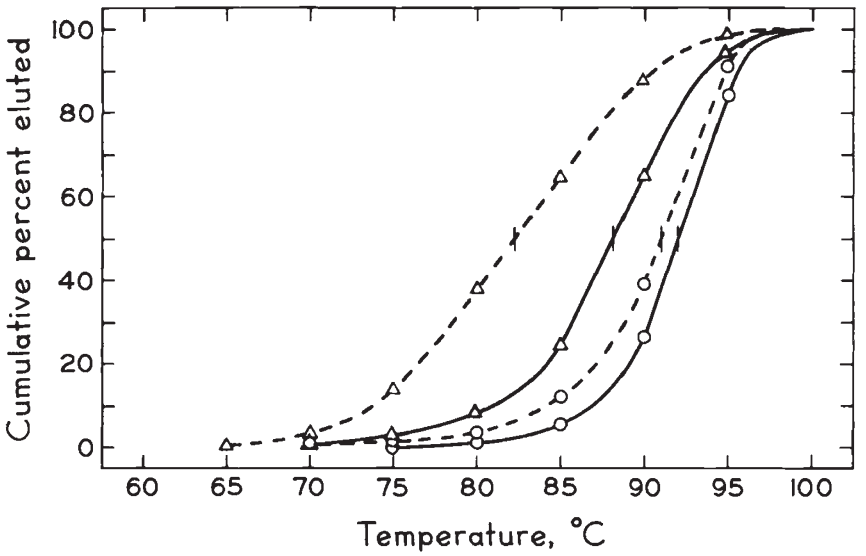

FIG. 1.—Thermal denaturation profiles for native and deaminated *E. coli* DNA in 120 mM and 75 mM PB. *E. coli* DNA which had been partially deaminated to introduce base pair mismatching (Ullman and McCarthy, 1973) was kindly supplied by R. E. Cuellar. Six replicate aliquots were heat-denatured and reassociated to equivalent Cot 200 in 0·6 M PB, 0·1 mM Na_2EDTA. After dilution into 120 mM PB and addition of tracer quantities of ^{14}C-labelled native *E. coli* DNA of about 250 NTP fragment length, the samples were applied to HAP at 60°C. Bound DNA was then eluted in 75 or 120 mM PB by raising the temperature in 5°C increments. Symbols: (△), deaminated *E. coli* DNA; (○), ^{14}C-labelled native *E. coli* DNA. Dashed lines indicate the thermal denaturation profiles obtained for 120 mM PB elution. Solid lines are profiles resulting from elution with 75 mM PB. ΔT_E values for the deaminated DNA were 8·8° in 120 mM PB and 3·9°C in 75 mM PB. When the same DNAs were melted in the spectrophotometer in 120 mM PB a ΔT_m of 4·0° was obtained from hyperchromicity measurements (R. E. Cuellar, personal communication).

1979). DNA sequence comparisons among Lathyrus species showed that divergence was indeed great enough to be detected among species within an angiosperm genus (Narayan and Rees, 1977).

2. MATERIALS AND METHODS

Plant material, DNA purification and single copy sequence preparation were as described (Belford and Thompson, 1980). Deaminated *E. coli* DNA was kindly supplied by R. E. Cuellar. DNA hybridization mixtures contained unlabeled total DNA as driver, a homologous single copy tracer and a heterologous single copy tracer in a ratio of 1 mg driver to 80,000 cpm ^{14}C-tracer and 400,000 cpm ^{3}H-tracer. Driver: tracer mass ratios were about 2000 : 1 and 9000 : 1 for ^{14}C- and ^{3}H-tracers, respectively. Driver and tracer DNAs were dissolved in 600 mM NaPB, *p*H 6·8, 0·1 mM Na_2 EDTA, to a final concentration of 4·5-5·0 mg DNA/ml. Aliquots were sealed in disposable glass capillaries, denatured 2-4 minutes in a polyethylene glycol bath at 104-110°C and reassociated for the required period in a circulating water bath at 66°C. The reaction was stopped by immersing the capillaries

in ice water followed by dilution into chilled 120 mM NaPB. The dilutions were stored on ice or frozen in liquid nitrogen and kept at −20°C until thermal denaturation.

Thermal denaturation of HAP*-bound DNA was performed as described by Britten *et al.* (1974) except that the elution buffer was 75 mM NaPB. This buffer molarity was chosen after constructing a "window diagram" as described by Martinsen and Wagenaar (1977) to ensure that only single-stranded DNA would be eluted from the HAP between 60 and 100°C. For each T_E determination, triplicate aliquots of reassociated DNAs were warmed to 60°C for several minutes, then loaded onto 0·25 ml HAP columns equilibrated in 120 mM NaPB at 60°C. After an initial wash with 120 mM NaPB to remove any unreassociated or degraded DNA, the HAP columns were equilibrated with 75 mM NaPB and thermally eluted in 5° steps. The presence of tracer DNAs was assayed by adjusting all column fractions to 2·5 ml and 120 mM NaPB, and counting in 9·4 ml Triton-X-100/toluene (1:2) scintillation fluid. The simultaneous use of ^{3}H- and ^{14}C-labelled tracers permitted each pair of homologous and heterologous tracer -driver duplexes to be formed in the same solution and analyzed on the same column.

3. Results

Historically, it has been standard practice to carry out thermal elution experiments with 120 mM NaPB. However, the results of Martinson and Wagenaar (1977) and our data (Belford, 1979) show that at temperatures above 60° some mismatched and/or short duplexes with low affinity for HAP can be eluted by 120 mM NaPB before the strands have completely dissociated. Because heterologous hybrids generally contain less well paired and possibly shorter duplex regions than homologous hybrids, premature elution of double strands would affect the apparent thermal stability of heterologous duplexes more than that of homologous duplexes and thus produce anomalously high ΔT_E values. 75 mM NaPB is near the center of the thermal elution "window" for well matched DNA and thus should minimize double-strand elution artefacts for interspecific hybrids (Martinsen and Wagenaar, 1977).

Thus we believe that our thermal elution data reflect actual thermal stabilities of hybrid duplexes more accurately than similar data obtained with 120 mM NaPB. This view is supported by the results of the experiment illustrated in fig. 1, in which artificially mismatched and control *E. coli* DNA samples were subjected to thermal elution in either 75 or 120 mM NaPB. The ΔT_E obtained in 75 mM NaPB was about half that obtained in 120 NaPB, and much closer to the ΔT_E measured by optical techniques. Similar ΔT_E ratios were obtained for interspecific hybrid duplexes between single copy tracers and total DNA of several Osmunda species (Stein *et al.*, 1979, and unpublished data). It is generally assumed that a 1° ΔT_E in 120 mM NaPB corresponds to about 1 per cent mismatched base pairs (*e.g.*, Britten *et al.*, 1974; Angerer *et al.*, 1976; Stein *et al.*, 1979). If we accept this assumption, it is reasonable to suppose that the same ΔT_E in 75 mM NaPB would correspond to about 2 per cent mispairing.

* Abbreviations: HAP, hydroxyapatite (Bio Rad HTP, Lot #16020); NaPB, equimolar Na_2HPO_4 and NaH_2PO_4, *p*H 6·8; NTP, nucleotide pairs; T_E temperature at which 50 per cent of the duplex DNA bound to HAP has eluted.

(i) *Atriplex thermal denaturation experiments*

Homologous and heterologous tracers were reassociated with excess unlabeled DNA to equivalent C_0t values of 880 and 28,000, bound to HAP, and denatured as described in Methods. In order to eliminate any effects on the melting profiles of contaminating repetitive sequence hybrids or intrastrand (foldback) duplexes in the *in vitro* labeled tracers, the C_0t 880

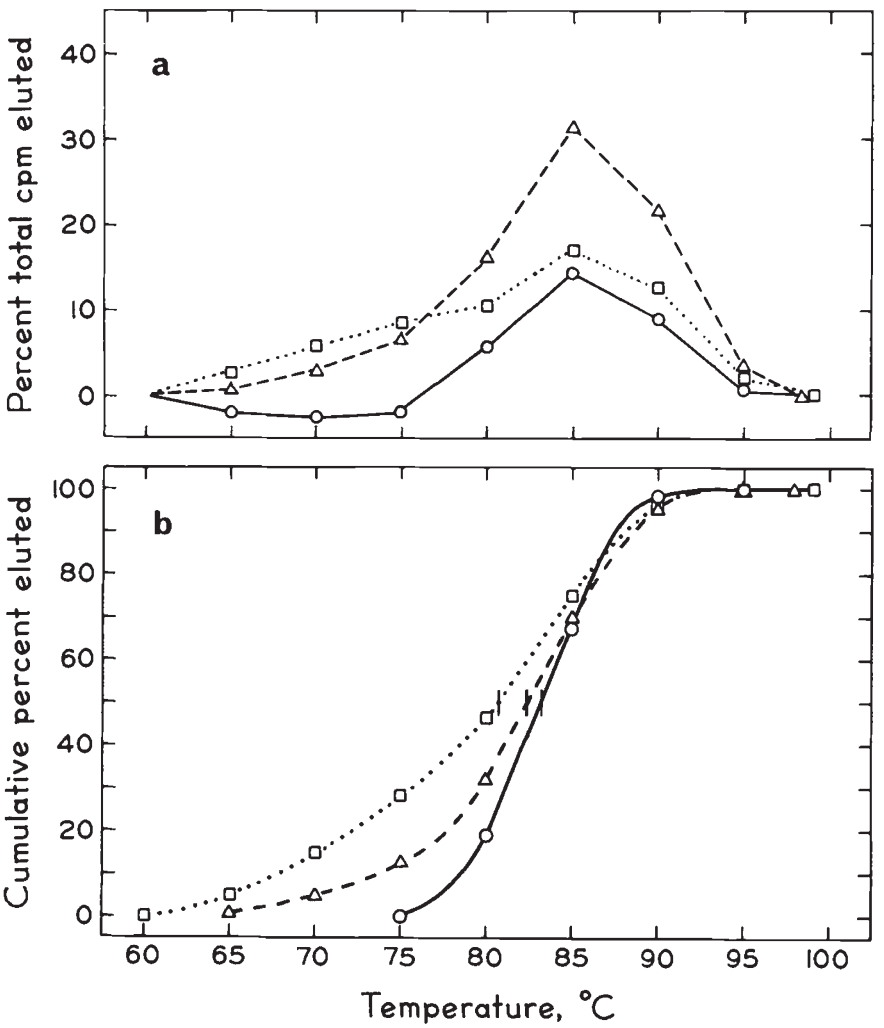

FIG. 2.—Thermal denaturation profiles of duplexes formed by ^{14}C-*A. serenana* single copy tracer reassociated with excess total *A. serenana* DNA. Reassociation and thermal elution procedures are described in the text. (· · · □ · · ·), Profile of the single copy tracer-driver DNA duplexes formed by reassociation to equivalent Cot 880. (– – △ – –), Profile of the duplexes formed by reassociation to ECot 28,000. The profile for duplexes formed between equivalent Cot values of 880 and 28,000 (—○—) was obtained by subtraction as described in Methods. In panel A, denaturation results are graphed as the percent of total ^{14}C-single copy tracer (HAP-bound and HAP-unbound fractions) eluted at each temperature. In Panel B, denaturation results are graphed as cumulative percent of HAP-bound ^{14}C-single copy tracer eluted. The T_E values for each curve are marked by vertical slashes.

denaturation profile was subtracted from the C_0t 28,000 profile as illustrated in fig. 2. Profiles thus corrected reflect the thermal stability of only those hybrids formed with single copy kinetics. The efficacy of this procedure was shown by using hybridization kinetics to detect the presence of repeated or foldback sequence contaminants and thus predict the result of the correction. Where a single copy tracer contains repeated sequences, the correction should result in an increase in T_E since repeated sequences (especially repeats remaining in single copy preparations isolated by kinetic fractionation) should form lower stability duplexes than single copy sequences. This effect is illustrated in figs. 2 and 3. On the other hand, if a tracer contains foldback sequences, the correction procedure should reduce the T_E by removing foldback duplexes. The majority of tracer profiles showed little or no change (±1°) in T_E when corrected by this procedure (table 1), indicating the purity and homogeneity of the tracer preparations. The few tracers showing a decrease of more than 1·5° in T_E after correction all contained significant percentages (*ca.* 30 per cent) of foldback sequences. Thus the correction procedure effectively removed repeated and foldback sequence contaminants from thermal stability profiles of homologous and heterologous tracer hybrids.

Table 1 lists both corrected and uncorrected ΔT_E values for all experiments. Corrected values were between 4·0 and 8·2°C. Except for the *A. rosea* × *A. serenana* values (discussed below), all replicates fall within 1·5° of each other. For reactions involving single copy DNA, reciprocal experiments (in which the same two tracers are reacted with driver DNA from one species in one reaction and with driver DNA from the second species in a second reaction) should give the same ΔT_E value. As can be seen in table 1, reciprocal values also generally fall within 1·5° of each other except for experiments involving spinach. So little hybridization occurred between spinach and Atriplex species (4-13 per cent after subtracting tracer duplexes formed before equivalent C_0t 880) that the differences in this case are not considered to be significant.

The *A. rosea* × *A. serenana* ΔT_E replicate difference of 3·0°C seems unexpectedly large (table 1). However, it is interesting that the 6·5° average ΔT_E for these reactions closely matches the reciprocal average of 6·7°. Thus the 3° difference between replicates may simply represent an extreme case of normal variation, a conclusion which emphasizes the usefulness of both replicate and reciprocal reactions.

4. Discussion

(i) *Intergeneric hybrids*

Atriplex-spinach ΔT_E values show poor reciprocity, differing by as much as 4°. We believe this reciprocity failure is due to the small amount of intergeneric duplex formation. Not only does the total single copy sequence cross reaction involve just 10-20 per cent of the available sequences, but furthermore, corrected ΔT_E values are based on duplexes formed between only 4-13 per cent of the single copy sequences. However, it is interesting that the ΔT_E values determined for spinach-Atriplex single copy sequence hybrids fall into the same general range as those determined for the Atriplex interspecific hybrids. The small amount of intergeneric cross reactivity,

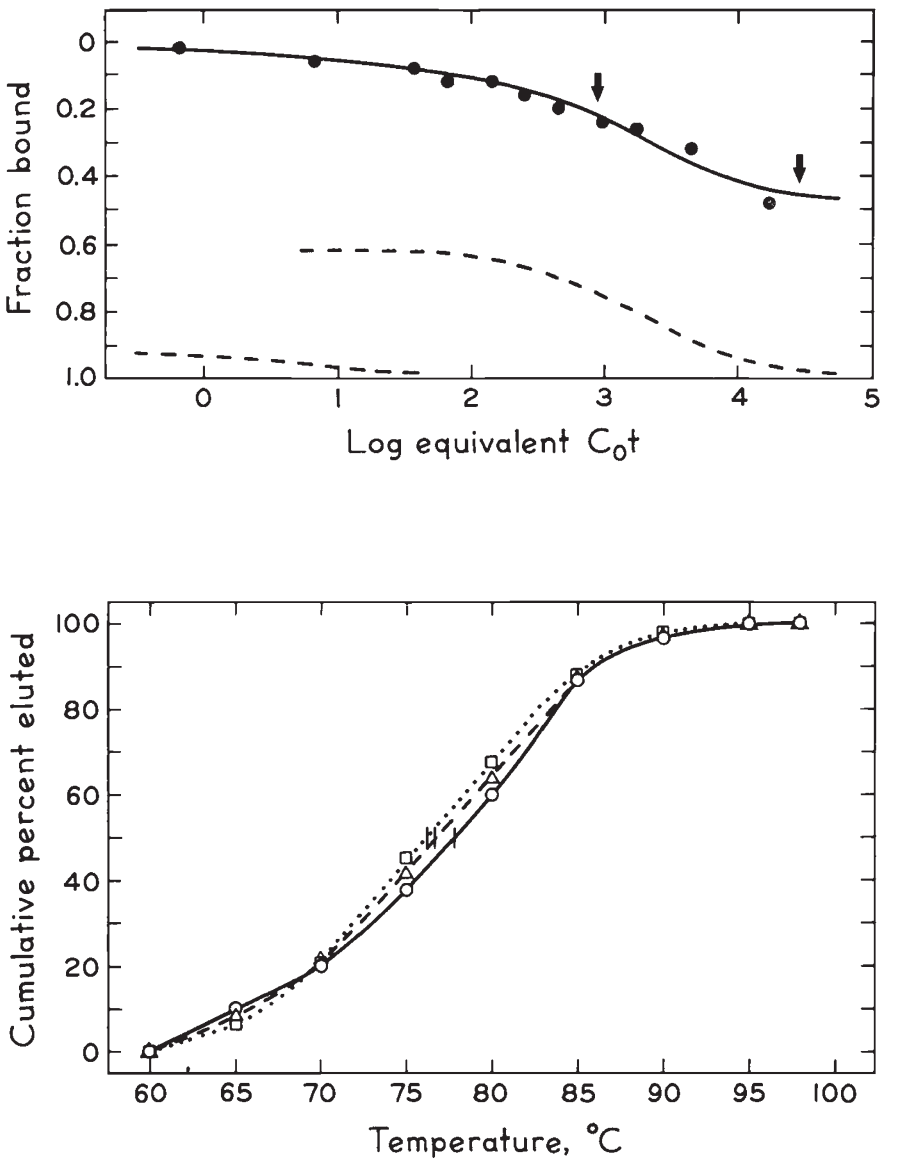

FIG. 3.—Reassociation kinetics and thermal denaturation profiles for the heterologous reaction between ^{3}H-*A. sabulosa* single copy sequence tracer hybridized with *A. serenana* total DNA. *Top Panel*: Kinetic data were obtained and fit by the retarded rate procedure for heterologous tracers as described (Belford and Thompson, 1980). The data gave a best fit with two components. The faster, repeated sequence component comprises approximately 9 per cent of the reactable heterologous tracer preparation. Arrows indicate the C_0t values to which tracer and driver DNAs were hybridized before thermal denaturation on HAP (EC_0t 880 and 28,000). Note that the repeated sequence component is completely reacted before EC_0t 880. *Bottom panel*: Thermal denaturation of HAP-bound tracer-driver duplexes was performed as described in the text. Denaturation profiles of ^{3}H-*A. sabulosa* DNA duplexes hybridized to EC_0t 880 (· · · □ · · ·) or EC_0t 28,000 (· · · △ · · ·) with *A. serenana* driver DNA were used to obtain the denaturation profile of tracer-driver duplexes between these two EC_0t values (– – ○ – –).

TABLE 1

Corrected and uncorrected ΔT_E values

Homologous Tracer	Heterologous Tracer	Exp.	ΔT_E, °C* Eq. Cot 880	Eq. Cot 28,000	Corrected
A. rosea	*A. sabulosa*	678	3·7	4·7	4·5
A. rosea	*A. triangularis*	678	6·2	5·7	5·0
A. rosea	*A. truncata*	778	5·5	5·5	5·2
A. rosea	*A. phyllostegia*	878	3·2	4·4	5·5
A. rosea	*A. hortensis*	778	4·5	5·6	6·2
A. rosea	*A. serenana*	578	5·0	6·3	8·0
A. rosea	*A. serenana*	678	4·7	4·7	5·0
A. rosea	*A. fruticulosa*	578	5·0	6·1	7·5
A. rosea	*A. fruticulosa*	678	4·7	5·7	7·0
A. rosea	Spinach	878	7·4	7·8	8·2
A. serenana	*A. fruticulosa*	177	1·5	2·5	4·7
A. serenana	*A. fruticulosa*	877	3·0	3·7	4·0
A. serenana	*A. truncata*	1078	4·1	4·5	5·0
A. serenana	*A. sabulosa*	277	4·5	4·7	5·0
A. serenana	*A. hortensis*	177	6·5	6·5	5·2
A. serenana	*A. phyllostegia*	377	6·5	5·7	5·5
A. serenana	*A. phyllostegia*†	1078	−4·0	−0·6	6·0
A. serenana	*A. rosea*	377	5·7	6·5	6·5
A. serenana	*A. rosea*	877	0·7	3·7	6·0
A. serenana	*A. rosea*	678	7·7	7·7	7·5
A. serenana	*A. triangularis*	277	5·0	6·5	8·2
A. serenana	Spinach	1078	−3·6	−2·5	4·4
A. fruticulosa	*A. rosea*	678	7·0	6·7	7·5
A. triangularis	*A. rosea*	678	7·3	6·7	6·5
A. sabulosa	*A. rosea*	678	4·5	4·2	4·0
A. hortensis	*A. rosea*	778	5·8	5·7	5·4
A. truncata	*A. rosea*	778	6·7	6·2	6·5
A. phyllostegia	*A. rosea*	787	6·5	5·4	4·2
Spinach	*A. rosea*	878	5·2	4·5	4·2
A. phyllostegia†	*A. serenana*	1078	8·6	8·0	4·7
A. truncata	*A. serenana*	1078	3·5	3·8	4·2
Spinach‡	*A. serenana*	1078	0	1·0	6·0

* Each value listed is the average of 3 replicate thermal elution profiles; standard deviations of replicate T_E values in a single experiment averaged <0·2°C. Corrected values were obtained by subtracting the low Cot profile from the high Cot profile as described in the text and fig. 2.

† The *A. phyllostegia* tracer used in these reactions contained about 30 per cent foldback sequences.

‡ The spinach and *A. serenana* tracers in these reactions both contained about 30 per cent foldback.

together with the improbability of spinach and *A. rosea*, or spinach and *A. serenana* having shared a common ancestor as recently as *A. rosea* and *A. serenana* or other Atriplex species pairs, leads us to hypothesize that the sequences showing intergeneric cross reaction represent a subset of evolutionarily conserved single copy DNA. Complexity estimates for this class (approximately 3×10^7 NTP; see Belford and Thompson, 1980) are well within the reported range for messenger RNA complexities in many organisms (*e.g.*, Kiper, 1979; Kamalay and Goldberg, 1980). A similarly conserved subset of single copy DNA in the rat and mouse genomes appears to be evolving at about the same rate as structural gene sequences (analyzed

by comparing single copy DNA and *c*DNA–DNA hybridizations at increasingly stringent reassociation criteria; Rosbash *et al.*, 1975).

The Atriplex-spinach results raise an important question concerning the general utility of the DNA hybridization technique in phylogenetic studies. The use of hybrid thermal stability as an index of divergence time depends upon the assumption that base substitution proceeds at a constant rate in the DNAs being compared. When hybridization drops to such a low level that a small fraction of selectively conserved sequences could represent a major part of the hybrids formed, one must wonder whether the resulting ΔT_E can be compared with those for species showing higher levels of cross reaction.

(ii) *Interspecific hybrids and phylogenetic considerations*

Within the genus Atriplex, cross reactivities were generally about 50 per cent or more (Belford and Thompson, 1980), thus reducing the uncertainty in interpreting ΔT_E measurements. The only exception was found in experiments with *A. phyllostegia* single copy sequences, where only about 30 per cent of the tracer was able to pair with *A. rosea* or *A. serenana* DNA. Perhaps because of this limited cross reaction, a greater than average reciprocal difference was observed for the *A. phyllostegia-A. rosea* cross reactivities and ΔT_E measurements.

The ΔT_E data show the lowest amount of base sequence divergence between the species pairs *A. serenana-A. fruticulosa* and *A. rosea-A. sabulosa*, a finding which agrees with the classical and experimental taxonomic evidence that these species are closely related pairs. Most other species pairs display ΔT_E values between 5 and 6·5° in 75 mM PB. These values would probably be about twice as high in 12° mM PB (see above and fig. 1). Thus the amounts of sequence divergence observed between Atriplex species are distinctly larger than intrageneric divergence values measured for species of sea urchin (Angerer *et al.*, 1976; Harpold and Craig, 1978), deer mouse (Rice, 1972), Junco (Shields and Straus, 1975), black flies (Sohn *et al.*, 1975) or salamander (Mizuno and MacGregor, 1974). Our divergence estimates for Atriplex are also greater than those reported for Lathyrus species (Narayan and Rees, 1977) and greater than or equal to those observed in the genus Osmunda (Stein *et al.*, 1979). Indeed, it appears that sequence divergence within Atriplex is more comparable to previously observed in *inter*generic values for animal DNA (Eden *et al.*, 1978; Rice, 1972; Shields and Straus, 1975; Laird *et al.*, 1969; Sohn *et al.*, 1975). No intergeneric divergence estimates have been published for higher plants other than the spinach × Atriplex data reported here. As noted above, these are not considered comparable to the intrageneric Atriplex data because of the low level of cross reaction on which they are based.

All species in this study were compared with the same two reference species. Possible divergence time relationships which can be derived from these data therefore fall into only three categories: a given lineage may have originated before the separation of lines leading to the reference species, at the same time as this separation, or at some later time. If genetic isolation was a unique and permanent event in each case, and mutations have accumulated at the same rate in different lines, then ΔT_E relationships should also fall into three classes. In the case of a lineage originating before the reference species, the ΔT_E to both references should be equal but

TABLE 2

Summary of ΔT_E measurements

Species A	Species B	ΔT_E, °C I	ΔT_E, °C II	Difference
A. rosea	*A. sabulosa*	4·5	4·0	0·5
	A. triangularis	5·0	6·5	1·5
	A. truncata	5·2	6·5	1·3
	A. phyllostegia	5·5	4·2	1·3
	A. hortensis	6·2	5·4	0·8
	A. serenana	6·5*	6·7*	0·2
	A. fruticulosa	7·2*	7·5	0·3
	Spinach	8·2	4·2	4·0
A. serenana	*A. fruticulosa*	4·3*	—	—
	A. truncata	5·0	4·2	0·8
	A. sabulosa	5·0	—	—
	A. hortensis	5·2	—	—
	A. phyllostegia	5·7*	4·7	1·0
	A. rosea	6·7*	6·5*	0·2
	A. triangularis	8·2	—	—
	Spinach	4·4	6·0	1·6

In column I, the ΔT_E values were obtained by comparing T_E values of single copy sequences of species A and B reassociated with total DNA of species A. In column II, the ΔT_E values were obtained from reciprocal reactions, that is, by comparing T_E values of species A and B single copy sequences reassociated with total DNA of species B. The differences between reciprocal values are listed in the third column. All data are corrected values. (*) indicates averages from two or more independent experiments.

greater than that between the reference species themselves. Species whose origin was contemporary with that of the references should also have equal ΔT_E values to each reference, but they should be the same as the ΔT_E between references. When a given lineage originated from one of the reference lines later in evolution its ΔT_E to that reference should be small compared to the ΔT_E observed with the other reference (which should still be equal to that between the references themselves).

These theoretical ΔT_E relationships are indicated by the heavy solid lines in the matrix diagram of fig. 4. The two arcs define points equidistant from each reference. Where these arcs intersect, the ΔT_E for each reference is the same as that between references. Dashed lines and circles have been used to indicate a 1° range of experimental error. Given this range all the Atriplex species can be considered to fit the predicted pattern for simple divergence relationships (although the fit for *A. triangularis* and *A. sabulosa* is only marginal; see below). *A. phyllostegia* and *A. hortensis* are located close to the intersection point, indicating they they became isolated from the *A. rosea* and *A. serenana* lines at about the same time as or shortly after these lineages separated from each other. The *A. truncata* lineage may have originated at about this time or perhaps somewhat later, while the placement of the *A. fruticulosa* range across the *A. serenana* leg of the matrix clearly suggests that *A. fruticulosa* diverged from *A. serenana* after the *A. serenana* line had separated from that of *A. rosea*.

The overlap of *A. triangularis* and *A. sabulosa* ranges with that of the *A. rosea* leg might also be interpreted to suggest that these species diverged from the *A. rosea* lineage after the *A. rosea* and *A. serenana* lines had

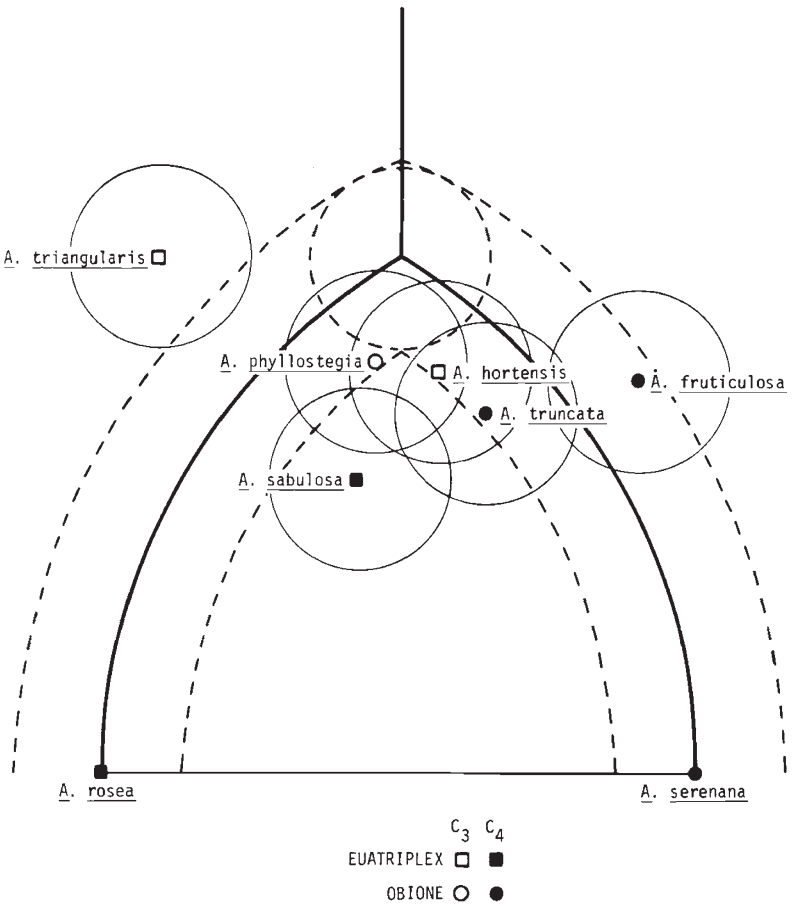

FIG. 4.—Location of the Atriplex species on the theoretical matrix described in the text. Linear distances from the reference species (*A. rosea* or *A. serenana*) are proportional to the average corrected ΔT_E values. Circles and dashed lines indicate a ±1° error range.

become independent. However, these overlaps are not great, which leads us to consider an alternative hypothesis involving secondary exchanges of genetic material. It may be that the *A. triangularis* line originated *before* the separation of *A. serenana* and *A. rosea*. At some later time, secondary gene exchange may have occurred between *A. triangularis* and *A. rosea*, reducing the apparent base-sequence divergence measurable for this species pair. Similarly, the *A. sabulosa* ancestor may have retained the ability to interbreed with both *A. rosea* and *A. serenana* lines for some time after the separation of the latter two species, so that the divergence between *A. sabulosa* and either reference appears to be less than the divergence between the references themselves.

Secondary gene exchange might occur by introgressive hybridization, as has been documented in many higher plant groups (Heiser, 1973), including Atriplex (Osmund *et al.*, 1980). Support for such an hypothesis comes from sexual hybridization data (Björkman *et al.*, 1973; Nobs, 1976) demonstrating the ability of both *A. sabulosa* and *A. triangularis* to form partially fertile

hybrids with *A. rosea*. Similarly successful sexual hybridizations were not obtained for other species used in our study except *A. fruticulosa* and *A. serenana*.

Phylogenetically, the introgression hypothesis parallels the simple divergence scheme in suggesting that the species near in the center of the diagram—*A. hortensis*, *A. truncata* and *A. phyllostegia*—represent lineages which arose at about the same time as those of *A. serenana* and *A. rosea*. Both hypotheses assume that *A. triangularis* and *A. sabulosa* have been fully separated from *A. serenana* for a longer time than from *A. rosea*, while *A. fruticulosa* has been isolated for a longer time from *A. rosea* than from *A. serenana*. The evolutionary mechanisms are at odds only on the question of whether a single and permanent divergence event has occurred.

Our hybrid thermal stability measurements indicate that lines leading to many present-day Atriplex species probably originated during a single period of rapid speciation. This group includes *A. rosea*, *A. serenana*, *A. hortensis*, *A. phyllosetegia*, and perhaps *A. truncata*; *A. triangularis* may have originated even earlier. Even the first four species, whose placement is most certain, include representatives of both subgenera. Thus, the subgeneric distinction proposed by Hall and Clements (1923), with its implication of a gradually reticulating phylogenetic "tree," is not supported by our data. Furthermore, since the same four species also include representatives of both C3 and C4 photosynthetic pathways, it appears logical to assume that C4 photosynthesis first appeared in Atriplex at about this time. In the context of the phylogenetic history derived from our molecular data, it is no longer necessary to postulate a polyphyletic origin for C4 photosynthesis within Atriplex.

If it is assumed that plant DNA base sequences diverge at rates comparable to those for animals, it is possible to construct an approximate time scale for Atriplex evolution. Estimates for base substitution rates in animal genomes mostly fall between 0·06 and 0·3 per cent per million years (Laird *et al.*, 1969; Fitch, 1976; Fitch and Langley, 1976; Kohne, 1970; Harpold and Craig, 1978; Angerer *et al.*, 1976; Galau *et al.*, 1976). We assume that a $1° \Delta T_E$ in the present experimental system corresponds to about 2 per cent base pair mismatch in interspecific hybrids, or about 1 per cent base substitution per lineage. Thus the ΔT_E of 6·5° between *A. rosea* and *A. serenana* would indicate about 6·5 per cent substitution in each genome. If we use a rate of 0·2 per cent substitution per million years, the major period of active speciation in Atriplex would have been roughly 30-35 million years ago. On the same basis, *A. fruticulosa* would have diverged from *A. serenana* about 20 million years ago. These estimates would place the major Atriplex speciation events in the Oligocene-Miocene epochs of the Tertiary period of geologic history. Although extremely tentative, this placement is not unreasonable in light of existing fossil and geologic evidence that with the recession of forested land, herbaceous plants were becoming increasingly abundant during the Oligocene, Miocene, and Pliocene epochs. Furthermore, since fossil evidence for C4 plants has been found in Miocene and Pliocene formations (Thomasson, 1978; Nambudiri *et al.*, 1978), it can be assumed that environmental conditions at that time were right for the appearance of C4 adapted species.

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