Genetics of Adaptive Radiation in Hawaiian and Cook Islands Species of Tetramolopium (Asteraceae). II. Genetic Linkage Map and Its Implications for Interspecific Breeding Barriers

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ABSTRACT

In a study of the genetic mechanisms associated with adaptive radiation in Hawaiian Tetramolopium, a genetic linkage map was constructed in an interspecific cross. A total of 125 RFLP and RAPD markers were mapped into 117 different loci on nine linkage groups for a map length of 665.7 cM. Segregation distortion occurred in 49% of the mapped probes, located primarily in four linkage groups. High percentages of one parental species genotype (*Tetramolopium rockii*) were recovered in three of these blocks and the second parental species (*T. humile*) in the remaining block. The high degree of distorted segregation suggests the buildup of internal crossing barriers, even though island plant species are typically characterized as highly cross compatible with few to no internal crossing barriers. This work and a review of previous crossing studies in island plants show that internal (postmating) crossing barriers do exist. The weak crossing barriers have likely been overlooked because the main focus has been on diversification and speciation through adaptation to extremely diverse environments.

THE proliferation of congeneric plant species on oceanic islands has been a fertile area of investigation for the study of rapid speciation and diversification. Carlquist (1974, 1980) has provided perhaps the most comprehensive background of the biology of island organisms and some key insights into their evolution. Today, studies in plant taxa are typically focused on the area of phylogenetic reconstruction and interpreting evolutionary patterns in light of island colonization, ecology, and diversification (*e.g.*, Wagner and Funk 1995; Francisco-Ortega *et al.* 1996; Kim *et al.* 1996). These studies are facilitated by the availability of molecular data that provide a wealth of information for phylogenetic reconstruction. Molecular markers may also be applied to the study of morphological evolution during diversification in island situations. They can give insights into the genetic basis of morphological changes (Doebley 1993; Whitkus *et al.* 1994; Bachmann and Hombergen 1997) and, ultimately, the genetic basis of the adaptations exhibited by taxa (Orr and Coyne 1992).

The genus Tetramolopium has several features that make it a model system for the study of the genetic processes associated with morphological diversification and adaptation in islands. Arriving in the Hawaiian Islands within the Pleistocene (Fosberg 1948; Smith 1977) from New Guinea (van Royen 1983), 11 well-defined morphological species radiated into lowland and upland habitats and have dispersed to the Cook Islands in the south Pacific (Lowrey 1986, 1995). Phylogenetic studies based on morphology (Lowrey 1995) and molecular markers (Okada *et al.* 1997) provide a well-resolved pattern of relationships. All Hawaiian species are diploid ($n = 9$), display some of the lowest levels of genetic diversity reported for island plant taxa (Lowrey and Crawford 1985; DeJoode and Wendel 1992; Okada *et al.* 1997; Gemmill *et al.* 1998), are short-lived perennials, and are cross compatible through the F_3 (Lowrey 1986; T. K. Lowrey and R. Whitkus, unpublished data). The current study reports on the construction of a molecular marker-based genetic linkage map in an interspecific cross in Tetramolopium. Although the map provides a framework for future genetic analysis of morphological diversification within the group, the results give an insight into the nature of reproductive isolation in Hawaiian Tetramolopium, a group characterized as having no internal barriers to crossing.

MATERIALS AND METHODS

Plants and cross: The mapping population was derived from a cross between *Tetramolopium rockii* var. *rockii* (monoecious, female) and *T. humile* ssp. *humile* (gynomonoecious, male). No reciprocal cross differences exist with regard to production of fertile F_1 in this combination (Lowrey 1986; T. K. Lowrey and R. Whitkus, unpublished data). Plants were grown in 4-inch pots in greenhouses at Riverside, California, using standard potting soil and ambient conditions. Crosses are made by removing the disk (male) florets in the capitula of *T. rockii* plants before anthesis and rubbing receptive capitula with *T. humile* in which disk florets are shedding pollen. All capitula used in crosses are covered with pollination bags to exclude

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small insects that can carry pollen, and to hold the achenes as they become mature.

An F_2 population was made by selfing a single F_1 plant from the interspecific cross. The F_1 plants show dominance to *T. rockii* with each capitulum producing ~50 ray florets (R. Whitkus, personal observation) so a few heads provide over 100 achenes. Achenes were sown in 4-inch pots and grown to obtain leaf material for genomic DNA.

RFLP and RAPD generation: Two random genomic libraries were prepared as a source of restriction fragment length polymorphism (RFLP) probes. The library designated TH was prepared from *T. humile*, and the library designated TR was from *T. rockii*. Methods for library construction, preparation and purification of probes, extraction, restriction, and Southern blotting of genomic DNA, and filter hybridizations and autoradiography have been described previously (Whitkus *et al.* 1992; Okada *et al.* 1997).

Additional RFLP probes were prepared from cloning of simple sequence repeat (SSR)-anchored fragments (Zietkiewicz *et al.* 1994). Genomic DNA from both parental plants was amplified with the primer $(CA)_8RG$ and amplification products cloned with the TA cloning system (Invitrogen Inc., Carlsbad, CA) following manufacturer's instructions. Subsequent probe preparation followed the procedure used for genomic probes.

Random amplified polymorphic DNA (RAPD) loci (Williams *et al.* 1990) were generated following the procedure outlined in de la Cruz *et al.* (1995) except 15 ng of genomic DNA and 1 unit of *Taq* DNA polymerase were used. Decamer primer sets A, B, C, D, E, F, G, K, and Z (Operon Technologies, Alameda, CA) were surveyed for polymorphism between the two parental DNAs.

Mapping: The mapping population consisted of 90 F_2 individuals. Clones / primers that proved polymorphic between the parental DNAs and in 5 randomly chosen F_2 were mapped in the entire population. Genotyping was based on identification of the parental bands in the F_2. In cases where scoring of an individual was questionable, the data point was recorded as absent. Errors were reduced by scoring genotypes twice on independent dates and then checking the database directly against the original data.

The map was constructed with MAPMAKER 3.0 (Lander *et al.* 1987; Lincoln *et al.* 1992). An initial map was constructed using GROUP, COMPARE, and TRY commands and two-point linkage criteria of a LOD of 3.5 and a maximum distance of 35 cM between marker loci.

The initial map was used as a check for potential genotyping errors by running the MAP command with error checking (Lincoln and Lander 1992) on each linkage group. Potential errors at a locus and the flanking loci were genotyped again from the original data, and errors corrected if found. This procedure was repeated to double-check the final database.

The final map was constructed using the automatic mapping features of MAPMAKER. Using the ORDER command (parameters set at seven informative loci, minimum LOD of 3.5, maximum distance of 35 cM, informativeness criteria of a minimum of 30 individuals, and a minimum distance of 5 cM), the most informative subset of loci and most likely order of all loci using multipoint linkage analysis was conducted on each initial group of loci. The final order of loci on a linkage group was checked with the RIPPLE command to identify alternate orders of loci up to 10 times less likely (LOD 1) than the best order. Haldane map distances were used in all analyses and reported on the map. Although the Haldane map function discounts interference, it is multilocus feasible and valid for multilocus analysis (Ott 1991).

Segregation distortion and recombination: Segregation distortion of genotypic frequencies was tested at each locus using a likelihood ratio test (*G*-test; Sokal and Rohlf 1981), adjusted to approximate the chi-square distribution following Williams (1976). Similar tests for gametic distortion were repeated on loci exhibiting significant genotypic segregation distortion.

The apparent number of recombinants was obtained through counts of the observed recombinants by examination of individual genotypes over each linkage group. In regions with runs of dominant markers the number of recombinants was doubled to account for the 50% reduction of observed recombination over dominant markers. The adjusted number of recombinants was divided by the average number of individuals used to map loci in the linkage group. This procedure gave an estimated crossover number for each linkage group.

Clustering of loci: Clustering of markers was tested using nearest neighbor analysis of points along a line, including the endpoints (Selkirk and Neave 1984). Randomly distributed points on a line have a scaled nearest neighbor value equal to or close to 0.5, overdispersed points give a value close to 1, and clustered points a value close to 0. Selkirk and Neave (1984) provide a formula for calculating approximate percentage values, given the number of points used in calculating the nearest neighbor distance. Scaled nearest neighbor distances were calculated for each linkage group and compared to the approximate one-tailed 0.05 percentage values to test for clustering.

The runs test (Neave and Worthington 1988) was used to determine whether dominant and codominant markers occur in a randomized pattern on each linkage group.

RESULTS

Polymorphism: Over 900 clones were picked from the *T. humile* (TH) and *T. rockii* (TR) genomic libraries. Dot blot experiments revealed 286 candidate low-copy or single-copy probes. Sixty-two of these provided usable polymorphisms (Table 1). Less than half (48%) of the polymorphic probes gave RFLPs for both restriction enzymes, while 21 were polymorphic with only *Eco*RV and 11 with only *Hin*dIII. Polymorphisms of cloned SSR-anchored fragments were found with *Eco*RV, with each parental DNA providing one polymorphic clone. The overall level of RFLP found in the survey was 21.4%. The RAPD primers provided a similar overall level of polymorphism (22.6%) with 30 polymorphic primers obtained from the 133 primers surveyed (Table 1). The number of polymorphic bands per primer ranged between one and five. Combining RAPDs and RFLPs, the overall polymorphism level obtained for the cross was 21.8%.

Segregation analysis revealed 64 codominant and 5 dominant RFLP marker loci (Table 1). Five genomic RFLP probes provided 2 loci, while a single locus was obtained for the remaining probes, even though additional bands may have been present on the autoradiograms. Four RAPD bands mapped as codominant alleles in 2 loci (primers B04 and E18). All remaining RAPD bands were interpreted as dominant loci in the F_2.

The number of polymorphic markers is related to the total genetic variability between the parents (species). Given the known low level of genetic variability in Tetra-

TABLE 1

Probe and primer survey results

Marker source	Number surveyed	Number polymorphic			Mapped		
		One enzyme	Two enzymes	Total	Codominant	Dominant	Total
Genomic RFLP	286	32	30	62	62	5	67
SSR-RFLP	13	2		2	2		2
RAPD primers	133	—	—	30	2	54	56

molopium (Lowrey and Crawford 1985; Okada *et al.* 1997), an interspecific cross was necessary to find sufficient polymorphism for mapping. In most instances, interspecific crosses provide 45% (*i.e.*, cotton; Reinisch *et al.* 1994) to over 90% (*i.e.*, sorghum; Chittenden *et al.* 1994) polymorphic probes or primers, although Mimulus is an exception with 28% RAPD primer polymorphism (Lin and Ritland 1996). The two species used in this study have the highest per species RFLP in Tetramolopium, with 39–46% polymorphic probes in *T. rockii* and 27–30% polymorphic probes in *T. humile* (Okada *et al.* 1997). The overall polymorphism level obtained in the Tetramolopium cross of 22% thus reflects the limited genetic variability and divergence at marker loci exhibited by members of the genus.

Linkage map: The Tetramolopium linkage map (Figure 1) consists of 125 markers in 117 different loci. The length of the map is 665.7 cM with an average distance of 6.5 cM between adjacent loci [665.7/(112 loci—9 ends)]. Nine linkage groups were found, composed of a total of 119 markers in 112 loci and corresponding to the haploid chromosome number for Tetramolopium. The nine linkage groups were obtained after 109 markers were mapped. The last 15 of 16 markers added loci to existing linkage groups and within 5 cM of existing loci (data not shown), while one marker (F09-1200) is unlinked. Six loci on the map are composed of probes with different autoradiographic and/or RAPD patterns that map to the same location. These are found on linkage groups B (TR544A/A03-800), G (F09-1500/TR-116; TH198/Z14-950; TR441/TR153), and H (TR453/TH225a; TH222/TH28/TR437). The different banding patterns for these multiple probe/primer marker loci suggest closely linked loci in which no recombination was observed in this cross.

A number of genotyping errors were found in the error-checking phase of map construction. However, the corrections did not result in large reordering of loci or changes in distances between loci of over 10 cM.

Six markers remained unlinked, although two RFLP probes (TH142 and TH187) map to one unlinked locus, despite exhibiting different autoradiographic patterns. An additional 175 cM (5 × 35 cM) would be required, at minimum, to join the unlinked loci into the map.

Segregation distortion: Evidence for skewed segregation (Table 2) exists at 57 loci (49% of the total loci). The majority of these (72%) show deviation toward the *T. rockii* genotype and are located mainly on the lower portion of linkage group B, and nearly all of linkage groups C (16 of 17 loci) and G (9 of 14 loci) (Figure 1). Loci showing an excess of alleles toward *T. humile* are located mainly on linkage group E (8 of 10 loci; Figure 1). Two loci (TH182a and TH142) have significant heterozygote excess. Almost all loci (86%) showing evidence of segregation distortion also have significant gametic deviations (Table 2).

Skewed segregation influences map construction by increasing the difficulty in determining linkages and estimating recombination frequencies (Wang *et al.* 1994; Paran *et al.* 1995; Sybenga 1996). On the Tetramolopium map the average distance between pairs of loci showing skewed segregation is less than those showing Mendelian expectations (5.2 *vs.* 6.9, respectively), but the difference is not significant (Mann-Whitney test, $P > 0.5$). Locus order in the 13 regions of the map in which alternate orders of loci were likely was not solely related to distorted segregation because 7 regions include loci with distorted ratios and 6 with Mendelian ratios. Finally, no significant correlation exists between the estimated number of crossovers (Table 3) and the percentage loci exhibiting segregation distortion on each linkage group ($P = 0.23$; Spearman rank correlation). These results suggest that distorted segregation has not contributed significantly to questionable locus order or recombination frequencies in the Tetramolopium map.

Clustering of loci: Nearest neighbor analysis revealed no significant clustering of loci on any of the linkage groups ($P > 0.05$, one-tailed test). Although clustering appears to be present on some linkage groups from visual inspection (*i.e.*, B and I), the sample sizes are likely too small to obtain significance. The runs test showed a significant run of locus type (dominant/codominant) on linkage groups B, G (runs = 5, $P_{0.05} \leq 5$), and C (runs = 4, $P_{0.05} \leq 5$).

Genome length: Three methods for estimating map length in Tetramolopium agreed in showing the observed map is smaller than expected. The first approach began by assuming Tetramolopium chromosomes behave normally during pairing and produce an average of one crossover per chromosome arm, which in turn results in a recombination fraction of 50% per arm

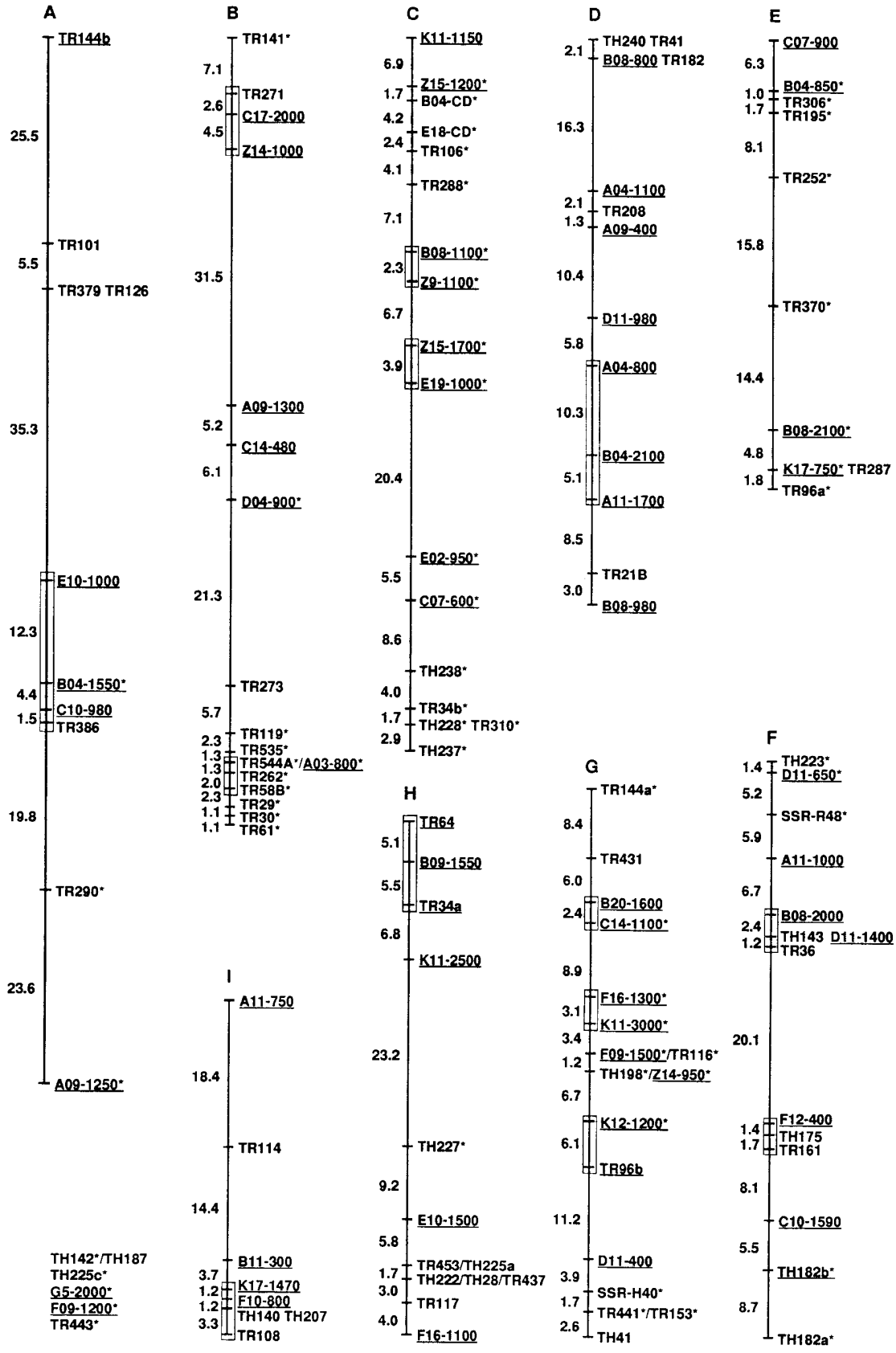

Figure 1.—Genetic linkage map for Tetramolopium. Markers appear to the right of each linkage group with a capital letter after a name indicating a marker derived from different probes and a small letter indicating alternate markers derived from a single probe. Names beginning with TH are from probes obtained from *T. humile* and those beginning with TR are probes obtained from *T. rockii.* Underlined names are dominant. Probe names separated by a slash (/) map to the same locus. Probe names on the same line separated by a space map within 1 cM of each other. An asterisk indicates a marker exhibiting distorted segregation. Boxes indicate regions of map where an alternate ordering of marker loci is possible but is up to 10 times less likely than given order. Haldane map distances are on the left of each linkage group.

TABLE 2

Loci showing distorted segregation and significant gametic deviation

Linkage group	Deviation[a]	Locus[b]
A	H	B04-1550, TR290*
	H/het	A09-1250
B	H	TR141*
	R	TR119*, TR535*, TR544A*/A03-800*, TR262*, TR58B*, TR29*, TR30*, TR61*
	R/het	D04-900
C	R	Z15-1200*, B09-CD*, E18-CD*, TR106*, TR288*, B08-1100*, TH238*, TR34b*, TH228*, TR310*, TR237*
	R/het	Z9-1100*, Z15-1700*, E19-1000*, E02-950*, C07-600*,
E	H	B04-850*, TR306*, TR195*, TR252*, TR370*, B08-2100, K17-750, TR96a*
F	R	TH223*, D11-650*, SSR-R48*
	het	TH182a*
	H/het	TH182b*
G	R	TR144a*, K11-3000*, F09-1500*/TR116*, TH198*/Z14-950*, K12-1200*, SSR-H40*, TR441*/TR153*
	R/het	C14-1100*, F16-3000*
H	R	TH227
Unlinked	H	G5-2000*
	R	TH225c*, TR443*
	het	TH142
	R/het	F09-1200

[a] Deviation of genotypes is toward *T. humile* (H), *T. rockii* (R), heterozygote excess (het) or the dominant genotype (H/het or R/het).

[b] Significant gametic deviation ($P < 0.05$) is indicated by an asterisk (*).

(Ott 1991; Sybenga 1996). This approach provides a minimum estimate of the genome-wide recombination fraction. The 18 chromosome arms in Tetramolopium give a total expected recombination fraction of 900 (18 crossovers). The total recombination fraction on the map of 585.7 (converted from map distances) is 65% of the expected 900. A second approach relies on the total number of estimated crossovers (Table 3). The obtained value of 12.9 is 72% of an expected 18.

A third estimate of the expected map length was obtained by method 4 of Chakravarti *et al.* (1991). Considering each linkage group to be a single chromosome and that marker loci are uniformly distributed, each linkage group is inflated by $(m + 1)/(m - 1)$, where m is the number of markers on the linkage group. The assumption of uniform locus distribution is supported by the nearest neighbor analysis failing to find significant clustering of loci. This procedure provides an estimated genome length of approximately 781 cM. The observed total map length is 85% of this value.

TABLE 3

Observed and adjusted number of recombinants and estimated number of crossovers on Tetramolopium map

Linkage group	Observed recombinants	Adjusted recombinants[a]	Average N	Estimated crossovers[b]
A	124	151.0	86.3	1.75
B	118	163.0	86.9	1.88
C	108	150.0	84.1	1.78
D	91	126.5	88.1	1.44
E	75	86.5	86.0	1.01
F	96	119.5	86.4	1.38
G	98	146.0	83.6	1.75
H	78	102.0	85.3	1.20
I	47	58.0	86.4	0.67

[a] The adjusted number of recombinants does not include potential double crossovers.

[b] The estimated number of crossovers is based on the average number of individuals used to construct each linkage group (N).

Based on the genome length estimates, the Tetramolopium map may near completion by linkage of the five unlinked loci. The additional 175 cM needed to link these loci would bring the total map length to 110% of the estimate provided by method 4 of Chakravarti *et al.* (1991), and bring the total recombination fraction to 80% of the expected 900. Similarities between genome length estimates and the total map length, apparent stability of the map from the last 16 markers, and density of loci argue that a large percentage of the genome has been mapped.

DISCUSSION

The construction of genetic linkage maps has become a routine procedure for examining genomic structure and locating trait loci: a powerful combination in evolutionary studies (Whitkus *et al.* 1994). In the present study, the fairly large number of loci exhibiting distorted segregation ratios provides some insight into genomic differentiation between the two parental species.

Distorted segregation ratios are a common observation in crosses between genetically divergent genomes (Zamir and Tadmor 1986; Gebhardt *et al.* 1991; Paterson *et al.* 1991; Kianian and Quiros 1992; Vallejos *et al.* 1992; Weeden *et al.* 1992; Menancio-Hautea *et al.* 1993; Wang *et al.* 1994; Bernacchi and Tanksley 1997). Reasons for skewed segregation ratios include genetic factors operating in pre- and postzygotic phases of reproduction (Zamir and Tadmor 1986; Cornu *et al.* 1989; Gebhardt *et al.* 1991), structural rearrangements (Stebbins 1950; Bonierbale *et al.* 1988; Kianian and Quiros 1992; Williams *et al.* 1995), or gametic selection (Zamir *et al.* 1982). Regardless of the cause for skewed segregation ratios, the outcome is a reduction in free recombination. Multiple, linked loci exhibiting distorted segregation on the Tetramolopium map (Table 2; Figure 1) thus indicate a degree of divergence between the two genomes that is not obvious from molecular-marker diversity studies.

The high level of segregation distortion obtained in this study indicates *T. rockii* and *T. humile* have genomes that are as genetically divergent as many continental plant species and expressed as a postmating mechanism. Postmating mechanisms arise between species as a byproduct of evolutionary divergence, typically after the erection of a premating barrier (Levin 1978; Grant 1981). Therefore, the similarity of the results obtained in this study and other interspecific crosses provides an insight into the nature of reproductive isolation between species in Tetramolopium.

Island plant taxa are considered rather atypical in regard to interspecific cross compatibility (Crawford *et al.* 1987). Oceanic island species are highly cross compatible with few to no internal barriers to crossing if no obvious chromosomal structural differences exist (Carlquist 1966; Gillett and Lim 1970; Rabakonandrianina 1980; Carr and Kyhos 1981; Borgen 1984; Ganders and Nagata 1984; Carr 1985; Carr and Baker 1988; Ganders 1989). Yet several studies have indicated a degree of cross incompatibility or reduction in free recombination. Carr and Baker (1988) reported that all hybrids observed in Hawaiian Hibiscadelphus have normal chromosome pairing and disjunction at meiosis, but exhibit variability in F_2 vigor and sterility, results the authors attributed to hybrid breakdown. In interspecific hybrids of Canary Island Argyranthemum, the only measurable difference was a slight depression in chiasma frequency when compared to parental populations (Humphries 1975). Gillett (1966) reported normal meiotic behavior in the hybrid *Scaevola gaudichaudiana* × *S. mollis* in Hawaii, but less than half of the observed microsporocytes had all bivalents "closed" (ring bivalents). The "open bivalents" were attributed to reduced chiasmata formation. In Hawaiian Bidens, Gillett and Lim (1970) reported several cases where artificially produced interspecific hybrids showed univalents or a reduction in fertility or vigor in some crosses. Finally, in Hawaiian Tetramolopium, Lowrey (1986) reported that all interspecific crosses had normal bivalents and disjunction in the F_1, and produced vigorous and fertile F_2. However, he found a range of values for pollen viability in the F_1 (60–100%). These examples illustrate that oceanic island plant taxa do exhibit some degree, albeit weak, of interspecific postmating reproductive isolation. However, researchers (including this author) have tended to emphasize the lack of genetic barriers between species. Although premating mechanisms related to adaptation to new habitats and genetic drift apparently predominate in the origin and isolation of island plant species (Crawford *et al.* 1987), the accumulation of postmating factors responsible for reproductive isolation (Levin 1978; Grant 1981) will still occur.

Extension of the results from this study to interpretation of an interspecific difference must be viewed with some caution. This report is based on a single cross, in one direction, and under one environment. Different results may be obtained with additional crosses or under different conditions. The greenhouse environment in which the current cross was performed and plants raised is very moderate compared to field conditions. If selection for particular gametic combinations produced the skewed segregation ratios seen in this cross, then we can expect more severe selection under field conditions. Lowrey (1986) reported a number of reciprocal interspecific cross differences as measured by pollen viability in F_1 hybrids. Interestingly, these occurred between sections Alpinum and Tetramolopium, the sections represented by *T. humile* and *T. rockii*, respectively. With intraspecific crosses, Lowrey (1986) found a surprising reduction in F_1 pollen viability in crosses involving the subspecies of *T. humile*, while all other intraspecific crosses proved as successful as intrapopulation crosses.

When combined with the current findings, the totality of results from crossing studies in Tetramolopium indicate there is a subtle and complex set of relationships among genomes of different species and within species. The results do not indicate, however, that there is a uniformly high level of crossing success within the Hawaiian group.

Few or no reproductive barriers are expected in island taxa because diversification of the species is very recent. High crossability among Tetramolopium species (Lowrey 1986) led to the assumption that there would be no crossing barrier between the two parents chosen for the mapping study. Yet the level of distorted segregation found in this investigation argues in favor of some form of a postmating barrier. The major feature of plant evolution on oceanic islands is adaptation to extremely diverse environments (Carlquist 1974; Crawford *et al.* 1987). In Tetramolopium the barrier may be related to protection of genomic regions from recombination as a means of maintaining coadapted gene complexes. Further work is needed to determine if this is indeed a factor involved with diversification in Hawaiian Tetramolopium.

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