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NOTES AND COMMENTS

MOLECULAR EVIDENCE FOR A MISSING WILD RELATIVE OF MAIZE AND THE INTROGRESSION OF ITS CHLOROPLAST GENOME INTO *ZEA PERENNIS*

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The unequivocal documentation of introgression can be a difficult task because alternative hypotheses, such as convergence or joint retention of the ancestral condition, are difficult to disprove. Similarly, the direction of introgression between two species can often be inferred, but more rarely proven. Molecular markers offer perhaps the best means of demonstrating introgression, because they are apt to be neutral and thus are less likely than morphological traits to converge under selection within a similar environment. Molecular markers also provide the opportunity to examine the occurrence of introgression for the independently inherited nuclear and cytoplasmic (chloroplast and mitochondrial) genomes. Cytoplasmic genomes present a particularly good opportunity to observe introgression, because they may contain multiple markers which, unlike nuclear markers, cannot recombine. This feature of cytoplasmic genomes allows introgression to be more readily distinguished from convergence. Nevertheless, there have been relatively few reports of introgression between plant species based on molecular markers (e.g., Rick et al., 1974; Doebley et al., 1984; Palmer et al., 1983), and there is at least one case in which molecular analyses have failed to confirm a suspected case of introgression (Rieseberg et al., 1988).

In this paper, I report a case of introgression of the chloroplast genome involving a perennial wild relative of maize, perennial teosinte (*Zea perennis* [Hitchc.] Reeves and Mangelsdorf). This case is of particular interest for several reasons. 1) The chloroplast genome type that has become incorporated into *Z. perennis* is unknown among the other species of the genus and, thus, represents a missing taxon. 2) *Zea perennis* is the only tetraploid in the genus, while the taxa of *Zea* whose chloroplast genomes most closely resemble the introgressive chloroplast genome are all diploids. This suggests that introgression may have occurred between taxa of different ploidy levels. 3) This case demonstrates how phylogenies based on chloroplast-genome studies alone could be misleading when performed in the absence of analysis of the nuclear genome.

MATERIALS AND METHODS

The genus *Zea* includes maize and its wild relatives, the teosintes. As treated by Iltis and Doebley (1980) and Doebley (1990), *Zea* contains four species divided between two sections. Section *Zea* contains one species, *Z. mays*, an annual diploid ($2n = 20$). This species is highly polymorphic, containing four subspecies: subsp. *mays* is the cultigen, corn or maize; subsp. *mexicana* is a wild taxon of teosinte of the Mexican central highlands; subsp. *huehuetenangensis* is a teosinte from the highlands of western Guatemala; and subsp. *parviglumis* is a teosinte of the river valleys of southwestern Mexico. Section *Luxuriantes* contains three wild species (teosintes). *Z. perennis* is a tetraploid ($2n = 40$) perennial teosinte of Jalisco, Mexico; *Z. diploperennis* is a diploid perennial teosinte also from Jalisco, but allopatric to *Z. perennis*; and *Z. luxurians* is an annual, diploid teosinte from southeastern Guatemala. The present study includes 39 accessions that represent the array of taxonomic variation in *Zea* (Doebley et al., 1987*b*; unpubl.). For comparative purposes, cpDNA was isolated from *Tripsacum dactyloides* and *T. pilosum*, which belong to the same tribe (Andropogoneae) of the grass family as *Zea*. For some samples, purified chloroplasts were prepared as described by Palmer (1986). DNA was isolated from the chloroplasts by phenol and chloroform extractions (Zimmer and Newton, 1982). For other samples, total cellular DNA was prepared by the method of Saghai-Maroof et al. (1984).

Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs, and the restriction reactions were carried out according to manufacturer's recommendations. Each of the 41 cpDNAs was digested with the following 21 restriction endonucleases: *Bam*H I, *Bcl* I, *Bgl* I, *Bgl* II, *Bst*E II, *Cfo* I, *Cla* I, *Dra* I, *Eco*R I, *Eco*R V, *Hae* III, *Hin*d III, *Kpn* I, *Nco* I, *Nru* I, *Nsi* I, *Pst* I, *Sac* I, *Ssp* I, *Stu* I, and *Xba* I. The DNAs were then electrophoresed in 0.8% agarose gels with a running buffer of 100 mM Tris-acetate, 1 mM EDTA, pH 8.1. DNA fragment sizes were estimated using the maximum-likelihood method with *Hin*d III-digested bacteriophage λDNA or *Hae* III-digested φX174 DNA as a standard (Schaffer and Sederoff, 1981). DNA fragments in the gels were denatured and transferred to a nylon hybridization membrane (Gene Screen Plus®) according to the manufacturer's (New England Nuclear) recommendations. Nylon filters were prehybridized overnight in a hybridization buffer of 10% dextran sulfate, 1 M NaCl and 1% SDS. Cloned DNA fragments were labeled with dATP[α-^{32}P] by nick translation as described by Maniatis et al. (1982). Nick-translated probes were separated from unincorporated dATP[α-^{32}P] on spun columns (Maniatis et al., 1982), denatured, and then added to the hybridization buffer. Hybridizations were carried out at 65°C overnight. Hybridization membranes were washed according to the manufacturer's instructions and exposed to X-ray film (Kodak XAR-5) at −80°C.

Cloned portions of the chloroplast genome were used to probe the nylon filters. These include: 1) plasmid clones of the sorghum chloroplast genome (pLD 7, 9,

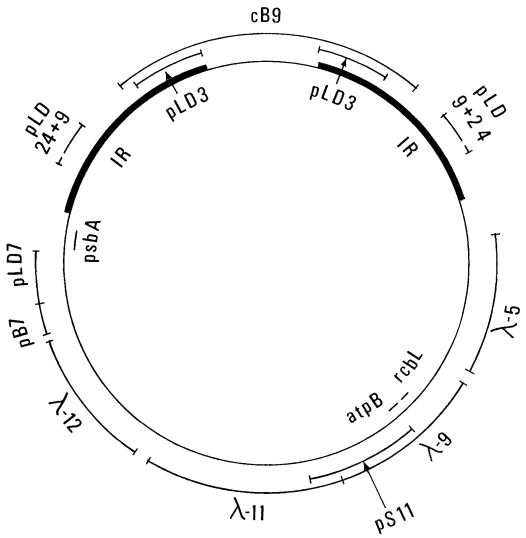

FIG. 1. Schematic diagram of the *Zea* chloroplast genome showing the location of cloned probes used in this study. Locations of the inverted repeats (heavy line), ATP synthase subunit b (*atpB*), large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*), and the 32-kd thylakoid membrane protein (*psbA*) are indicated.

and 24; Dang and Pring, 1986); 2) charon 4A clones of maize cpDNA (λ-5, λ-9, λ-11, and λ-12; Larrinua et al., 1983); 3) a single cosmid clone of maize cpDNA (cB9; D. Lonsdale, unpubl.); and 4) a 12.5-kb *Sma* I fragment cloned from *Z. perennis* (pS11) and a 3.5-kb *Pst* I clone from *Z. mays* (pB7). The positions of all clones are indicated on the maize chloroplast genome map (Fig. 1).

A Wagner parsimony phylogenetic tree for the taxa was constructed with the Mix program of the Phylogenetic Inference Package version 2.9, created and made available by J. Felsenstein (Department of Genetics, University of Washington, SK-50, Seattle, WA 98195).

RESULTS

The survey of restriction-site variation in the chloroplast genome among the 41 accessions revealed a total of 43 restriction-site polymorphisms and six insertion/deletion events. Most of these mutations have been previously described (Doebley et al., 1987*a*, 1987*b*). The full set of 49 mutations was used to construct a Wagner parsimony phylogenetic tree (Fig. 2). In all aspects except one, the topology of this tree agrees well with several other lines of evidence including morphology (Doebley, 1983), allozymes (Doebley et al., 1984), and rDNA analysis (Zimmer et al., 1988). The single difference lies in the positioning of *Z. perennis* in two separate locations: 1) near *Z. diploperennis* and 2) near the subspecies of *Z. mays*. The positioning near *Z. diploperennis* is in agreement with considerable

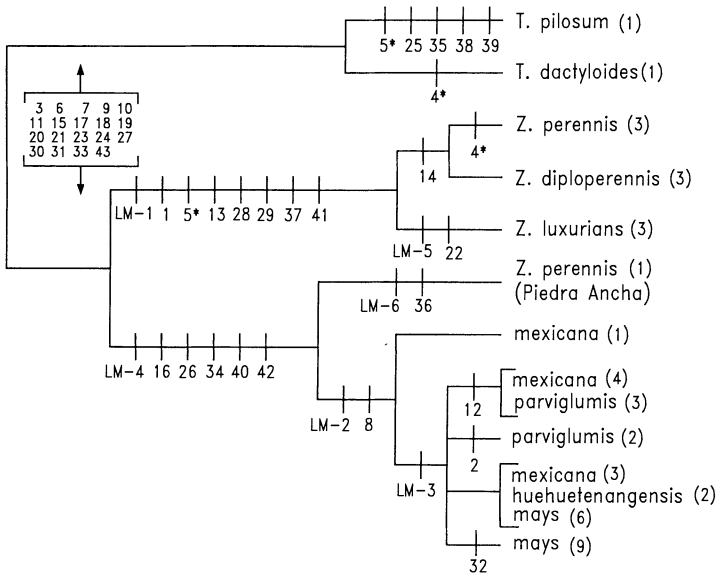

FIG. 2. Wagner parsimony tree for *Zea* chloroplast-genome types based on 43 restriction-site loss/gain and six insertion/deletion mutations. The identification numbers of restriction-site and length (insertion/deletion) mutations appear along the branch segments (Doebley et al., 1987*a*, 1987*b*; unpubl.). Only two of the 43 restriction-site mutations (numbers 4 and 5) are homoplasious, each involving the parallel loss of a *Dra* I site. The number of collections possessing a particular genome type appears parenthetically after the specific or subspecific epithets.

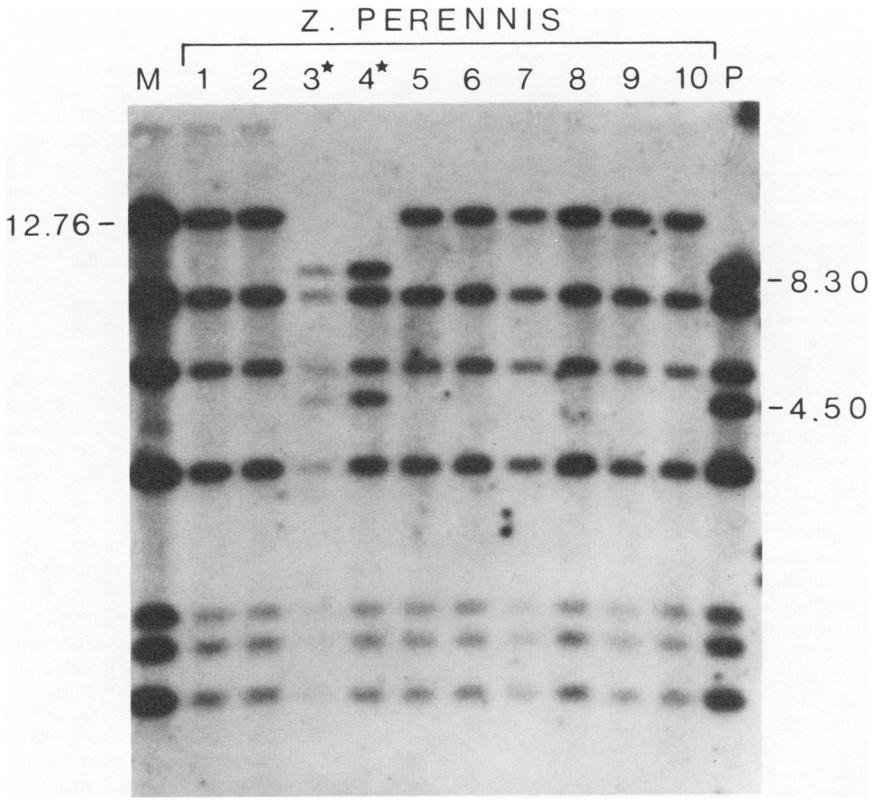

FIG. 3. Autoradiograph showing variation in the chloroplast genome among ten individuals of the Piedra Ancha population of *Z. perennis*. The DNAs were digested with *Bam*H I, and the filter was probed with cB9 (Fig. 1). Samples of *Z. mays* (M) and the Los Depositos population of *Z. perennis* (P) flank the ten Piedra Ancha samples for comparison. A single restriction-site mutation splits the 12.76-kb fragment of *Z. mays* into 8.3- and 4.5-kb fragments in typical *Z. perennis*. Only two of the ten individuals from the Piedra Ancha population possess the chloroplast genome type typical of their species.

biosystematic data which show that *Z. perennis* resembles *Z. diploperennis* and, to a lesser extent, *Z. luxurians* in its morphology (Doebley, 1983), allozyme constitution (Doebley et al., 1984), chromosome structure (Pasapuleti and Galinat, 1982), and rDNA (Zimmer et al., 1988). The latter position, on the other hand, is in complete contrast with all other information concerning this species, and in this sense, it is atypical.

The collection of *Z. perennis* possessing the atypical chloroplast genome (*Iltis et al. 1050,* Piedra Ancha) and one collection with the typical (*Guzman s.n.,* Los Depositos) chloroplast genome were examined in more detail. Ten additional individuals from each were assayed for several mutations that define these two genome types. For the Los Depositos collection, all ten individuals possessed the typical *Z. perennis* genome. For the Piedra Ancha collection, eight individuals possessed the atypical genome type, while two plants possessed the typical *Z. perennis* chloroplast genome (Fig. 3). In order to determine whether the eight individual plants with the atypical genome type contained the normal *Z. perennis* nuclear genome, each was grown to maturity for morphological examination. All eight plants were morphologically typical of their species, having the diagnostic male tassel characteristics and stout rhizomes. The chromosomes of two of these plants were examined to determine whether they were tetraploid like *Z. perennis* or diploid like all other taxa of *Zea*. Both plants were tetraploid with $2n = 40$ chromosomes. This same collection (although not these same plants) had been previously analyzed for its allozyme constitution and was in no way distinct from other *Z. perennis* in this regard (Doebley et al., 1984).

DISCUSSION

All lines of evidence indicate that the atypical chloroplast genome type found in eight of ten plants of the Piedra Ancha collection represents a foreign cytoplasmic genome that has become incorporated into the nuclear background of *Z. perennis*. As is apparent in Figure 2, this atypical genome type has not been found in any other *Zea*. Although this genome type shows its closest relationship to the subspecies of *Z. mays*, it is distinguished from *Z. mays* by five or six mutations. This number of mutations is about equivalent to the number (four) distinguishing the chloroplast genomes

of *Z. perennis* and *Z. luxurians,* which are very distinct taxa, and it is notably more than the number (one or two) among the subspecies of *Z. mays.* This suggests that the foreign chloroplast genome found in the Piedra Ancha collection of *Z. perennis* came from a taxon other than those represented in our sample. As our sample of taxa represents all known taxa of *Zea,* this foreign chloroplast genome must have come from a missing taxon. Further, as part of a survey of chloroplast-DNA diversity in *Zea,* more than 140 collections of *Z. mays* (sensu lato) have been analyzed and none possessed the atypical genome found in *Z. perennis* (unpubl.).

This discovery has several important implications. First, it provides an obvious case of introgression, and one in which the direction of introgression is clearly identified. Moreover, the fact that the two chloroplast genomes are distinguished by 15 restriction-site mutations and three insertion/deletion events (Fig. 2) indicates that the two species involved were quite distinct. Their placement in Figure 2 would indicate that they belong to separate sections of the genus. Second, this discovery provides evidence that there exists or previously existed another taxon in *Zea.* The fact that the Piedra Ancha population remains polymorphic for the typical and atypical genomes suggests that the introgression event was relatively recent, rather than ancient. If this is true, then the missing taxon may still exist and could potentially be located in the region surrounding the Piedra Ancha population. Third, *Z. perennis* is a tetraploid, while all other taxa in the genus are diploids. Available cytological evidence suggests that *Z. perennis* is an autotetraploid derivative from a *Z. diploperennis*-like ancestor (Shaver, 1962). The atypical chloroplast genome type found in the Piedra Ancha population is phylogenetically allied with the subspecies of *Z. mays* (Fig. 2), all of which are diploids. Thus, it seems likely that the missing taxon was also a diploid. If this is true, then the introgression event occurred between taxa of different ploidy levels. It is possible to cross *Z. perennis* and *Z. mays,* and although the triploid hybrid has very low fertility, it takes only a few generations of back-crossing to the *Z. mays* parent to restore fertility. Finally, this example of introgression aptly demonstrates a potential danger of constructing phylogenies based on chloroplast-DNA data in the absence of the analysis of the nuclear genome. If we had included only the Piedra Ancha population of *Z. perennis* and had surveyed only one or two individuals, it is likely that we would have misjudged the phylogenetic relationship between *Z. perennis* and the other taxa of *Zea.* The danger of this type of error is particularly acute in studies that examine only a single population or single individual per taxon. There would appear to be a need for more surveys of chloroplast-DNA diversity that include numerous populations per species and numerous individuals per population (Banks and Birky, 1985).

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EVOLUTION OF THE BREEDING SEX RATIO UNDER PARTIAL SEX CHANGE

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One of R. A. Fisher's (1930) major insights on evolution of the sex ratio was that differential sex-specific mortality after the period of parental care would not affect the favored primary sex ratio. Many explicit population-genetic models support this conclusion (e.g., Leigh, 1970; Shaw and Mohler, 1953; Karlin and Lessard, 1986). The meaning of this is quite straightforward: the adult or breeding sex ratio is not itself a target of natural selection; it is simply the ratio that results from selection on the primary sex ratio (usually for ½) combined with the prevailing sex-specific mortality and maturation schedules. This lack of direct natural selection on the breeding sex ratio is perhaps nearly universal for typical dioecious species. Interestingly, this result is not true for sex-reversing organisms. While the usual sex-allocation problem is here taken to be natural selection acting on the age or size at sex transformation (Leigh et al., 1976; Warner, 1988*a*, 1988*b*; Charnov, 1982*a*), alteration of that age also changes the breeding sex ratio. It is a fair statement that, under sex reversal, natural selection acts directly on the breeding sex ratio (Charnov, 1982*a*; Charnov and Bull, 1989).

This paper deals with evolution of the adult sex ratio in partially sex-changing species. Consider protogyny in which an individual reproduces first as a female, then changes sex to reproduce for the rest of its life as a male. Upon closer examination, many protogynous fish species have been found to have populations that consist of sex changers and pure males (Warner and Robertson, 1978; Robertson and Warner, 1978; Choat and Robertson, 1975). The typical life history is illustrated in Figure 1. The young fish may be either male or female; the older ones are male. The initial phase may often have different coloration from the terminal phase; initial-phase males usually use different reproductive tactics than terminal-phase males (Warner and Hoffman, 1980). Charnov (1982*a*), building on the pioneering work of Warner and Hoffman (1980), showed that the ESS proportion of males among the initial-phase fish (P) could be written as:

$$P = \frac{h}{1 + h} \tag{1}$$

where h is the proportion of the females who mate with initial-phase males. While the original derivation of (1) assumed no sex differences during the initial phase in growth, mortality, or age at change to terminal phase, later work showed that the result was fairly robust to violations of these assumptions, at least for h not near 1 (Charnov, 1982*b*). Let T be the proportion of the breeding population in the terminal phase and let r be the proportion of males among the breeders (the adult sex ratio). Then, r is of course

$$r = T + (1 - T)P;$$

or, from (1), we have

$$r = \frac{h + T}{1 + h}. \tag{2}$$

If h and T can take on any values from 0 to 1, then virtually all sex ratios are possible. However, there is one further constraint that limits combinations of h and T. Protogynous sex reversal is only stable if males gain substantial reproductive ability with age (or size) (Warner, 1988*a*, 1988*b*). At the minimum each terminal-phase male must mate with more females per unit time does each initial-phase male. If the breeding population is of size N, this restriction is that

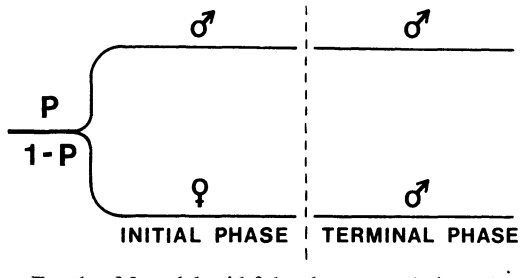

FIG. 1. Many labroid fishes have populations that consist of a mixture of protogynous sex changers and pure males. The fish often come in two color forms, initial phase (IP) and terminal phase (TP). IP fish may be male or female, while all TP fish are male. P is the proportion of the IP fish who are male.