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Evolution

Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*

(restriction endonucleases/slow rate of evolution/chloroplast DNA phylogeny/intraspecific variation/red fruit color)

JEFFREY D. PALMER* AND DANIEL ZAMIR†‡

*Department of Plant Biology, Carnegie Institution of Washington, Stanford, California 94305; and †Department of Vegetable Crops, University of California, Davis, California 95616

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ABSTRACT **Chloroplast DNA was purified from 12 accessions that represent most of the species diversity in the genus *Lycopersicon* (family Solanaceae) and from 3 closely related species in the genus *Solanum*. Fragment patterns produced by digestion of these DNAs with 25 different restriction endonucleases were analyzed by agarose gel electrophoresis. In all 15 DNAs, a total of only 39 restriction site mutations were detected among 484 restriction sites surveyed, representing 2,800 base pairs of sequence information. This low rate of base sequence change is paralleled by an extremely low rate of convergent change in restriction sites; only 1 of the 39 mutations appears to have occurred independently in two different lineages. Parsimony analysis of shared mutations has allowed the construction of a maternal phylogeny for the 15 accessions. This phylogeny is generally consistent with relationships based on morphology and crossability but provides more detailed resolution at several places. All accessions within *Lycopersicon* form a coherent group, with two of the three species of *Solanum* as outside reference points. Chloroplast DNA analysis places *S. pennellii* firmly within *Lycopersicon*, confirming recent studies that have removed it from *Solanum*. Red-orange fruit color is shown to be a monophyletic trait in three species of *Lycopersicon*, including the cultivated tomato, *L. esculentum*. Analysis of six accessions within *L. peruvianum* reveals a limited amount of intraspecific polymorphism which, however, encompasses all the variation observed in *L. chilense* and *L. chmielewskii*. It is suggested that these latter two accessions be relegated to positions within the *L. peruvianum* complex.**

Several properties make the chloroplast genome particularly well suited for comparative restriction endonuclease analysis as a means of assessing evolutionary relationships among plants. The chloroplast genome of vascular plants consists of a single molecular species, which is sufficiently small [120–180 kilobase pairs (kb) (1)] to permit resolution of all the fragments produced by many six-base enzymes yet large enough to allow rapid sampling of a great many restriction sites by using just a moderate number of enzymes. Major sequence rearrangements are generally quite rare during chloroplast genome evolution (1), so that changes in restriction patterns can usually be interpreted as the consequence of base substitutions, rather than DNA rearrangements. The high degree of base sequence conservation of chloroplast DNA (1, 2) should facilitate comparisons of chloroplast genomes from widely divergent plants, not only among genera within a family (3, 4), but also between families and even orders of flowering plants.

Previous studies of chloroplast DNA variation have not, however, fully exploited the suitability of the chloroplast genome for evolutionary studies. Most of these studies (4–9) have examined only a few DNAs and have utilized relatively few restriction enzymes (sometimes only one). Most importantly, the amount of sequence variation has generally been assessed only by qualitative comparisons, with no attempt being made to relate the observed pattern differences to specific mutational events. Two recent studies (3, 10) have analyzed chloroplast DNA variation among species within *Nicotiana* and related genera of the Solanaceae in terms of specific mutations, primarily base substitutions. However, in both studies the ability to draw phylogenetic relationships critically was severely limited by the relatively few enzymes or species surveyed.

In this study we demonstrate that comprehensive examination of sequence variation within a set of chloroplast DNAs, from the genera *Lycopersicon* and *Solanum*, does permit critical evaluation of evolutionary relationships among plant species. While an extremely limited amount of sequence divergence is observed among these DNAs, by using a large number of restriction enzymes we demonstrate that sufficient variation can be detected to permit construction of a detailed chloroplast DNA phylogeny that is essentially free of ambiguity. We discuss the implications of this phylogeny for our understanding of evolutionary relationships in *Lycopersicon* and *Solanum*.

MATERIALS AND METHODS

Chloroplast DNA was purified from a single plant of the species listed in Table 1 according to the sucrose gradient method described by Palmer (11). Restriction endonucleases were purchased from Bethesda Research Laboratories and New England BioLabs, and digestions were performed according to the supplier's instructions. Electrophoresis was on 0.7%–1.5% horizontal agarose slab gels of 0.4 × 20 × 22–40 cm in 100 mM Tris·HCl, pH 8.1/12.5 mM NaOAc/0.25 mM EDTA.

RESULTS

Nature of Chloroplast DNA Variation. Fig. 1*A* displays the fragments produced by digestion of chloroplast DNA from samples 1–10 with the restriction endonuclease *Kpn* I. The *Kpn* I fragments shown, plus an additional doublet fragment of 0.8 kb that was not retained on this particular gel, sum in size to 158 kb. The only differences in these patterns are the disappearance in samples 3–10 of a 7.7-kb fragment that is present in samples 1 and 2, and the appearance in samples 3–10 of 4.8- and 2.8-kb fragments that are absent from samples 1 and 2. Within the limits of error of mobility measurements in agarose gels 4.8 and 2.8 kb sum in size to 7.7 kb. Therefore, we conclude that the most likely explanation for the observed differences is a mutation, probably a single base substitution, that has created a new *Kpn*

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Abbreviation: kb, kilobase pair(s).

‡ Present address: Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel.

Table 1. Source of chloroplast DNAs

Species	Accession no.	Site of collection	Province
1 *Solanum lycopersicoides*	LA 1964	Chupapalca	Tacna
2 *S. juglandifolium*	LA 2134	Tinajillas	Morona Santiago
3 *Lycopersicon peruvianum*	LA 1969	Los Calavaritos	Arequipa
4 *L. chilense*	LA 460	Palca	Tacna
5 *S. pennellii*	LA 716	Atico	Arequipa
6 *L. hirstum*	LA 1777	Rio Casma	Ancash
7 *L. chmielewskii*	LA 1305	Tambo	Ayacucho
8 *L. esculentum*	T6	UC Davis	California
9 *L. pimpinellifolium*	LA 722	Trujillo	La Libertad
10 *L. cheesmanii*	LA 1401	Isabela	Galapagos
11 *L. peruvianum*	PI 126435	Matucana	Lima
12 *L. peruvianum*	LA 1032	Aricapampa	La Libertad
13 *L. peruvianum*	LA 1955	Matarani	Arequipa
14 *L. peruvianum*	PI 127832	West of Arequipa	Arequipa
15 *L. peruvianum*	LA 1945	Caraveli	Arequipa

All accessions were from Peru, except nos. 2 and 10 (Ecuador) and 8 (University of California, Davis, CA).

I restriction site within the 7.7-kb fragment.

The *Sac* I patterns shown in Fig. 1*B* demonstrate variation not only in chloroplast DNA, but also in contaminating nuclear and mitochondrial DNA. A single *Sac* I restriction site mutation has occurred within the 9.9-kb chloroplast DNA fragment of samples 2–7 to produce fragments of 8.8 and 1.1 kb in samples 8–10. Bands of variable intensity and position that result from nuclear and mitochondrial DNA contamination are also visible. Nuclear ribosomal DNA bands (R. A. Jorgensen, personal communication) are particularly prominent in samples 4 and 5. The intensity of the ribosomal bands correlates with the prominence of the background smear of nuclear DNA in these samples. In addition, a series of very faint bands is visible in the region of the gel between 7 and 20 kb in both Fig. 1 *A* and *B*. These bands arise from a small amount of mitochondrial DNA contamination of the chloroplast DNA preparations, as judged by comparison with restriction profiles of purified mitochondrial DNA from *L. esculentum* (data not shown).

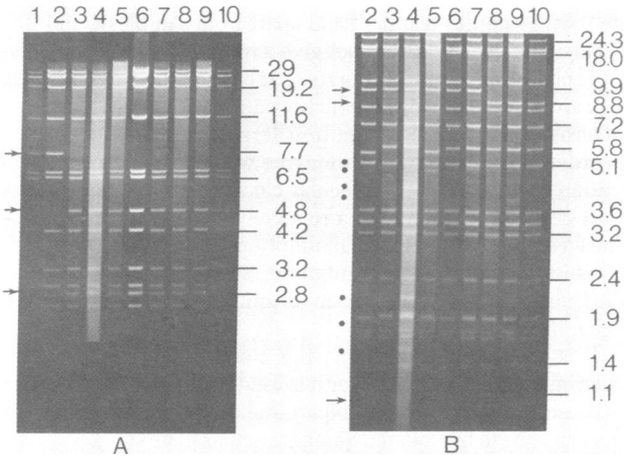

FIG. 1. Electrophoresis in 0.8% agarose gels of *Kpn* I (*A*) and *Sac* I (*B*) digests of chloroplast DNA samples 1–10 (see Table 1). Numbers on right are size in kb of various of the *Kpn* I and *Sac* I fragments, based on other gels in which the samples were sized relative to *Eco*RI, *Hin*dIII, *Sma* I, and *Sal* I digests of phage λ DNA. Arrows indicate fragment size differences ascribable to restriction site changes (Table 2). Dots indicate *Sac* I bands of variable intensity and location that hybridize to nuclear ribosomal DNA (R. A. Jorgensen, personal communication).

An additional source of variation in the chloroplast DNA *Sac* I patterns is the increased mobility of a 5.8-kb fragment in samples 2 and 5. We believe that this size variation reflects two independent deletions in the 5.8-kb fragment, which is slightly smaller in sample 2 than in sample 5, rather than the creation of new restriction sites near the ends of the fragment. The same relative pattern of increased mobility of the same fragment in both samples 2 and 5 has been observed with a number of enzymes. Moreover, hybridization experiments indicate that all such size variants in samples 2 and 5 that are produced by several different enzymes map to a single region on the genome (unpublished data). These two deletions are the only detected deletions/additions larger than 50 base pairs in these DNAs.[§]

Table 2 summarizes all the apparent restriction site mutations that we were able to detect in digesting the 15 DNAs with 25 restriction enzymes. Given the large number of enzymes (25) and fragments (484) surveyed, we have made no attempt to map all the mutations with restriction endonucleases. Restriction mapping is necessary for DNAs that have accumulated significant numbers of base substitutions (12, 13), but it is not necessary for such highly conserved DNAs (<1% sequence divergence) as these chloroplast DNAs. We have, however, constructed maps for the *Pvu* II, *Bgl* I, and *Hpa* I sites of *L. esculentum* chloroplast DNA, and in Fig. 2 we indicate the map locations of the single *Bgl* I and *Pvu* II mutations that were found.

Although we have not determined the exact location of any of the other mutations, we can at least differentiate between mutations on the inverted repeat and on the single copy regions (Fig. 2). Only a single mutation (*Bgl* II: 1.05 kb = 0.68 kb + 0.37 kb) appears to be located within the inverted repeat as judged by the doublet intensities of the observed bands (Table 2).

Chloroplast DNA Phylogeny. The mutations presented in Table 2 were used to build a chloroplast DNA phylogeny according to the parsimony principle (13, 16). Fourteen of the 39 restriction site phenotypes are phylogenetically informative—i.e., are shared by two or more DNAs—whereas 25 are unique to

[§] In certain digests very small size differences of 10–50 base pairs were observed that we were unable to analyze further as a consequence of the extremely small mobility differences involved. We have assumed that these differences reflect small deletions/insertions rather than restriction site changes, and we have not considered them in constructing phylogenies.

Table 2. Chloroplast DNA restriction site mutations

Enzyme	No. bands scored	Changed fragments, kb: Losses	Changed fragments, kb: Gains	Mutated samples
Kpn I	13	4.9 + 2.8	7.7	1, 2
Pvu II	12	21.4 + 6.0	27.4	2
Bgl I	11	30	27 + 3.1	2
Pst I	13	15.0	9.0 + 6.0	2
Bal I	15	27.0	17.0 + 10.0	2
Nru I	12	7.2	5.9 + 1.3	1
*Bam*HI	30	2.2	1.4 + 0.85	9, 10
*Eco*RI	39	5.8 + 2.2	8.1	8–10
Cfo I	36	2.25 + 1.8	4.0	3–7, 11–15
Bcl I	32	8.5 + 3.1	11.7	8
Sal I	11	15.3 + 4.1	19.4	1
		2.6 + 0.6	3.1	1
Xho I	21	5.4 + 5.2	10.7	5
		11.0 + 8.1	19.2	1
Stu I	14	10.6 + 1.9	12.5	2
		9.5 + 6.1	15.4	1
*Hin*dIII	24	15.0	11.0 + 4.0	8–10
		11.6 + 7.5	19	2
Xba I	29	2.6	1.5 + 1.1	6
		18 + 1.3	19	2
Sac I	21	9.8	8.7 + 1.1	8–10
		18	17.5 + 0.5	1
		25	2.3 + 0.3	1
Pvu I	21	14.8	11.7 + 3.1	8–10
		8.8 + 3.3	12.2	1, 2, 9
		25 + 10.0	34	8
		7.8 + 1.4	9.3	6
Bgl II	38	3.3	2.7 + 0.6	8–10
		4.2	3.3 + 0.9	3, 4, 7, 11–15
		0.68* + 0.37*	1.05*	3, 4, 7, 11–15
		1.9 + 1.3	3.2	1, 2
		3.1 + 2.0	5.0	1
		2.9	1.65 + 1.3	4, 7, 12–15
*Eco*RII	32	7.7	4.5 + 3.1	12
		2.9	1.5 + 1.4	2
		5.2	3.3 + 1.75	1, 2
		2.9 + 1.6	4.6	6
		4.1 + 2.9	7.1	1
		1.3 + 0.7	2.0	7

The evolutionary direction of restriction site changes is given with reference to the ancestral pattern for each enzyme based on the root of the tree drawn in Fig. 3. The direction of mutations *at* the root of the tree—i.e., those that distinguish samples 1, 2, or 1 + 2 from the rest of the samples—cannot be determined; such mutations have been arbitrarily assigned to samples 1, 2, or 1 + 2. No mutations were detected with *Bst*EII (10 bands), *Sac* II (9 bands), *Tth* I (8 bands), *Sma* I (12 bands), *Sph* I (11 bands), and *Hpa* I (10 bands). Each sample was digested with all 25 enzymes, except for sample 10, which was tested only with *Cfo* I, *Sac* I, *Kpn* I, *Bam*HI, *Bcl* I, and *Pvu* I. The *Eco*RI, *Hin*dIII, and *Bgl* II mutations indicated for sample 10 have been inferred on the basis of its relationship to samples 8 and 9 (Fig. 3). The number of bands scored is given for sample 8 and represents all those bands, starting in order from the largest band, that could confidently be separated and analyzed on a series of agarose gels of different percentages.

* Doublet bands.

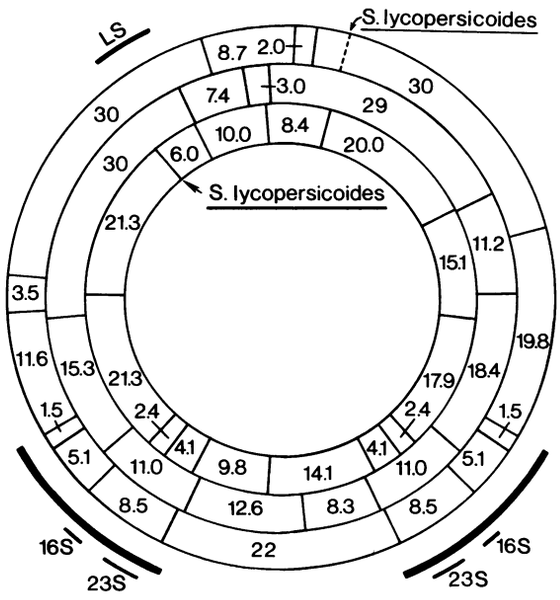

FIG. 2. Location of mutations on the chloroplast DNA restriction map of *L. esculentum*. Restriction sites, 16S and 23S ribosomal RNA genes, and large subunit (LS) gene of ribulose-1,5-bisphosphate carboxylase were mapped by comparative restriction analysis of chloroplast DNA from *L. esculentum* and the closely related chloroplast DNAs from *Nicotiana tabacum* and *Petunia hybrida*, for which restriction and gene maps have been published (14, 15). The two long, heavy black lines represent the minimal extent of the inverted repeat. Arrows indicate restriction site changes between *L. esculentum* and *S. lycopersicoides*. Fragment sizes are given in kb. *Bgl* I fragments are shown on the outer circle, *Hpa* I fragments on the middle circle, and *Pvu* II fragments on the inner circle.

a single DNA. The most parsimonious tree (Fig. 3) requires a minimum of 15 independent mutations to account for the observed distribution of the 14 phylogenetically informative DNA phenotypes. This tree postulates only a single case of a convergent or parallel restriction site change. This is the *Pvu* I mutation (12.2 kb = 8.8 kb + 3.3 kb) that is present in samples 1, 2, and 9. This is a particularly clear case of a convergent mutation because no less than five other mutations group sample 9 with samples 8 and 10 and apart from samples 1 and 2.

Parsimony analysis does not give a rooted tree. *A priori*, one could choose any group as the root, or base, of the tree and work outward to produce a tree which would have no more internal inconsistencies than any other tree derived in the same manner. In order to choose a root for our tree we have assumed the operation of a chloroplast molecular clock—i.e., that the chloroplast genome is evolving at a relatively constant rate in all the lineages under study. This assumption leads to a tree (Fig. 3) in which the numbers of mutations, starting from the base of the tree and proceeding to each terminal point, are most nearly equal in all lineages.

It is essential to determine the mode of inheritance of the chloroplast genome whenever it is used in phylogenetic studies, because many examples of biparental, as well as maternal, inheritance have been found among vascular plants (17). We have isolated chloroplast DNA from three different *Lycopersicon* F_1 hybrids. In each case the maternal parent was *L. esculentum*, and the paternal parents were *L. hirsutum*, *L. chmielewskii*, and *S. pennellii*. We digested chloroplast DNA from a single F_1 plant from each of these three crosses, along with their parental DNAs, with enzymes that distinguish the parental genomes (Table 2). In all three cases the F_1 hybrid DNA was identical to that of the maternal parent, *L. esculentum* (Fig. 4). Thus our chloroplast DNA phylogeny represents a maternal phylogeny.

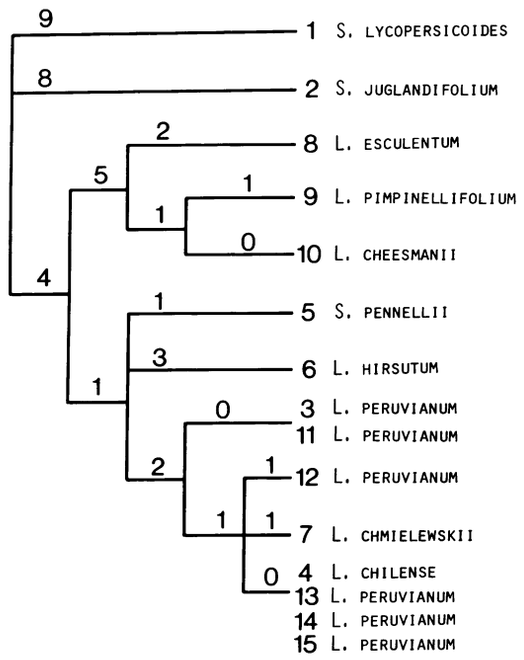

FIG. 3. Chloroplast DNA phylogeny. Numbers at termination of branches indicate accessions (Table 1), and numbers on the branches indicate the number of mutations specific to each branch (Table 2; see text).

DISCUSSION

Nature of Variation in the Chloroplast Genome. We have observed significant size variation in only a single region (*Sac* I 5.8-kb fragment, Fig. 1) of these chloroplast genomes; all other restriction fragment changes can be attributed to base substitutions. Because only two of the site changes have been completely mapped (Fig. 2) it is impossible to generalize about the distribution of base substitutions in the genome. However, it appears significant that only one of the 40 site changes has occurred within the inverted repeat, which constitutes 20–30% of the genome. It is known that the chloroplast ribosomal RNA genes, which occupy 20–30% of the inverted repeat (Fig. 2), are extremely conserved in base sequence among angiosperms (18). We can offer no speculations about selective constraints that might be operating in the rest of the inverted repeat, because little is known about coding functions in that region.

The direction of 19 of the restriction site changes can be determined (Table 2) on the basis of the phylogeny drawn in Fig. 3. Nine of these changes are site gains and 10 are site losses. This distribution is to be expected if the average restriction site density, and therefore base composition, of the chloroplast genome does not change with time.

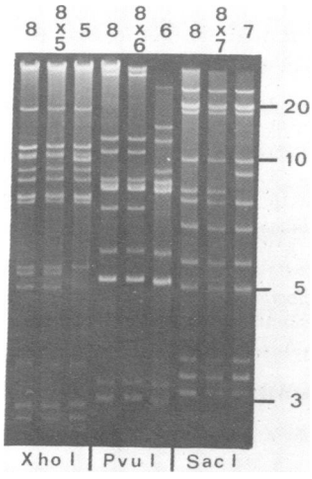

FIG. 4. Inheritance of chloroplast DNA in *Lycopersicon*. Lanes marked 8×5, 8×6, and 8×7 indicate chloroplast DNA from F_1 hybrids for which sample 8 was the maternal parent and samples 5, 6, and 7, respectively, were the paternal parents. Size scale at right is in kb.

Conservative Evolution of Chloroplast DNA. The largest number of site changes observed between any pair of DNAs is 20, for samples 1 and 8, and 1 and 9. On the other hand, several of the DNAs are identical in all 2,800 base pairs compared. *P*, the estimated pairwise percent sequence divergence (12), ranges between 0% and 0.7% for these chloroplast DNAs. These divergence values are considerably less than those measured in interspecific comparisons of mitochondrial DNA from the rodent genera *Rattus* (19), *Geomys* (20), and *Peromyces* (21) and are generally less than the intraspecific variation found in the same studies. Indeed, the range of chloroplast DNA variation observed in this study is most similar to that found in a study of mitochondrial DNA sequence polymorphisms among individual humans (22).

Part of the explanation for the much lower amount of chloroplast DNA sequence variation relative to that in animal mitochondrial DNAs is that the taxonomic ranks used for the different groups may not be equivalent in terms of age; the plants examined in this study may form a much younger group than the various rodent groups. The genus *Lycopersicon* (≈10 species) is significantly smaller than the closely related genus *Solanum* (≈1,400 species) into which it is sometimes placed (23), and one might expect to find more extensive variation within a much larger genus such as *Solanum*. Indeed, in unpublished studies we have found significantly greater sequence variation in the genus *Atriplex* (≈250 species) than we report here for *Lycopersicon*.

An additional explanation is that the base substitution rate may be lower in the chloroplast genome than in the animal mitochondrial genome. An absolute base substitution rate has been calculated for animal mitochondrial DNA on the basis of fossil and protein data (12), but comparable reliable data are not available for angiosperms, and thus an absolute estimate for the rate of chloroplast DNA evolution is not possible at this time. Primate mitochondrial DNA has been estimated to have a base substitution rate that is 5 to 10 times higher than that of primate single-copy nuclear DNA (12). No equivalent studies have been performed with the nuclear DNA of the species examined in this study, although there is clearly much more variation in nuclear DNA-encoded isozymes in species of *Lycopersicon* than in the corresponding chloroplast genomes (see below). Preliminary investigation of chloroplast DNA variation within *Atriplex* suggests that the chloroplast genome *is* evolving significantly more slowly than single-copy nuclear DNA (24) from the same species. It is well established that at least one component of the chloroplast genome, the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase, is evolving significantly more slowly than its nuclear DNA-encoded counterpart, the gene for the small subunit of the same enzyme (25). Thus it appears that the chloroplast genome is evolving quite slowly relative to plant nuclear DNA and probably also relative to animal mitochondrial and nuclear DNAs.

We find it impossible to draw any conclusions at this time regarding the constancy of chloroplast DNA evolution. The paucity of mutations in these DNAs precludes performance of a statistically reliable relative rate test in order to test the molecular clock hypothesis (26).

Phylogenetic Relationships in *Lycopersicon* and *Solanum*. The low amount of sequence variation in these chloroplast DNAs is reflected by the near absence of convergent mutations. This enables a phylogeny to be drawn that is ambiguous only in the intrinsic property of choosing a root for the tree (see *Re-*

sults). The relationships expressed in our chloroplast DNA phylogeny agree quite well with those inferred from other criteria, such as morphology and crossability (27). Our phylogeny places two of the three *Solanum* species (*S. lycopersicoides* and *S. juglandifolium*) clearly outside the entire group of *Lycopersicon* accessions. *S. lycopersicoides* does hybridize, although unilaterally, with some species of *Lycopersicon*, but chromosome pairing is poor and the hybrids are sterile (27). In contrast, hybrids between species of *Lycopersicon* exhibit complete chromosome pairing and complete to somewhat impaired fertility (27). Our analysis indicates that *S. pennellii* clearly belongs to *Lycopersicon*, in agreement with other studies (27) that have removed it from *Solanum*.

Red-orange fruit color, the result of various pigmented carotenoids, is clearly a monophyletic trait in this group of plants. One of the most distinct lineages in the chloroplast DNA phylogeny is that of the only three species in our survey—*L. esculentum* (the cultivated tomato), *L. pimpinellifolium*, and *L. cheesmanii*—that feature red-orange fruits.

Intraspecific Polymorphism. We have found a limited amount of chloroplast DNA polymorphism among the six accessions of *L. peruvianum* examined. This is not unexpected, given the extreme variation in morphology and isozymes found at all levels in this species, even between individuals of the same population (27). The contrast between a high level of variation of nuclear traits, isozymes in particular, and a low level of chloroplast DNA variation reinforces our earlier conclusion regarding the conservatism of the chloroplast genome.

The chloroplast DNA polymorphism encountered within *L. peruvianum* encompasses all the variation observed in *L. chilense* and *L. chmielewskii* (Fig. 3). On this basis alone it seems reasonable to relegate these species to a rank, perhaps of subspecies, within the *L. peruvianum* complex. Subsumption of *L. chilense* and *L. chmielewskii* into the *L. peruvianum* complex solely on the basis of common chloroplast DNAs need not also imply common pathways of acquisition of these DNAs. Because chloroplast DNA is maternally inherited in *Lycopersicon*, it is reasonable to postulate that *L. chilense* and *L. chmielewskii* have arisen by introgression of a member of the *L. peruvianum* complex (*sensu stricto*) into a more or less unrelated *Lycopersicon* species.

Classical taxonomic data would suggest that introgressive hybridization is a more likely explanation for the origin of *L. chmielewskii*. On the basis of crossability and morphological relationships, Rick (27) has placed *L. chmielewskii* in a group that includes *Lycopersicon* species 5, 6, and 8–10, apart from a group that contains *L. peruvianum* and *L. chilense*. On the other hand, recent isozyme studies (S. Tanksley, personal communication) group *L. chmielewskii* and its sibling species *L. parviflorum* together with *L. peruvianum* in the same manner as suggested by our chloroplast DNA analysis. Similar contrasts between morphological–organismal data and molecular data relating to rates, if not branching orders, of evolution have led Wilson and co-workers (26, 28) to suggest that organismal evolution often proceeds at varying rates independent from the more or less constant clocklike rate with which molecular evolution proceeds.

An alternative explanation for the distribution of chloroplast DNA polymorphisms is that the mutations which group these three species are actually independent. We consider this possibility very unlikely, given the extremely low amount of sequence variation observed in these DNAs. Moreover, two of the three mutations that group either some or all of these accessions are site gains, which are much less likely to occur in a convergent manner than site losses (22).

That intraspecific polymorphism for chloroplast DNA does exist, and that this polymorphism does, in the one instance examined, encompass the variation observed in two other species, raises the possibility that with more extensive comparisons one might find sufficient intraspecific variability as to obliterate completely the relationships shown in Fig. 3. We consider this unlikely, given the large number of mutations that define the other major divisions in our phylogeny relative to the small number of mutations found in the entire *L. peruvianum–chilense–chmielewskii* complex.

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