mitochondrial and chloroplast DNA-based phylogeny of Pelargonium (Geraniaceae)

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Overall phylogenetic relationships within the genus Pelargonium (Geraniaceae) were inferred based on DNA sequences from mitochondrial(mt)-encoded nad1 b/c exons and from chloroplast(cp)-encoded trnL (UAA) 5‘-exon–trnF (GAA) exon regions using two species of Geranium and Sarcocaulon vanderetiae as outgroups. The group II intron between nad1 exons b and c was found to be absent from the Pelargonium, Geranium, and Sarcocaulon sequences presented here as well as from Erodium, which is the first recorded loss of this intron in angiosperms. Separate phylogenetic analyses of the mtDNA and cpDNA data sets produced largely congruent topologies, indicating linkage between mitochondrial and chloroplast genome inheritance. Simultaneous analysis of the combined data sets yielded a well-resolved topology with high clade support exhibiting a basic split into small and large chromosome species, the first group containing two lineages and the latter three. One large chromosome lineage (x = 11) comprises species from sections Myrrhidium and Chorisma and is sister to a lineage comprising P. mutans (x = 11) and species from section Jenkinsonia (x = 9). Sister to these two lineages is a lineage comprising species from sections Ciconium (x = 9) and Subsucculentia (x = 10). Cladistic evaluation of this pattern suggests that x = 11 is the ancestral basic chromosome number for the genus.

Key words: chloroplast DNA; Geraniaceae; mitochondrial DNA; nad1; Pelargonium; phylogeny; trnL–F.

The genus Pelargonium L’Hér. (Geraniaceae) has been the subject of a range of systematic and taxonomic studies based on data from morphology, palynology, karyology, phytochemistry (e.g., Albers and van der Walt, 1984; Gibby and Westfold, 1986; van der Walt, 1990; Albers, Gibby, and Austmann, 1992; Dreyer et al., 1992; Gibby et al., 1996; Stafford and Gibby, 1992; van der Walt et al., 1995), and DNA sequences (Jones and Price, 1996; Bakker et al., 1998, 1999). Overall phylogenetic relationships across the genus, however, are not clear. Pelargonium comprises in total over 250 species, the majority of which occur in the South African Cape region, and is currently grouped into 16 infrageneric sections. The last formal revision of the genus was by Knuth (1912).

In a previous study (Bakker et al., 1999), based on cpDNA trnL(UAA) 5‘-exon–trnF (GAA) sequences, we focused on phylegetic relationships among 68 species of Pelargonium with small chromosomes, mostly <1.5 μm in length (Albers and van der Walt, 1984; Gibby and Westfold, 1986; Gibby, Albers, and Prinsloo, 1990). This monophyletic group was found to consist of two main lineages and comprises over 80% of the genus (and 11 of the 16 recognized sections), including woody shrubs, stem succulents, geophytes, and herbaceous annuals. The remainder of the genus comprises ~55 species with chromosomes of 1.5–3.0 μm long (Albers and van der Walt, 1984; Gibby and Westfold, 1986; Gibby, Albers, and Prinsloo, 1990). Here we shift the emphasis to overall phylogenetic relationships within Pelargonium, represented by five small chromosome and 20 large chromosome species and using two species of Geranium and Sarcocaulon vanderetiae as outgroups. Based on partial sequences from mitochondrial(mt)-encoded nad1 b/c exons and from chloroplast(cp)-encoded trnL–F regions we identify a basic division in the genus into a small and a large chromosome species group, the latter consisting of three lineages.

Plant mitochondrial DNA sequences have only rarely been used as a source of phylogenetic markers (e.g., Davis et al., 1998; Duff and Nickrent, 1999) because of their presumed slow rate of nucleotide substitution, considered to be of limited value for lower level plant phylogenetic reconstruction (Palmer, 1992). In this paper, however, we show that mtDNA nad1 b/c exons do contain levels of variation suitable for species-level reconstruction in Pelargonium. We also compare mtDNA- and cpDNA-based phylogenies in an attempt to test for linkage of organellar genome inheritance.

MATERIALS AND METHODS

Table 1 lists all 31 taxa used in the analysis together with their authority, accession and voucher details and basic chromosome number. Plant material was obtained largely from living collections at Chelsea Physic Garden (London), Stellenbosch University (RSA), Kirstenbosch Botanic Garden (RSA), and the Botanical Gardens of the Westfälische Wilhelms-Universität Münster (Germany). Voucher specimens are deposited in BM, MSUN, NBG, RNG, and STEU (Table 1).

DNA extraction and PCR—Double-stranded PCR (polymerase chain reaction) was carried out according to Demesure, Sodzi, and Petit (1995) for the mtDNA nad1 b/c exons using their primers nad1 b (5'-GCAATACGTCTGAGCTCTCA-3') and nad1 c (5'-GGAGCTCGAT-
Table 1. List of taxa and sequences used.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Chr. number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source&lt;sup&gt;b&lt;/sup&gt;</th>
<th>nadL&lt;sup&gt;c&lt;/sup&gt;</th>
<th>trnL-F&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geranium pusillum L.</td>
<td>x = 13&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Reading, UK; Pankhurst 1 (RNG)</td>
<td>GBAN-AF167131</td>
<td>GBAN-AF167151</td>
</tr>
<tr>
<td>Geranium robertianum L.</td>
<td>x = 16&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Reading, UK; Pankhurst 2 (RNG)</td>
<td>GBAN-AF167114</td>
<td>GBAN-AF167127</td>
</tr>
<tr>
<td>Pelargonium alternans Wendl.</td>
<td>x = 11</td>
<td>Worcester, W Cape, RSA; STEU 4355 (STEU)</td>
<td>GBAN-AF167126</td>
<td>GBAN-AF167152</td>
</tr>
<tr>
<td>P. articulatum (Cav.) Willd.</td>
<td>x = 11</td>
<td>Kanopoort, W Cape, RSA; STEU 2309 (STEU)</td>
<td>GBAN-AF167105</td>
<td>GBAN-AF167144</td>
</tr>
<tr>
<td>P. australis Willd.</td>
<td>x = 11</td>
<td>Tasmania, Australia; CPG 8829 (BM)</td>
<td>GBAN-AF167129</td>
<td>GBAN-Z95280</td>
</tr>
<tr>
<td>P. caucalioides (Cav.) Salisb.</td>
<td>x = 11</td>
<td>Rheinsonderrand, W Cape, RSA; STEU 1191 (STEU)</td>
<td>GBAN-AF167114</td>
<td>GBAN-AF167141</td>
</tr>
<tr>
<td>P. caucalioides (Cav.) Salisb.</td>
<td>x = 9</td>
<td>Madagascan; CPG 8842 (BM)</td>
<td>GBAN-AF167106</td>
<td>GBAN-AF036087</td>
</tr>
<tr>
<td>P. elongatum (Cav.) Salisb.</td>
<td>x = 4</td>
<td>Simonstown, Cape Peninsula, RSA; CPG 8890 (BM)</td>
<td>GBAN-AF167108</td>
<td>GBAN-AF167146</td>
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<td>Reading, UK; Pankhurst 1 (RNG)</td>
<td>GBAN-AF167112</td>
<td>GBAN-AF167139</td>
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<td>P. elongatum (Cav.) Salisb.</td>
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<td>GBAN-AF167139</td>
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<td>GBAN-AF167139</td>
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</table>

<sup>a</sup> Basic chromosome number.
<sup>b</sup> CPG = Chelsea Physic Gardens, London, UK; STEU = Stellenbosch University, Stellenbosch, Republic of South Africa; voucher locations are indicated in parentheses.
<sup>c</sup> GenBank accession number (the prefix GBAN- has been added to link the online version of *American Journal of Botany* to GenBank but is not part of the actual GenBank accession number).
<sup>d</sup> From van Loon (1984).
<sup>e</sup> From Albers (1990).
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Fig. 1. Mitochondrial DNA nad1 b/c intron loss in Geraniaceae: alignment of nad1 b/c exon boundaries in angiosperms and Pinus sylvestris, with translated sequence shown on top. Nucleotide substitutions are shaded. First and last 30 bp of the group II intron in non-Geraniaceaeangiosperms are inserted. Pau = Pelargonium australe, Pgr = Pelargonium griseum, Pex = Pelargonium exstipulatum, Pto = Pelargonium tongaense, Psp = Pelargonium spinosum, Gpu = Geranium pulmonatum, Gro = Geranium robertianum, Sva = Sarcocaulon vanderetiae, Obe = Oenothera bertiana, Ath = Arabidopsis thaliana, Tae = Triticum aestivum, Cla = Citrullus lanatus, Phy = Petunia hybrida, Psy = Pinus sylvestris.

Phylogenetic analysis—Phylogenetic analysis and tests for clade support were performed using PAUP 3.1.1. (Swofford, 1993), MacClade 3.04 (Maddison and Maddison, 1992), and Parsimony Jackknife 4.22 (Farris et al., 1996).

RESULTS

For all species included in this study (see Table 1) PCR amplification using the mitochondrial nad1 b and c primers yielded fragments ~200 bp long, which were sequenced on both strands. Sequences have been deposited in GenBank (see Table 1; the prefix GBAN- has been added to link the online version of American Journal of Botany to GenBank but is not part of the actual GenBank accession number). All sequences could be aligned unambiguously with the corresponding exon sequences from other angiosperms (Oenothera bertiana M63033, Petunia hybrida X60401, Arabidopsis thaliana X98301, Triticum aestivum X57976, Citrullus lanatus X04130, and Pinus sylvestris AJ223312), but, whereas these taxa contain a 500–2000 bp long group II intron between nad1 exon b and c, the Geraniacean nad1 b and c exons are uninterrupted (Fig. 1). Intron loss has not been reported from this region in angiosperm mtDNA.

The nad1 b/c alignment (203 positions in total) contained 63 positions of the 3' end of the nad1 exon b and 140 positions of the 5' end of exon c. It contained 32 phylogenetically informative and three variable but uninformative positions in total. A heuristic parsimony search of this alignment (1000 cycles of random stepwise addition with one tree held at each step, STEEPEST DESCENT; ACCTRAN, MULPARS, COLLAPSE in effect) yielded 32 most parsimonious trees (MPTs) of 48 steps with CI (consistency index) = 0.81 and RC (rescaled consistency index) = 0.78. Support for basal nodes as measured by 10000 replicates of jackknife analysis was high (Fig. 2).

The plastid trnL–F alignment (893 positions) contained 63 positions of the 3' end of the nad1 exon b and 140 positions of the 5' end of exon c. It contained 32 phylogenetically informative and three variable but uninformative positions in total. A heuristic parsimony search of this alignment (1000 cycles of random stepwise addition with one tree held at each step, STEEPEST DESCENT; ACCTRAN, MULPARS, COLLAPSE in effect) yielded 32 most parsimonious trees (MPTs) of 48 steps with CI (consistency index) = 0.81 and RC (rescaled consistency index) = 0.78. Support for basal nodes as measured by 10000 replicates of jackknife analysis was high (Fig. 2).

The plastid trnL–F alignment (893 positions) contained 192 phylogenetically informative characters, including 14 phylogenetically informative indels (insertions or deletions) that were recoded as single binary
Fig. 2. *Pelargonium* nad1 b/c (left) and trnL–F (right) jackknife trees in which nodes with support values <50% have been collapsed. Jackknife support values (10,000 replicates) are indicated above the nodes.

characters, irrespective of indel length. The alignment also contained 142 variable positions that were uninformative. A Branch and Bound parsimony search of this alignment (initial upper bound: compute via stepwise; addition sequence: furthest) yielded 18 MPTs of 469 steps with CI = 0.86 and RC = 0.75. Clade support as measured by 10,000 replicates of jackknife analysis was high both for terminal and basal nodes (Fig. 2). The overall topology of the *trnL–F* tree was congruent with that of the *nad1* b/c tree, only the position of *P. karooicum* differed in the two phylogenies (Fig. 2).

The combined mtDNA/cpDNA data set (1110 characters) contained 233 phylogenetically informative characters; a Branch and Bound parsimony search (using the search settings above) yielded nine MPTs of 520 steps, CI = 0.85, and RC = 0.76. In order to reduce the influence of "noisy" characters, successive weighting (Farris, 1969) was performed in which characters were reweighted based on their rescaled consistency index on the nine MPTs with a base weight of 1000. Successive rounds of heuristic searches were performed until treelength stabilized for two successive searches. This yielded six MPTs with CI = 0.95 and RC = 0.92. Length of these six trees on the equally weighted matrix was 520 steps, which is the same length as the equally weighted MPTs. Strict and semistrict consensus tree topologies were identical for both the six successively weighted and the nine equally weighted trees. In summary, the effect of successive weighting was a general increase in support values (see Figs. 3 and 4). Clade support was measured by 1000 replicates of bootstrapping using heuristic search with ten replicates of random sequence addition and TBR branch swapping. Support was high, both for basal and more terminal nodes, underlining the value of combining data sets in a simultaneous analysis (Carpenter and Nixon, 1997). In general, jackknife values for nodes in common between mtDNA and the cpDNA phylogenies were higher for those nodes in the combined analysis. One of the six most parsimonious reconstructions is shown as a phylogram in Fig. 4.

Basic chromosome number of all taxa included was treated as an unordered multistate character and optimized onto the *Pelargonium* phylogeny in Fig. 3 using the "show reconstructions" option in PAUP 3.1.1. This
Fig. 3. Pelargonium combined nad1 b/c trnL–F semistrict consensus tree of the six successively weighted MPTs. Bootstrap support values (1000 “full heuristic” replicates) are indicated above nodes; when two values are given the first refers to bootstrap analysis of the equally weighted matrix whereas the second refers to the successively weighted analysis; one value is given when both analyses yielded the same result. Basic chromosome numbers of all taxa are given at the terminals, whereas reconstructions of basic chromosome numbers for internal nodes (using the “show reconstructions” command in PAUP) are boxed and given only for nodes that differ in state from those immediately preceding them. Pelargonium sectional classification (with total number of recognized species for each section in brackets) and outgroup assignment are indicated by shading.

yielded $x = 11$ as most parsimonious reconstruction for the ingroup node, both under ACCTRAN and DELTRAN optimization (Fig. 3).

DISCUSSION

Pelargonium contains two main lineages that correlate with chromosome size. This basal split into small and large chromosome species is well supported not only by our DNA sequence data but also by evidence from hybrid formation, since difference in chromosome size rather than in basic chromosome number appears to be a greater barrier to interspecific hybridization (Gibby et al., 1996). Whereas proliferation within the small chromosome lineage has been extensive, including a range of growth forms and 11 recognized sections (covering ~80% of species), the large chromosome lineage of Pelargonium contains only (sub)shrubs, some of them with succulent stems, and one or two herbaceous annual species. Biogeographically, the contrast between the two lineages is extended further in that the small chromosome lineage is largely confined to the South African Western Cape region, whereas large chromosome Pelargonium species occur mainly in the Eastern Cape, tropical east Africa, the Arabian Peninsula, Madagascar, and Asia Minor (van der Walt and Vorster, 1983). In a previous study (Bakker et al., 1999) we discussed the hypothesis that the proliferation of lineages in the small-chromosome clade may have been triggered by the late Pliocene aridifications and establishment of a winter rainfall climate in the Western Cape region. According to this scenario, the acquired stem succulence in this clade correlates with a radiation into a range of arid niches. Evolution in the large-chromosome lineage exhibits a different pattern with no extensive radiations, higher karyological differentiation, and generally longer branch lengths in molecular phylogenies (Bakker et al., unpublished data).

We consider the successively weighted topology and its support values (Fig. 3) as the best representation of our data because of the lower weight given to cladistically unreliable characters, i.e., characters (mostly rapidly changing) that are not likely to reflect correctly the phylogenetic history of the taxa because of chance correlations (Farris, 1969). The topology as depicted in Fig. 3 will therefore be the basis for the rest of this discussion.

The large-chromosome sublineage with basic chromosome number $x = 11$ comprises sections Myrrhidium and Chorisma and is sister to a lineage comprising section Jenkinsonia and P. mutans. Section Myrrhidium contains short-lived perennial subshrubs (van der Walt and Boucher, 1986), whereas section Chorisma contains...
Pelargonium combined nad1 b/c trnL-F analysis: one out of six most parsimonious reconstructions calculated from the combined and successively weighted data set (see text). The scale bar indicates 5 (successively weighted) steps. Bootstrap values at the nodes are as explained in Fig. 2. Sectional assignments: C = Ciconium, Ss = Subsucculentia, J = Jenkinsonia, Ch = Chorisma, M = Myrrhidium.

Fig. 4. Pelargonium combined nad1 b/c trnL-F analysis: one out of six most parsimonious reconstructions calculated from the combined and successively weighted data set (see text). The scale bar indicates 5 (successively weighted) steps. Bootstrap values at the nodes are as explained in Fig. 2. Sectional assignments: C = Ciconium, Ss = Subsucculentia, J = Jenkinsonia, Ch = Chorisma, M = Myrrhidium.

(sub)shrubs, sometimes with root tubers and with herbaceous to succulent stems (Albers et al., 1995). Our data confirm the recognition of section Chorisma sensu Albers et al. (1995) and indicate that it is sister to the lineage containing species from section Myrrhidium, represented here by P. caulisfolium Jacq. and the east African P. whytei Bak., a presumed close relative of P. multicaule Jacq. (van der Walt and Boucher, 1986).

Monophyly of species belonging to section Jenkinsonia is supported by our data. This section comprises mostly perennial (sub)shrubs with rather woody stems (van der Walt et al., 1997) and with basic chromosome number $x = 9$. Pelargonium mutans appears to be the sister group of the sect. Jenkinsonia clade. Pelargonium mutans was described by Vorster (1992), who placed it in section Ciconium, although the floral morphology differs markedly from other species in Ciconium. The basic chromosome number for P. mutans ($x = 11$; Gibby, unpublished data) differs from that for species of sect. Ciconium ($x = 9$) and for species of its sister group, the sect. Jenkinsonia clade. The phylogenetic evidence presented here suggests that P. mutans could be assigned to section Jenkinsonia.

Sister to the Chorisma/Myrrhidium and Jenkinsonia sublineages is a lineage comprising sections Ciconium and Subsucculentia. Section Ciconium (as given by van der Walt and Vorster, 1988, p. xxiii) consists of herbaceous shrubs mostly with slightly succulent stems and is represented here by six species. The most frequent basic chromosome number in this section is $x = 9$, though $x = 4$ and $x = 8$ are recorded for two taxa (the latter is not included here). From cytological and morphological evidence, Albers, Gibby, and Austmann (1992) suggested that P. articulatum ($x = 9$), previously classified in sect. Ligularia, may be more closely related to species of section Ciconium, and our data show it to be nested within the main Ciconium clade. Pelargonium elongatum ($x = 4$) appears to be sister to an unresolved clade that includes P. peltatum, P. tongaense, P. multibracteatum, and P. articulatum, all with $x = 9$. Both P. elongatum and P. multibracteatum were previously placed in section Eumorpha (e.g., Knuth, 1912), but the transfer of at least P. multibracteatum to Ciconium is supported here. The position of P. caylae ($x = 9$), a tetraploid from southeastern Madagascar, in a different lineage from the main Ciconium clade is substantiated by its different petal color and leaf shape (Gibby, Albers, and Prinsloo, 1990). Its
position sister to *P. karooicum* (*x = 10*) is unexpected since the two species differ not only in basic chromosome number but are also quite different in both floral and leaf morphology. Support for this position is low and comes exclusively from homoplasiously reconstructed changes in three different nucleotide positions in the *trnL–F* data set. Subsequently, in contrast with most other nodes, bootstrap support for this node decreases from 74 to 53% after successive weighting (Figs. 3 and 4).

Section *Subsucculentia* of van der Walt et al. (1995) appears therefore to be paraphyletic (Fig. 3), with *P. karooicum* in a different lineage from *P. spinosum* and *P. grandicalcaratum*. This section includes (sub)shrubs with succulent or somewhat woody stems, but *P. karooicum* is distinguished from the three other species of section *Subsucculentia* by leaf morphology, habit, number of fertile stamens, and in lacking the flavonols quercetin and kaempferol (van der Walt et al., 1995). Extended taxonomic sampling around these lineages using nuclear-encoded DNA sequences is likely to clarify the position of *P. karooicum* further. The topological incongruence of this species between the two organellar phylogenies as shown in Fig. 2 may indicate chloroplast capture from *P. caylae* given that the mitochondrial sequence correlates with cytological data.

**Karyological evolution**—In order to reconstruct karyological evolution in *Pelargonium*, we treated basic chromosome number here as an unordered multistate character, although, admittedly, homology of the different karyotypes is debatable. Optimization onto the overall *Pelargonium* phylogeny yielded *x = 11* as the most parsimonious reconstruction for the ingroup node, both under ACCTRAN and DELTRAN optimization (see Fig. 3). The implication is that the occurrence of *x = 11* in the *Chorisma/Myrhrhdium* clade represents a plesiomorphic condition for the large chromosome clade in total and that two lineages with *x = 9*, i.e., the Jenkinsonia clade and the *Ciconium/Subsucculentia* clade, have developed independently from a lineage containing *x = 11*. The lineages containing *x = 10* (the paraphyletic section *Subsucculentia*) are in our reconstruction interpreted to have been derived from a lineage containing *x = 9*. Likewise, the occurrence of *x = 4* in *Pelargonium elongatum* probably reflects an extreme case of chromosome fusion based on *x = 9*. Gibby et al. (1996) suggested mechanisms such as chromosome fusion or Robertsonian exchange to explain patterns of karyological change observed within *Pelargonium* sect. *Hoarea*, resulting in reduction of basic chromosome number from *x = 11* → *10* → *9*. At the main lineage level in *Pelargonium* we see a pattern of reduction in chromosome number where *x = 9* developed twice from *x = 11*. The latter is reconstructed most parsimoniously as the ancestral state for the genus, which was already proposed by Albers and van der Walt (1984) based on the observation that *x = 11* is the most frequently occurring basic chromosome number in the genus. These authors also proposed that small chromosome size is ancestral in *Pelargonium*, but this is neither supported nor refuted by our data, since, with regard to chromosome size, the phylogeny presented here comprises a two-taxon statement.

**Pelargonium mtDNA intron loss**—Whereas intron losses have been found in several genes in the angiosperm chloroplast genome and have proven to be useful molecular phylogenetic markers (Downie and Palmer, 1992; Doyle, Doyle, and Palmer, 1995; Downie, Llanas, and Katz-Downie, 1996; Wallace and Cota, 1996; Bailey et al., 1997), intron loss (without loss of adjacent exons) in the angiosperm mitochondrial genome has so far been described only for *nad4* (Gass, Makaroff, and Palmer, 1992; Geiss, Abbas, and Makaroff, 1994), *nad1 d/e intron* and *cox2* (Qiu et al., 1998; Palmer, 1992). The *nad1 b/c intron* loss described in this study is therefore the first recorded in vascular plants. Our evidence suggests that the *nad1 b/c intron* has been lost from the entire Geraniaceae clade, although *Geranium* is represented here by only two species and *Sarcocaulon* by one. Intron loss for *Erodium*, which was not included in this study, was confirmed by diagnostic PCR amplification.

The combined use of mtDNA and cpDNA sequences in phylogenetic reconstruction allows testing of hypotheses regarding inheritance of organellar genomes such as the “paternal leakage” of chloroplast genomes. Both chloroplast and mitochondrial genomes have been observed in pollen tubes of *P. zonale* and the possibility of biparental inheritance of both genomes has been suggested (Kuroiwa et al., 1993; Nagata et al., 1997). Tilney-Bassett (1963) provided evidence for biparental inheritance of chloroplasts, which would suggest that cpDNA is actually transmitted into following generations. If biparental inheritance of mitochondrial and chloroplast genomes occurred frequently but episodically in *Pelargonium*, we would expect topological incongruencies between mtDNA- and cpDNA-based phylogenetic reconstructions, unless inheritance of the two genomes is linked. The fact that the phylogenies proposed here are largely congruent lends support to the idea that inheritance of the two organellar genomes is linked, a finding that was also reported in *Quercus* (Dumolin-Lapègue, Pe monge, and Petit, 1998).

**LITERATURE CITED**


