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MITOCHONDRIAL AND CHLOROPLAST DNA-BASED PHYLOGENY OF *PELARGONIUM* (GERANIACEAE)¹

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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 phylogenetic 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 exon sequences 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, Sodji, and Petit (1995) for the mtDNA *nad1* b/c exons using their primers *nad1* b (5'-GCATTACGTCTGCAGCTCA-3') and *nad1* c (5'-GGAGCTCGAT-

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TABLE 1. List of taxa and sequences used.

Taxon	Chr. number ^a	Source ^b	nacl b/c ^c	trnL-F ^c
<i>Geranium pusillum</i> L.	x = 13 ^d	Reading, UK; Pankhurst 1 (RNG)	GBAN-AF167131	GBAN-AF167151
<i>Geranium robertianum</i> L.	x = 16 ^d	Reading, UK; Pankhurst 2 (RNG)	GBAN-AF167132	GBAN-AF167152
<i>Pelargonium alternans</i> Wendl.	x = 11	Worcester, W Cape, RSA; STEU 4355 (STEU)	GBAN-AF167126	GBAN-AF036052
<i>P. articulatum</i> (Cav.) Willd.	x = 9	Karopoort, W Cape, RSA; STEU 2309 (STEU)	GBAN-AF167105	GBAN-AF167144
<i>P. australe</i> Willd.	x = 11	Tasmania, Australia; CPG 8829 (BM)	GBAN-AF167129	GBAN-Z95280
<i>P. caucasicum</i> Jacq.	x = 11	Riviersonderend, W Cape, RSA; STEU1191 (STEU)	GBAN-AF167114	GBAN-AF167141
<i>P. corymbosum</i> Humbert	x = 9	Madagascar; CPG 8842 (BM)	GBAN-AF167106	GBAN-AF036087
<i>P. elongatum</i> (Cav.) Salisb.	x = 4	Simonstown, Cape Peninsula, RSA; CPG8890 (BM)	GBAN-AF167108	GBAN-AF167146
<i>P. exilifolium</i> Vorster	x = 11	Grahamstown, E Cape, RSA; STEU 1889 (BM)	GBAN-AF167112	GBAN-AF167139
<i>P. fulgidum</i> (L.) L'Hérit.	x = 11	Elandsbaai, W Cape, RSA; G&C 33 (BM)	GBAN-AF167124	GBAN-AF036056
<i>P. grandicaule</i> Knuth	x = 10	Botterkloof, W Cape, RSA; STEU 2055 (STEU)	GBAN-AF167110	GBAN-AF167148
<i>P. griseum</i> Knuth	x = 9	Steynsberg, E Great Karoo, W Cape, RSA; CPG 8518 (BM)	GBAN-AF167119	GBAN-AF036091
<i>P. grossularioides</i> (L.) L'Hérit.	x = 19	Hogsback, E Cape, RSA; STEU 1872 (MSUN/STEU)	GBAN-AF167130	GBAN-Z95289
<i>P. karooicum</i> Compton	x = 10	W Cape, RSA; STEU 2967 (BM)	GBAN-AF167111	GBAN-AF167149
<i>P. mollicomum</i> Fourcade	x = 11	Grahamstown, E. Cape, RSA; STEU 3282 (BM)	GBAN-AF167113	GBAN-AF036090
<i>P. multibracteatum</i> Hochstetter ex A. Rich.	x = 9	Dallil, Yemen; CPG 7757 (BM)	GBAN-AF167107	GBAN-AF167145
<i>P. mutans</i> Vorster	x = 11	Mkuze river, Veerplaats, Natal, RSA; GBB 513 (BM)	GBAN-AF167122	GBAN-AF167138
<i>P. nanum</i> L'Hérit.	x = 8	Gydopass, Ceres, W Cape, RSA; H875 (MSUN)	GBAN-AF167128	GBAN-Z95296
<i>P. pelattum</i> (L.) L'Hérit.	x = 9	S Cape, RSA; CPG 5685 (BM)	GBAN-AF167103	GBAN-AF167143
<i>P. praemorsum</i> Andr. (Diétr.)	x = 9	Namaqualand, N Cape, RSA; STEU 1575 (BM)	GBAN-AF167121	GBAN-AF167137
<i>P. redactum</i> Vorster	x = 9	8 km W of Aus on B 4, Namibia; AL 2738 (MSUN)	GBAN-AF167120	GBAN-AF167134
<i>P. senecioides</i> L'Hérit.	x = 11	Piketberg, W Cape, RSA; SL 17300 (MSUN)	GBAN-AF167127	GBAN-AF036080
<i>P. sericifolium</i> J.J.A van der Walt	x = 9	Leipoldville, Cape, RSA; CPG 8855 (BM)	GBAN-AF167117	GBAN-AF167135
<i>P. spinosum</i> Willd.	x = 11	Spektakel Pass, N Cape, RSA; STEU 2068 (BM/STEU)	GBAN-AF167123	GBAN-AF036057
<i>P. tetragonum</i> (L.f.) L'Hérit.	x = 10	Desert Mts N of Steinkopf, N Cape, RSA; CPG 8470 (BM)	GBAN-AF167109	GBAN-AF167147
<i>P. tongaense</i> Vorster	x = 11	Worcester, SW Cape, RSA; CPG 7993 (BM)	GBAN-AF167116	GBAN-AF167140
<i>P. trifidum</i> Jacq.	x = 9	Pongola river, McKane's point, Natal, RSA; STEU 3074 (BM)	GBAN-AF167104	GBAN-AF036089
<i>P. triste</i> (L.) L'Hérit.	x = 9	Cape, RSA; STEU 2730 (BM)	GBAN-AF167118	GBAN-AF167136
<i>P. whytei</i> Bak.	x = 11	W Cape, RSA; STEU 2234 (BM/STEU)	GBAN-AF167125	GBAN-AF036045
<i>Sarcocaulon vanderretiae</i> L. Bol.	x = 11 ^e	Mozambique; CPG 8634 (BM)	GBAN-AF167115	GBAN-AF167142
		Cookhouse, E. Cape, RSA; CPG 8471 (BM)	GBAN-AF167133	GBAN-AF167150

^a Basic chromosome number.^b CPG = Chelsea Physic Gardens, London, UK; STEU = Stellenbosch University, Stellenbosch, Republic of South Africa; voucher locations are indicated in parentheses.^c 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).^d From van Loon (1984).^e From Albers (1990).

mtDNA *nad1* b/c
Jackknife tree

cpDNA *trnL-F*
Jackknife tree

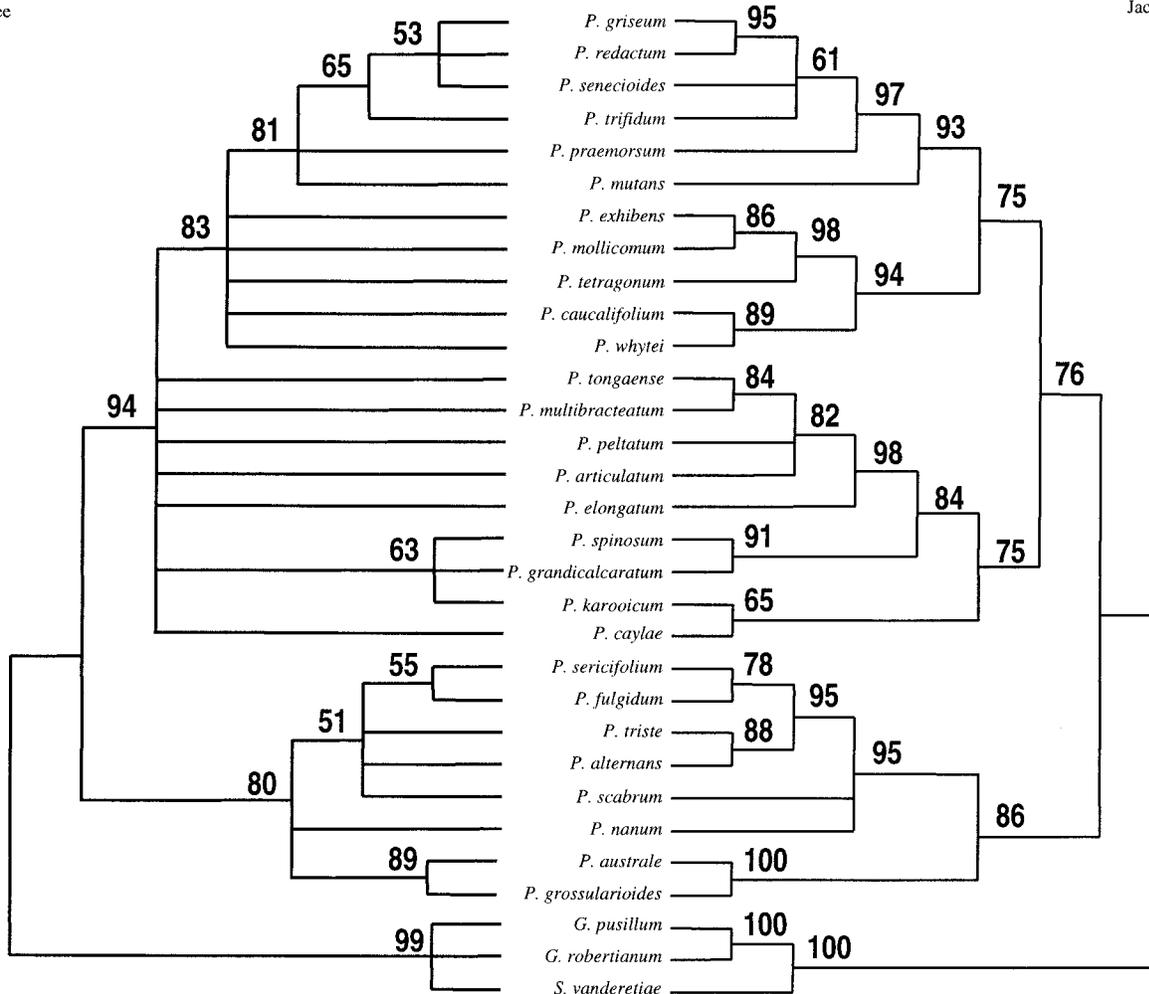


Fig. 2. *Pelargonium nad1* b/c (left) and *trnL-F* (right) jackknife trees in which nodes with support values <50% have been collapsed. Jackknife support values (10 000 replicates) are indicated above the nodes.

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

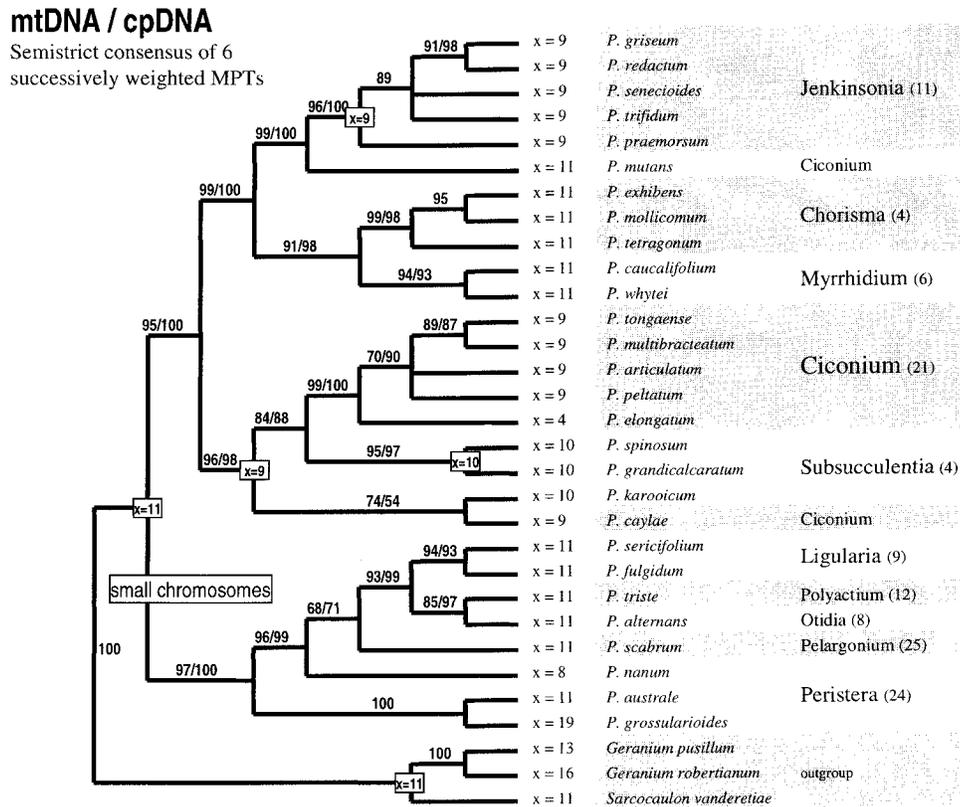


Fig. 3. *Pelargonium* combined *nad1* b/ c/ *trnL-F* semistrict consensus tree of the six successively weighted MPTs. Bootstrap support values (1000 “full heuristic” replicates) are indicated above nodes; when two values are given the first refers to bootstrap analysis of the equally weighted matrix 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 recognised 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

mtDNA / cpDNA

One of six successively weighted
most parsimonious trees

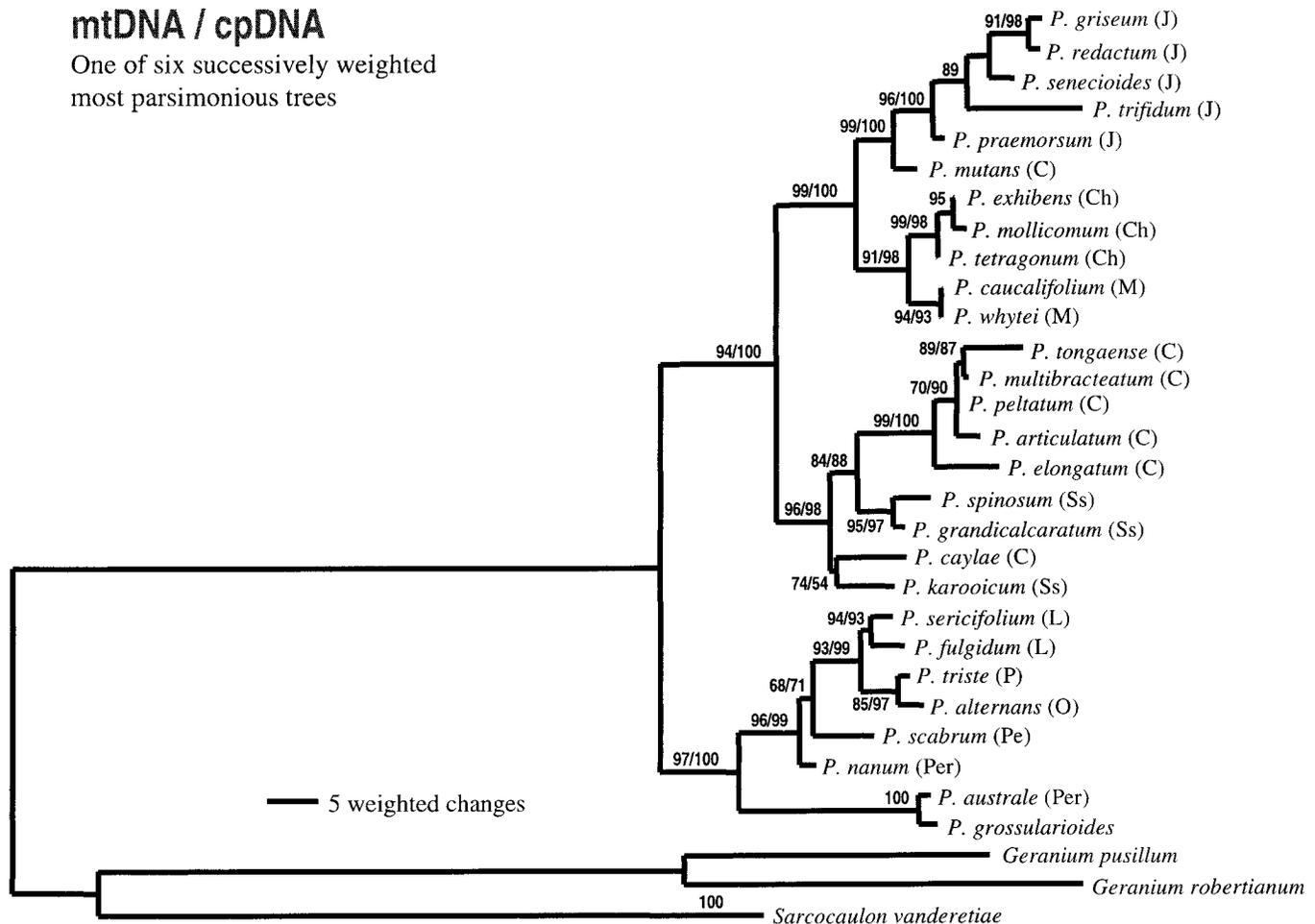


Fig. 4. *Pelargonium* combined *nad1* b/ c/ *trnL-F* analysis: one out of six most parsimonious reconstructions calculated from the combined and successively weighted data set (see text). The scale bar indicates 5 (successively weighted) steps. Bootstrap values at the nodes are as explained in Fig. 2. 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. caucalifolium* 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 section *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. karoicum* ($x = 10$) 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. karoicum* in a different lineage from *P. spinosum* and *P. grandicalcaratum*. This section includes (sub)shrubs with succulent or somewhat woody stems, but *P. karoicum* 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. karoicum* 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/Myrrhidium* 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 $x = 11 \rightarrow 10 \rightarrow 9$. 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, Pemonge, and Petit, 1998).

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