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MOLECULAR PHYLOGEOGRAPHY, RETICULATION, AND LINEAGE SORTING IN MEDITERRANEAN *SENECIO* SECT. *SENECIO* (ASTERACEAE)

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Abstract.—The Mediterranean species complex of *Senecio* serves to illustrate evolutionary processes that are likely to confound phylogenetic inference, including rapid diversification, gene tree-species tree discordance, reticulation, interlocus concerted evolution, and lack of complete lineage sorting. Phylogeographic patterns of chloroplast DNA (cpDNA) haplotype variation were studied by sampling 156 populations (502 individuals) across 18 species of the complex, and a species phylogeny was reconstructed based on sequences from the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. For a subset of species, randomly amplified polymorphic DNAs (RAPDs) provided reference points for comparison with the cpDNA and ITS datasets. Two classes of cpDNA haplotypes were identified, with each predominating in certain parts of the Mediterranean region. However, with the exception of *S. gallicus*, intraspecific phylogeographic structure is limited, and only a few haplotypes detected were species-specific. Nuclear sequence divergence is low, and several unresolved phylogenetic groupings are suggestive of near simultaneous diversification. Two well-supported ITS clades contain the majority of species, amongst which there is a pronounced sharing of cpDNA haplotypes. Our data are not capable of diagnosing the relative impact of reticulation versus insufficient lineage sorting for the entire complex. However, there is firm evidence that *S. flavus* subsp. *briviflorus* and *S. rupestris* have acquired cpDNA haplotypes *and* ITS sequences from co-occurring species by reticulation. In contrast, insufficient lineage sorting is a viable hypothesis for cpDNA haplotypes shared between *S. gallicus* and its close relatives. We estimated the minimum coalescent times for these haplotypes by utilizing the inferred species phylogeny and associated divergence times. Our data suggest that ancestral cpDNA polymorphisms may have survived for ca. 0.4–1.0 million years, depending on molecular clock calibrations.

Key words.—Chloroplast DNA, ITS, lineage sorting, molecular clock, phylogeography, reticulation, *Senecio*.

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Testing historical hypotheses concerning the partitioning of genetic variation within and among species is critical to the study of evolution and, in particular, to our understanding of the patterns and processes involved in species diversification. When studying relationships among recently separated groups of plant species, two evolutionary processes often need to be taken into account: (1) reticulation, that is, natural hybridization, introgression, as well as homo- and polyploid speciation (Abbott 1992; Arnold 1997; Rieseberg 1997, 1998); and (2) incomplete lineage sorting, that is, the persistence and retention of ancestral polymorphisms through multiple speciation events (Avise 1994, 2000). This concern is particularly noticeable for large nonrecombining gene segments, such as the (mainly uniparentally inherited) chloroplast genome, because the effects of reticulation are potentially retained through subsequent generations, namely, speciation events (Doyle 1992; Wendel and Doyle 1998). This, for example, is aptly illustrated by the many interspecific molecular studies related to reticulate evolution in angiosperms that have inferred chloroplast DNA (cpDNA) capture through introgressive hybridization (e.g., Soltis and Kuzoff 1995; Wolfe and Elisens 1995), often in the apparent absence of introgression of biparentally inherited nuclear genes (Rieseberg and Wendel 1993; Soltis and Soltis 1995). The possibility that cpDNA /nuclear gene tree incongruencies arise through the retention of ancestral cpDNAs from polymorphic ancestors has received far less attention than has reticulate evolution. This might relate to the fact that the magnitude and extent of cpDNA polymorphism has long been regarded as being insufficient for intraspecific studies, thereby prompting plant molecular systematists to sample a single or only a few individuals per species for phylogenetic analysis. Recently, however, more comprehensive surveys have found substantial inter- and intrapopulational variation in cpDNA (e.g., Mason-Gamer et al. 1995, 1999; Bain and Jansen 1997, 2000; Dumolin-Lapègue et al. 1997; Comes and Abbott 1998, 1999; but see Jordan et al. 1997).

To find a solution to the problems associated with plant groups that are in the process of differentiating and/or hybridizing it is important to sample molecular data across a large number of loci, for example, distinct gene trees (Pamilo and Nei 1988; Doyle 1992; Soltis and Kuzoff 1995). Moreover, detailed geographical sampling of molecular variation within and between species is mandatory. With respect to cpDNA, plant evolutionary biologists are becoming increasingly aware of the potential of ''phylogeographic'' (sensu Avise 1994) sampling strategies (e.g., Demesure et al. 1996; Soltis et al. 1997; Comes and Abbott 1998, 1999; Abbott et al. 2000a; Maskas and Cruzan 2000; Matos and Schaal 2000). However, only a few such analyses have yet been conducted that have involved this level of analysis within an entire species complex (*Senecio* [*Packera*] spp.: Bain and Jansen 1997, 2000; *Quercus* spp.: Dumolin-Lapègue et al. 1997). Whereas phylogeographic cpDNA studies may provide useful information on intraspecific genetic differentiation, they have limited potential in resolving the times and orders of evolutionary divergence. Hence, additional tools, such as nuclear DNA sequencing, are needed to build up a solid phylogenetic framework and introduce a temporal dimension to the description of the genetic structure.

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TABLE 1. *Senecio* taxa included in the cpDNA restriction analysis, together with their simplified distribution ranges, life habit, and ploidy level (2x, 4x). The number of populations and the total number of individuals surveyed per taxon are indicated. Voucher specimens and seed samples of all populations are stored at the St. Andrews (STA) herbarium.

Senecio taxa[1]	Distribution[2]	Life habitat, ploidy[3]	Number of populations	Number of individuals haplotyped
S. aegyptius subsp. *aegyptius*	Egypt; Sudan	A, 4x	1	2
S. aegyptius subsp. *thebanus*	Egypt	A, 4x	1	1
**S. aethnensis*	Sicily, Mt. Etna	P, 2x	1	1
**S. chrysanthemifolius*	Sicily	P, 2x	1	1
S. flavus subsp. *breviflorus*	Egypt; Near East; Arabia	A, 4x	5	5
S. flavus subsp. *flavus*	Canary Islands; SW, N Africa; Near East	A, 2x	1	1
S. gallicus	S France; Iberia; Algeria (?)	A, 2x	14	115
S. glaucus	Canary Islands; N Africa; Sicily; Cyprus; SW Asia and W Himalayas	A, 2x	23	152
S. hesperidium	SW Morocco	A, 2x	2	2
S. leucanthemifolius	W, C Mediterranean; Crete	A, 2x	17	30
S. lividus	W, C Mediterranean; Turkey	A, 4x	8	10
**S. nebrodensis*	N, C, S Spain	P, 4x	6	11
S. petraeus	S Spain	A, 2x	1	1
S. rodriguezii	Balearic Islands	A, 2x	1	1
S. rupestris	SC, SE Europe; local in C and W Europe	P, 2x	21	28
**S. squalidus* subsp. *squalidus*[4]	British Isles	P, 2x	1	1
S. squalidus subsp. *araneosus*[5]	N Morocco, Rif Mts.	P, 2x	1	1
S. sylvaticus	Europe	A, 4x	4	5
**S. vernalis*	NE, C, SE Europe; SW Asia	A, 2x	15	90
S. viscosus	Europe	A, 4x	6	7
S. vulgaris var. *vulgaris*	Cosmopolitan	A, 4x	19	19
S. vulgaris subsp. *denticulatus*	Spain; Sicily; Channel Islands	A, 4x	8	11
Outgroup				
S. malacitanus	S, SE Spain; Balearic Islands; Morocco	P, 4x	1	1
Total			157	503

[1] Asterisks refer to taxa initially screened for RFLP variation over 18 enzymes × 15 *Lactuca sativa* cpDNA probes.
[2] Data are from Alexander (1979); Kadereit (1984b, 1991); Liston and Kadereit (1995); Coleman et al. (2001); Comes and Abbott, pers. obs.
[3] A, annual, P, biennial or short-lived perennial; ploidy data are from Alexander (1979) and Chater and Walters (1976), except for *S. flavus* subsp. *breviflorus* (see Coleman et al. 2001).
[4] This taxon as found in the British Isles is often considered conspecific with *S. rupestris* from southern-central and southeastern Europe (e.g., Alexander 1979). However, in accordance with the views of Crisp (1972) and Walters (1963), here we treat *S. squalidus* as a separate taxon due to its likely homoploid hybrid origin from *S. aethnensis* and *S. chrysanthemifolius* (Abbott et al. 2000b).
[5] The taxonomic status of this subspecies needs careful reassessment. Preliminary herbarium surveys indicate that morphologically it falls within the range of variation seen in *S. rupestris* rather than *S. squalidus* subsp. *squalidus* (M. Coleman, pers. comm.).

By using the Mediterranean species complex of *Senecio* sect. *Senecio* (Asteraceae) as a model system, the present study aims to illustrate evolutionary processes that are likely to confound phylogenetic inference, including rapid diversification, reticulation, and incomplete lineage sorting. The complex, as recognized here (cf. Alexander 1979), contains certain annual and short-lived perennial species of *Senecio* sect. *Senecio* (Jeffrey et al. 1977; Jeffrey 1979) that have long been considered ''closely allied'' with the type species of the genus, *S. vulgaris* (Alexander 1979; Kadereit 1984a). Species within the complex are primarily distributed across countries bordering the Mediterranean (see also Table 1). However, several taxa are also more widely distributed throughout the more temperate regions of central, northern, and eastern Europe, or show a distribution pattern extending far into the steppe and (semi-) desert provinces of southwestern and inner Asia. The type species, *S. vulgaris*, is cosmopolitan in distribution.

Following the latest revision by Alexander (1979), the group consists of approximately 23 diploid and tetraploid species and three hexaploid species, with $2n = 20$, 40, and 60 chromosomes, respectively. The recognition of the Mediterranean species complex of *Senecio* sect. *Senecio* as a single evolutionary entity by most researchers implies that it is monophyletic relative to the other (more than 100) species presently recognized within the section (Jeffrey 1992), although this has never been explicitly tested.

Notably, with a few exceptions, the tetraploids and hexaploids tend to be highly inbreeding, associated with reduced or absent outer ray florets, whereas the diploids tend to have long ray florets and are outbreeding (Alexander 1979). The group is well known for its amenability to experimental studies of genecology and plant evolution, but is notorious for its taxonomic complexity (Walters 1963). Some of the taxonomic confusion within the group might be attributed to relatively high levels of species interfertility, the origin of polyploid, and probably also diploid species following interspecific hybridization (Abbott 1992; Abbott et al. 2000b), continued hybridization and introgression in areas of sympatry or parapatry (e.g., Comes and Abbott 1999), and/or the shared presence of ancestral similarities due to recent differentiation.

Much of the confusion within the species complex has been clarified by recent molecular investigations into the origin and evolutionary history of the diploid *S. squalidus* in the British Isles (Abbott et al. 2000b) and several polyploid species (*S. vulgaris*: Ashton and Abbott 1992a; Harris and Ingram 1992a; Comes et al. 1997; *S. nebrodensis* and *S. viscosus*: Kadereit et al. 1995; Purps and Kadereit 1998; *S. cambrensis*: Ashton and

Abbott 1992b; *S. teneriffae*: Lowe and Abbott 1996). However, comparatively little is known about the phylogenetic relationships among the majority of the widespread diploid taxa within the species group (*S. gallicus, S. glaucus, S. leucanthemifolius, S. rupestris, S. vernalis*), various geographically restricted diploid (*S. aethnensis, S. chalureaui, S. chrysanthemifolius, S. hesperidium, S. rodriguezii, S. petraeus*) and polyploid endemics (*S. aegyptius, S. hoggariensis, S. massaicus*), and the relationship of all these species to (1) a supposedly monophyletic group of glandular-hairy tetraploids, comprising *S. nebrodensis, S. lividus, S. sylvaticus,* and *S. viscosus* (Kadereit et al. 1995); and (2) a morphologically rather distinct species of probably Southwest African origin (*S. flavus*; Alexander 1979; Liston and Kadereit 1995). A robust phylogenetic hypothesis for the species complex is still elusive, and more evidence for the overall degree to which rapid diversification, reticulation, and incomplete lineage sorting have affected the entire group is needed.

Here we take up this complex matter using a large analysis of cpDNA restriction site and length variation (RFLPs) and sequence variation of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) within and among closely related taxa of Mediterranean *Senecio* sect. *Senecio*. In addition, our analysis includes preliminary data on randomly amplified polymorphic DNAs (RAPDs) for a subset of species, providing reference points for comparison with the cpDNA and ITS datasets. The specific objective of this study was to determine (1) the order and times of evolutionary divergence; (2) the occurrence of reticulation; and (3) the effects of interspecies transfer of cpDNA in relation to shared ancestral cpDNA polymorphism. Finally, an attempt was made to empirically assess the time for which cpDNA polymorphisms could have survived multiple speciation events by using the phylogeny of *Senecio* species estimated from ITS sequence data.

Materials and Methods

Collection of Plant Material and DNA Isolation

A total of 156 *Senecio* populations (502 individuals) from 18 species in the complex were sampled from locations throughout the Mediterranean region and Europe (Fig. 1; because two accessions of unknown locality were included, only 154 are shown). This material was employed in a survey of cpDNA RFLP variation using southern blot analysis (Table 1), whereas subsamples were also scored for cleaved-amplified polymorphisms (CAPs) in cpDNA, ITS sequences, and presence/absence of randomly amplified polymorphic sequences (RAPDs). The individuals analyzed for cpDNA and ITS variation represent nearly all of the named species in the group (according to Alexander 1979) with the exception of the geographically highly restricted taxa *S. chalureaui, S. hoggariensis,* and *S. massaicus* and two reported allohexaploids (*S. cambrensis* and *S. teneriffae*; Lowe and Abbott 1996). As an outgroup in the phylogenetic analyses we used *S. malacitanus*, a long-lived perennial that is not a member of the species complex (Alexander 1979). Further details on the geographic distribution, life habit, and ploidy level of the taxa analyzed are given in Table 1.

Material for DNA analysis was generally obtained from greenhouse plants raised from seed collected in the field. For a very few accessions, silica gel-dried material was used. Whole genomic DNAs were isolated from leaf tissue using a CTAB-based protocol (Abbott et al. 1995) followed by an RNase A treatment (Boehringer, Mannheim, Germany) and a final ammonium acetate precipitation. Some DNA samples, including all those from silica dried materials, were purified on a CsCl gradient as described in Abbott et al. (1995).

Restriction Fragment-Length and Site Polymorphisms (RFLPs) of cpDNA

Since we were primarily interested in intraspecific cpDNA polymorphism across the entire species complex, typically only one or two individuals per population were analyzed (Table 1). This allowed us to maximize the number of populations and species we could survey. However, several widespread species (*S. gallicus, S. glaucus, S. vernalis*), which contained reasonably high levels of cpDNA polymorphism in a first screening, were more extensively sampled. Their distribution of cpDNA haplotype variation within and among populations has been detailed elsewhere (Comes and Abbott 1998, 1999), but will be summarized here for comparison. Chloroplast DNA haplotype data previously reported for *S. nebrodensis*/*S. viscosus* (Kadereit et al. 1995) and *S. vulgaris* (Comes et al. 1997) will also be included for comparative analysis. Because of its large size, a complete list containing detailed geographic information on the location of all collection sites and the number of individuals of each haplotype per site is available at the population genetic database at http://seahorse.louisiana.edu/PGDBdata/comes2001.html

Methods for cpDNA restriction enzyme digestion, agarose electrophoresis, southern blotting, and hybridization were as described in Harris and Ingram (1992b) and Comes et al. (1997). Filters were hybridized with cloned fragments of ^{32}P- or digoxigenin-labeled cpDNA from *Lactuca sativa* (Jansen and Palmer 1987), and hybridized probes were visualized via autoradiography, or chemiluminescence according to the manufacturer's (Boehringer, Mannheim, Germany) specifications. For a preliminary survey, five species, embracing much of the morphological diversity within the complex (marked with an asterisk in Table 1), were screened for cpDNA restriction site and length variation over a wide range of probe-enzyme combinations (15 probes × 18 enzymes; Abbott et al. 1995; Abbott, unpubl. data). This survey resolved seven polymorphisms (asterisked in Table 2) across the five species studied. The subsequent full analysis of all 503 sampled plants (representing 19 species, and including the outgroup) was limited largely to those probe-enzyme combinations that had identified the seven polymorphisms in this preliminary survey, but also included two additional enzymes for probes pLsC 8–11 (i.e., *Hin*DIII and *Xba*I).

Haplotypes identified were further characterized by a survey of RFLP variation within the PCR amplified region of the chloroplast *trn*K gene for 39 individual plants from all species, except *S. aethnensis, S. chrysanthemifolius,* and *S. hesperidium*. Amplifications were performed following a protocol described in Schwarzbach and Kadereit (1995). The primers used were *trn*K-2621 (5′-AACTAGTCGGATGGAG-

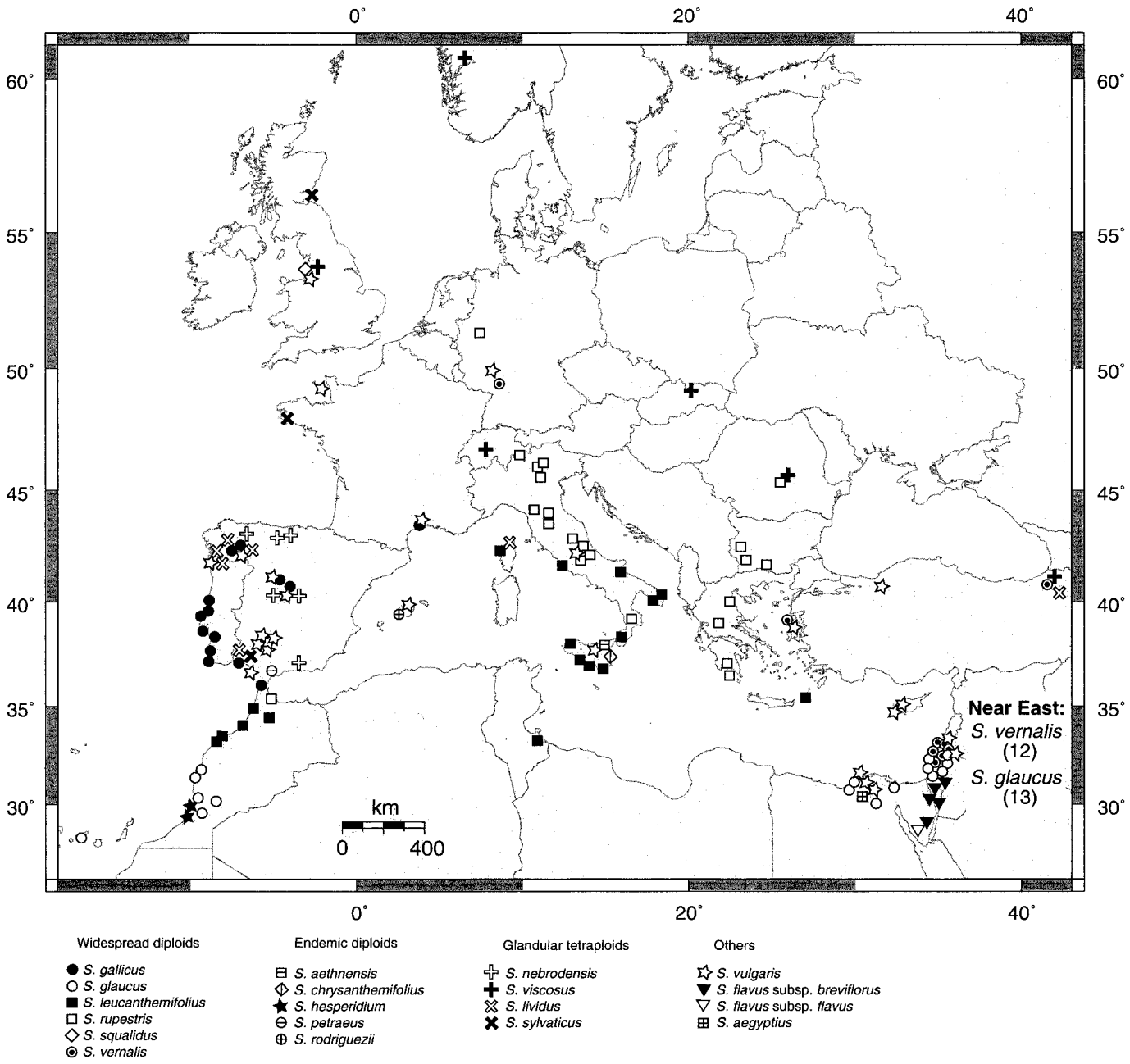

FIG. 1. Map of the sampled populations for 18 species of the Mediterranean *Senecio* complex used in this study for cpDNA haplotype analysis. For two densely collected species from the Near East (*S. vernalis* and *S. glaucus*), numbers in parentheses refer to the actual numbers of populations sampled. See Table 1 for approximate distribution ranges of the species and subspecies analysed.

TAG-3′) and *trn*K-11 (5′-CTCAACGGTAGAGTACTCG-3′). PCR products (2560 bp) were individually digested with three restriction enzymes (*Bst*OI, *Hpa*II, *Cfo*I) shown to yield polymorphisms in an initial survey of 19 accessions from 13 representative species, and using a series of 13 additional enzymes (data not shown). Restriction fragments were separated by electrophoresis in 1.4% agarose gels.

ITS Amplification and Sequencing

The entire ITS1-5.8S-ITS2 region was sequenced in 37 individuals from 18 species in the complex. Full collection details of these individuals, together with their cpDNA haplotype, are given in the Appendix I.

All amplifications were performed in 100 μl volumes containing 75 μl dionized sterile water, 2 μl of 25mM $MgCl_2$ solution, 10 μl 10 × Dynazyme™ buffer (100 mM Tris pH 8.8, 15mM $MgCl_2$, 500 mM KCl_2, 1% Triton X-100), 10 μl of a 2mM dNTP solution in equimolar ratio, 0.5 μl each of primers ITS4 and ITS5 (White et al. 1990) at 25 pmol/μl, 2 units (1 μl) Dynazyme™ polymerase (Finnzymes OY, Flowgen Laboratories, Lichfield, U.K.), and 1 μl genomic DNA (approx. 25 ng). Double-stranded DNA templates were pro-

TABLE 2. Chloroplast DNA polymorphisms detected in the *Senecio* material studied. Character states were polarized as ancestral (0) or derived (1) by outgroup comparison with *S. malacitanus* (see also Appendix II).

Polymorphism[1]	Enzyme	Probe/product[2]	Mutation (fragment size in kb)[3]		Type[4]
			0	1	
*1	*Pvu*II	4+5	14.0	9.1 + 4.9	site gain
*2	*Hae*III	4+5	—	−0.045	deletion
3	*Hae*III	4+5	1.5	absent	?
4	*Hae*III	4+5	—	+0.035	insertion
5	*Hae*III	4+5	—	−0.04	deletion
6	*Hae*III	4+5	—	+0.02	insertion
7	*Cla*I	6	11.1	absent	?
*8	*Cla*I	6	—	−0.33/0.35	deletion
*9	*Cla*I	6	3.1 + (0.2)	3.3	site loss
10	*Cla*I	6	—	−0.7	deletion
*11	*Pst*I	1–3	2.8	absent	?
12	*Pst*I	1–3	—	−0.05	deletion
*13	*Cfo*I	6	8.4	7.0 + 1.4	site gain
14	*Cfo*I	6	—	+0.12	insertion
*15	*Eco*RI	8–11	2.0 + (0.4)	2.4	site loss
16	*Eco*RI	8–11	—	−0.06	deletion
17	*Eco*RI	8–11	4.9 + 1.1	6.0	site loss
18	*Eco*RI	8–11	—	−0.1	deletion
19	*Hin*DIII	8–11	3.5	absent	?
20	*Hin*DIII	8–11	10.9	4.2 + 2.5 + (4.2)	site gain
21	*Xba*I	8–11	—	+0.7	insertion
22	*Xba*I	8–11	absent	6.5	?
23	*Hpa*II	*trn*K	2.6	2.4 + 0.2	site gain
24	*Hpa*II	*trn*K	2.4	2.0 + 0.4	site gain
25	*Bst*OI	*trn*K	1.5	1.1 + 0.4	site gain
26	*Bst*OI	*trn*K	—	+0.14	insertion
27	*Cfo*I	*trn*K	—	+0.02	insertion

[1] Asterisks indicate seven RFLP polymorphisms resolved by an initial wide screen across five *Senecio* taxa with 18 enzymes × 15 *Lactuca sativa* cpDNA probes (see Materials and Methods).
[2] Probe designation follows Harris and Ingram (1992a, b) and Abbott et al. (1995).
[3] A line indicates no equivalent band detected. Fragment sizes in parentheses refer to presumed but undetected bands.
[4] See Results for details of unknown (?) mutation types.

duced in a thermal cycler set at an initial 1 min at 97°C, followed by 26 cycles of 97°C for 1 min, 48°C for 1 min, 72°C for 3 min, with a final incubation of 7 min at 72°C. PCR products were cleaned with the Wizard™ Kit (Promega, Madison, WI).

PCR products were cycle sequenced in forward and reverse reactions using the ABI PRISM™ Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) with AmpliTaq-DNA polymerase FS, 0.5 μl of 8 pmol primer (5ng/μl of either ITS4 or ITS5), and 1.3 or 2.5 μl template DNA (approx. 30 or 60 ng) for ITS4 and ITS5, respectively, all in a total volume of 5 μl. Thermocycling profiles were set at 26 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Single-stranded, dye-labeled DNAs were precipitated with 1.0 μl 3M NaOAc (pH 5.6), left in 25 μl 96% ethanol at RT for 5 min, followed by chilling on ice for 10 min, and washed in 300 μl 70% ethanol. Sequences were detected on an ABI PRISM 377 DNA automated sequencer. Boundaries of the two ITS regions and the three coding regions (18S, 5.8S, and 26S) were determined by comparison with sequences from *Krigia* (Kim and Jansen 1994).

Sequences of the two allohexaploids, *S. cambrensis* and *S. teneriffae* showed multiple (''additive'') sequence signals at their variable sites, and thus were excluded from phylogenetic analyses. For all other samples we found no evidence of intra-individual ITS polymorphism (Hershkovitz et al. 1999); all PCRs yielded single products and all direct-sequencing electropherograms were interpretable as unique sequences.

Randomly Amplified Polymorphisms of DNA (RAPDs)

RAPD variation was screened over a subset of 18 individuals (representing 10 species of the complex) that were also examined for ITS and/or cpDNA variation (Appendix I). Seventeen 10-mer random primers were used (Operon Technologies, Alameda, CA: OPH-01–09, OPH-11–15, OPH-17–19). Amplifications were performed in 25 μl volumes containing 17.55 μl dionized sterile water, 2.5 μl 10 × Dynazyme™ buffer (see above), 2 μl of a 2mM dNTP solution in equimolar ratio, 0.2 μl of primer at 5 pmol/μl, 0.5 unit Dynazyme™ polymerase, and 2.5 μl genomic DNA (approx. 12.5 ng). Thermocycling profiles were set at an initial 3 min for 94°C, followed by 45 cycles of 94°C for 30 sec, 36°C for 45 sec, 72°C for 1.5 min, with a final extension of 4 min at 72°C. Amplification products were visualized on 1.4% agarose gels stained with ethidium bromide and photographed under UV light. All amplifications were repeated twice to check the stability of amplification products. A total of 362 fragments were scored, 358 of which varied among accessions. The complete data matrix is available upon request from the first author.

Analyses

cpDNA haplotypes

To ascertain genealogical relationships among cpDNA haplotypes, an initial unweighted maximum-parsimony (MP) analysis was performed using PAUP* (test versions 4.0b3a–5a, Swofford 2001) with a ''branch-and-bound'' search and the following options in effect: MULTREES and SIMPLE addition of haplotypes. To assess node support, bootstrap values (BV) from 1000 replicates were calculated using the FULL HEURISTIC option with CLOSEST addition sequence of haplotypes. Subsequent analyses using different gain : loss weighting schemes (1.3:1, 2:1) for restriction sites resulted in identical cladogram topologies (not shown). Haplotypes were then assembled into an unrooted maximum-parsimony gene tree, which could be constructed by hand because of the low level of homoplasy (see also Olsen and Schaal 1999).

ITS sequences

Multiple sequences were aligned using CLUSTAL V (Higgins et al. 1992), with minor manual adjustments. Phylogenetic trees were generated from the combined ITS-1 and ITS-2 regions (excluding the 5.8S gene) by Fitch parsimony and the heuristic search strategy in PAUP*. The following conditions were in effect: MULTREES, tree bisection-reconnection (TBR) swapping, STEEPEST DESCENT, and 500 replications of RANDOM sequence addition. In these searches, gaps (indels) were scored as missing sites, with potentially informative indels superimposed onto the resulting trees. Bootstrap values were estimated from 100 replicate FULL HEURISTIC analyses with CLOSEST addition sequence.

PAUP* was used to calculate uncorrected pairwise (*p*) distances among species, as well as maximum-likelihood (ML) distances (Swofford et al. 1996) under a TrNef + Γ model of sequence evolution (see Tamura and Nei 1993). Parameter estimates for ML were calculated using the topology of one of the most parsimonious trees. The TrNef + Γ substitution model best described the sequence data after performing a series of hierarchical log likelihood-ratio (LR) tests as implemented by the program MODELTEST (vers. 3.0, Posada and Crandall 1998). The TrNef + Γ model assigns different substitution rates to pyrimidine and purine transitions, as well as a third rate to all transversions; it uses equal base frequencies and allows different rates at different sites based on a discrete gamma distribution (Γ) with four rate categories for the variable sites and with estimated shape parameter, α. The ML distance matrix was clustered by the neighbor-joining (NJ) procedure (Saitou and Nei 1987), and a bootstrap analysis of 10,000 replicates was performed. Full sequences (including 5.8S) have been deposited in the GenBank database under accession numbers AJ400777–AJ400813 (see also Appendix I).

One strongly deviant ITS sequence (*S. flavus* subsp. *flavus*, no. 5) was further characterized by examining its secondary structure stability and substitution patterns in order to test the hypothesis of this sequence being a pseudogene. Plant ribosomal pseudogenes should exhibit (1) low secondary structure stability and high free folding energy (ΔG) due to the accumulation of random substitutions and the destabilization of hair pins; and (2) high levels of C $\rightarrow$ T substitutions ($\geq$40% of all substitutions), possibly reflecting methyl-cytosine deaminations and relaxed selective constraints for chromatin condensation (Buckler et al. 1997). For various taxa, therefore, we compared ΔG (in kcal/mol; at 37°C) of the entire ITS sequence (including 5.8S) with the estimated ΔG of the reshuffled sequence using the computer program RNADRAW (vers. 1.1, Matzura and Wennborg 1996). The following accessions were included in the secondary structure analysis: 1, 5, 7, 15, 25, 27, 30, 39, 48, and 49 (see Appendix I for accession numbers). The relative frequency of C $\rightarrow$ T substitutions in *S. flavus* subsp. *flavus* was estimated by tracing unambiguous character-state changes onto one of the most parsimonious trees using PAUP*.

RAPDs

Amplified RAPD band states (362) were scored as present (1) or absent (0). Mean character differences between RAPD accessions were calculated using PAUP*, and a NJ tree computed based on 1000 bootstrap replicates. Analyses were repeated by calculating genetic distances from the complementary value of Jaccard's (J) similarity coefficient (1-J) as implemented by NTSYS-pc (vers. 1.60, Rohlf 1990). Jaccard's coefficient explicitly excludes the number of shared absence of bands. Because both coefficients gave almost identical tree topologies, only the phenogram based on PAUP* mean distances is presented.

Molecular clock analysis

Rate constancy of the ITS sequence data was evaluated by relative rate tests (Wu and Li 1985) as implemented by the program K2WULI (vers. 1.0, Jermiin 1996). After discovery of lineage rate constancy among two major subclades, approximate divergence times were estimated utilizing ITS clock calibrations for other Asteraceae/Senecioneae (Sang et al. 1994, 1995). To allow direct comparison with these latter studies, we employed pairwise Kimura (1980) two-parameter (K2P) distances as the measure of differences between sequences. The Kimura two-parameter statistic assumes equal base frequencies, and allows different substitution rates for transitions and transversions. Ninety-five percent confidence intervals for sequence divergence estimates were then approximated as $\pm$2 SE of the K2P distances.

RESULTS

Chloroplast DNA Polymorphisms

The survey of a total of 503 individuals from 157 populations with eight probe/enzyme combinations revealed 22 polymorphisms (Table 2), which allowed identification of 16 haplotypes. A subset of 39 individuals representing the complete collection of haplotypes was further studied with the *trn*K primer pair and three selected enzymes. Although this analysis detected five additional polymorphisms, none distinguished additional haplotypes (see also the matrix in the Appendix II).

Among the 27 polymorphisms resolved, nine resulted from restriction site gains or losses, while 13 were apparently due to insertions or deletions. The exact cause of the remaining

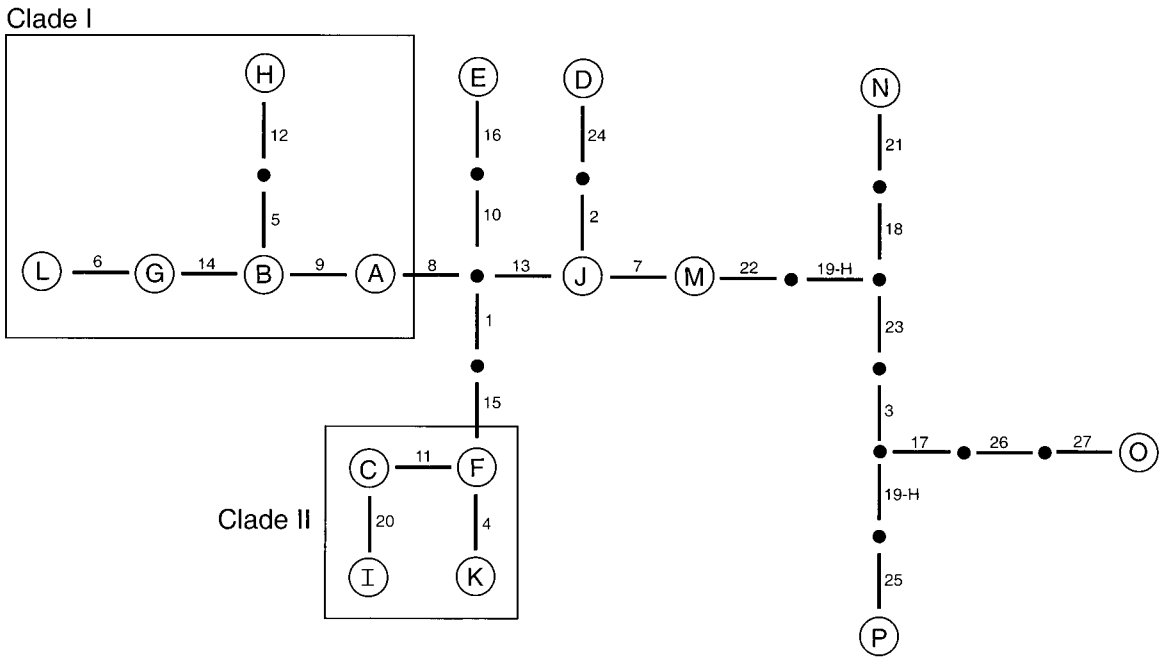

FIG. 2. Maximum-parsimony gene tree for cpDNA haplotypes of *Senecio*. Each branch represents a single mutational step, with numbers indicating the polymorphism involved (designated as in Table 2). Unobserved intermediate haplotypes are denoted by black dots. For branches of more than one mutational step and without observed haplotypes (or side branches), the relative placement of the polymorphisms along the branches is arbitrary. Polymorphism no. 19 is marked as homoplasious (H). The two major clades identified are confirmed by bootstrap values of 64% and 89%, respectively, in the corresponding cladogram rooted by *S. malacitanus* (haplotype *P*).

polymorphisms could not be determined in that they were due to the presence/absence of a single fragment. However, in each case a larger deletion or rearrangement could be ruled out because relative differences in fragment lengths were not similar for any of several other restriction enzymes (data not shown).

Maximum-parsimony analysis of cpDNA polymorphisms generated a single most parsimonious cladogram (length 28 steps; consistency index, excluding autapomorphies [CI]: 0.923; retention index [RI]: 0.964) that could be arranged into an unrooted haplotype tree (Fig. 2). Only one of these polymorphisms (no. 19) was homoplasious, meaning that the same mutational step had to be mapped onto the tree more than once. Two main clades, I and II, are evident in Figure 2, and these were also confirmed by modest and robust bootstrap values (BV) of 64% and 89%, respectively, in the corresponding MP cladogram rooted by *S. malacitanus*.

The cpDNA results presented here along with earlier results (Kadereit et al. 1995; Comes et al. 1997; Comes and Abbott 1998, 1999) indicate considerable intraspecific cpDNA polymorphism. Moreover, there is a considerable sharing of cpDNA haplotypes among the widespread diploid taxa, involving cpDNAs from both the I and II clades designated above (Table 3). Much of this diversity also embraces the variation present in most of the endemic diploids, and that found within the widespread tetraploid *S. vulgaris*. More samples are still necessary in the case of the endemic diploid *S. petraeus*, collected in only one population (in southern Spain, Sa. de Nieves) but represented by a new haplotype (*L*), which itself is a derivative of haplotype *G*, unique to *S. gallicus* from Spain (Fig. 2; Table 3). The glandular tetraploid species have a rather divergent set of cpDNAs (*D* and *M*) that did not form a distinct group in the haplotype tree. Only *S. nebrodensis* was polymorphic for cpDNA, whereas *S. viscosus* and *S. lividus*/*S. sylvaticus* were monomorphic for haplotypes *D* and *M*, respectively. It is noteworthy that two radically different sets of haplotypes (*N* vs. *F* and *K*) were found in individuals of *S. flavus* subsp. *flavus* and subsp. *breviflorus*, respectively, with haplotype *F* being also present in the widespread diploids (Table 3). Previously, Liston and Kadereit (1995) had also reported considerable cpDNA restriction-site differentiation between these two subspecies.

Figure 3A provides a summary of the geographical distribution of haplotypes found across all widespread and endemic diploid species (as identified in Table 3), as well as *S. flavus*; that is, those species exhibiting a pronounced pattern of shared interspecific cpDNA polymorphism, with the exception of the tetraploid, cosmopolitan *S. vulgaris*. Despite some major gaps in our geographical sampling and unequal within-population sample sizes, certain haplotypes appear to predominate in particular areas. Moreover, haplotypes belonging to the same clade were often located in the same part of the range (Fig. 3B). This is most obvious for clade I haplotypes, of which the most widespread ones (*A, B*) are predominant in the western and central Mediterranean region and shared between a large number of species. All other haplotypes of clade I are restricted to the Iberian Peninsula, where they either occur in *S. gallicus* (*G, H*) or *S. petraeus* (*L*). In contrast, for clade II, the broadly distributed and transspecific haplotypes (*C, F*) are predominant in North Africa and the Near East, while two rare haplotypes are entirely confined to the Near East, where they are found in *S. flavus*

TABLE 3. Number of individuals per cpDNA haplotype for each *Senecio* species surveyed. Clades were identified by maximum-parsimony methods as shown in Figure 2. Haplotypes *A–H* correspond to those reported in previous intraspecific studies (Abbott et al. 1995; Kadereit et al. 1995; Comes et al. 1997; Comes and Abbott 1998, 1999). Note that haplotypes are indicated separately for two subspecies of *S. flavus*.

	cpDNA haplotype[1]																
	Clade I					Clade II				Others							
Senecio taxa	*A*	*B*	*H*	*G*	*L*	*C*	*I*	*F*	*K*	*E*	*J*	*D*	*M*	*N*	*O*	*P*	Total
Widespread diploids:																	
S. gallicus	12	56	3	38				6									115
S. glaucus	5	5				118		4			20						152
S. leucanthemifolius	13	10				2		4			1						30
S. rupestris	16	6				6											28
S. squalidus		2 (21)															2
S. vernalis		6				74		6			4						90
Endemic diploids:																	
S. aethnensis	1 (2)	(3)															1
S. chrysanthemifolius		2 (11)															2
S. hesperidium						6											6
S. petraeus					1												1
S. rodriguezii						1											1
Glandular tetraploids:																	
S. nebrodensis												8	3				11
S. viscosus												7					7
S. lividus													10				10
S. sylvaticus													5				5
Others:																	
S. vulgaris	8	5				13	1			3							30
S. flavus subsp. *flavus*														1			1
S. flavus subsp. *breviflorus*								2	5								7
S. aegyptius															3		3
Outgroup																	
S. malacitanus																1	1
Total	55	92	3	38	1	220	1	22	5	3	25	15	18	1	3	1	503

[1] Numbers in parentheses refer to Abbott et al. (1995), based on the following numbers of populations analyzed: *S. squalidus* subsp. *squalidus* (20); *S. aethnensis* (1); *S. chrysanthemifolius* (11).

subsp. *breviflorus* (*K*) or *S. vulgaris* (*I*; not shown in Fig. 3A). The visual assessment of a geographical association among related clade I versus II haplotypes around the Mediterranean basin (Fig. 3B) is also confirmed by a permutational chi-square analysis using GEODIS software (vers. 2.0, Posada et al. 2000), which strongly rejects the null hypothesis of no association (Comes and Abbott, unpubl. data). Barring selection, these findings are suggestive of separate west/central Mediterranean and North African/Near East origins of clade I and II haplotypes, respectively, perhaps reflecting a deep vicariance event or two ancient waves of colonization along the northern and southern coasts of the Mediterranean Sea. A full nested clade analysis (Templeton 1998), often useful for phylogeographic cpDNA studies at the intraspecific level (e.g., Maskas and Cruzan 2000), was considered inappropriate for the present data set because of the pronounced sharing of haplotypes between species.

ITS Sequence Data

Nuclear sequences of the combined ITS regions (ITS-1 and ITS-2) resulted in the alignment of 487 nucleotides, which contained 92 (18.9%) variable and 43 (8.8%) parsimony informative sites, as well as three cladistically informative indels (see below). The G + C content of the two ITS regions averaged 50.3% (26.2% + 24.1%), which is at the lower end of the range of proportions (50–75%) observed for most angiosperm ITS sequences (Jobst et al. 1998; Hershkowitz et al. 1999). Applying maximum-likelihood (ML) under a TrNef + Γ model over the most parsimonious trees (see below), and without a molecular clock enforced, we calculated a transition/transversion ratio of 2.15, with a marked bias towards pyrimidine substitutions (i.e., the pyrimidine/purine transition ratio was 2.89). The ML estimate of the gamma shape parameter (α) was 0.370. Based on parsimony, across the entire data matrix, only seven of 487 sites changed three times and one changed four times. This is suggestive of a lack of saturation of base mutations and a relatively recent common ancestry, which is further supported by the high transition/transversion bias (e.g., Albert et al. 1994).

Average pairwise sequence divergences between accessions (measured as uncorrected *p* distances) ranged from 0% to 11.8%, among which the distances between *S. flavus* subsp. *flavus* and all other accessions were the largest (9.9–11.8%). Excluding this divergent taxon, the average *p* distance was 2.9% (range 0–5.6%). In fact, the ITS sequences of several samples surveyed were found to be the same, including those of different species and multiple intraspecific accessions.

Further examination of the secondary structure of the deviant *S. flavus* subsp. *flavus* sequence revealed that it was substantially more stable than the conformation of its reshuffled sequence ($\Delta G = -162.53$ vs. -145.19 kcal/mol). The average ΔG difference across the nine additional *Senecio* sequences examined was 21.27 ± 6.34 kcal/mol (estimate ± SD), and thus fully encompassed the difference found in *S.*

flavus subsp. *flavus* (17.34 kcal/mol). Based on parsimony, the percentage of C → T transions in this taxon was 18.8% of all substitutions (48 in total), and thus considerably lower than in putative ITS pseudogenes (i.e., ≥40%; Buckler et al. 1997). Meanwhile, five other subsp. *flavus* ITS sequences of Canary Island, Moroccan, and Sinai origin have become available, with PCR products cloned prior to sequencing (M. Coleman, St. Andrews University, unpubl. data). Except for a single substitution, all these sequences proved identical and resulted in a 99.7% base-match when compared to the one reported in the present paper (M. Coleman, pers. comm. 2001). Overall, we conclude that our subsp. *flavus* sequence (no. 5, from Sinai) exhibits little evidence for being a pseudogene or artifact, but rather seems to be representative of the rDNA present in this morphologically rather divergent taxon of probably Southwest African origin (Alexander 1979; Liston and Kadereit 1995).

Maximum-parsimony analysis found two shortest trees of 117 steps (CI = 0.863, RI = 0.929), one of which is shown in Figure 4. In both trees, *S. aegyptius* emerged as sister to the other ingroup species (96% BV, and further supported by a one-base deletion), followed by *S. flavus* subsp. *flavus*, which fell sister to a monophyletic clade (78% BV) of all the remaining accessions. Three major subclades were evident within this latter clade: a largely unresolved group, subclade A (96% BV), of mainly west and central Mediterranean species, but also including *S. glaucus* (nos. 13–15) and *S. flavus* subsp. *breviflorus* from the Near East; and a second group, subclade B (88% BV, one eight-base deletion, one two-base insertion), containing *S. vernalis*, *S. vulgaris*, and *S. rupestris* from Greece (no. 34). The third subclade contained two of the four glandular tetraploids, *S. nebrodensis* and *S. viscosus* (77% BV). Unfortunately, the relationship of the three subclades to each other and to the remaining glandular tetraploids, *S. sylvaticus* and *S. lividus*, remained problematic because of poorly (<50% BV) supported branches (Fig. 4). The second minimum-length tree only differed in placing *S. chrysanthemifolius* as sister to *S. gallicus* from Spain with 66% bootstrap support (data not shown).

In a second phylogenetic analysis we used the neighbor-joining (NJ) algorithm in conjunction with estimates of ML distance relationships under the TrNef + Γ model of evolution (with gamma shape parameter, α, given above). The resulting NJ-ITS tree (Fig. 5) mainly differed from the most parsimonious trees in that all glandular tetraploids formed a single, albeit weakly supported cluster (<50% BV). Based on morphological, karyological and crossing data (Alexander 1979; Kadereit 1984a) the glandular tetraploids have been thought to form a monophyletic group, being reproductively isolated from species presently assigned to ITS subclades A and B. The molecular evidence inferred from their distinct set of cpDNA haplotypes (Table 3), as well as RAPD characteristics (see below; Fig. 6) does not contradict this view. Thus, the NJ-ITS tree in Figure 5 reflects our preferred phylogenetic hypothesis of overall relationships among subclades. Nonetheless, bootstrap analysis of the NJ tree still fails to convincingly resolve the relationships between the ''glandular'' clade and subclades A and B.

ITS Sequence and cpDNA Data

Mapping the distribution of cpDNA haplotypes found within sequenced individuals (and the same or adjacent populations) onto the ITS maximum-parsimony tree (Fig. 4) indicates two major instances in which the ITS and cpDNA data contrast strongly with species taxonomy. *Senecio flavus* subsp. *breviflorus*, as well as a particular *S. rupestris* accession possess ITS sequences and cpDNA haplotypes which otherwise are characteristic of species they co-occur with. Specifically, *S. flavus* subsp. *breviflorus* from Israel (no. 5) shares a very similar ITS sequence and an identical cpDNA haplotype (*F*) with *S. glaucus* from Israel (no. 15). Likewise, the Greek accession of *S. rupestris* (no. 34) has the same ITS sequence and the same cpDNA haplotype (*C*) as *S. vernalis* from Continental Europe (no. 41, of German origin).

RAPD Data

Figure 6 presents a NJ tree based on RAPD mean character differences between all pairwise combinations of 10 selected *Senecio* species (18 accessions, plus *S. malacitanus*), and calculated from 358 polymorphic markers. Overall, the RAPD tree is entirely consistent with intraspecific groupings and largely congruent with suspected species relationships based on morphological characters. The tree based on Jaccard distances (not shown) was identical except for identifying *S. nebrodensis* and *S. viscosus* as sister taxa. An incongruence between Figure 6 and the ITS trees, from both MP- and ML-distance analyses, is that *S. flavus* is sister to subclade A rather than being divided into one lineage within subclade A and another that falls outside the larger clade containing subclades A, B, and the glandular tetraploids. However, the most prominent feature of the RAPD phenogram is that Israeli *S. flavus* subsp. *breviflorus* clusters with subsp. *flavus* from Sinai (100% BV) and not with *S. glaucus* sampled from both Israel and Egypt (compare Figs. 4 and 6).

Rate Constancy and Estimated Divergence Times between ITS Sequences

Relative rate tests based on pairwise comparisons of K2P distances clearly rejected the hypothesis of equal evolutionary rates among ITS sequences of the ingroup taxa (excluding identical sequences) in comparison with a third, more distantly related outgroup taxon (*S. malacitanus*). Of the 253 comparisons made, 64 (25.3%) showed significant rate heterogeneity at the $P = 0.05$ level (z scores $> |1.96|$). Subsequent exclusion of the divergent *S. flavus* subsp. *flavus* still resulted in significant rate heterogeneity (18.2%), mainly due to the deviant behaviour of *S. aegyptius*, *S. sylvaticus*, and *S. lividus*. However, because we were mainly interested in divergence times of two terminal ITS subclades (A and B) that share a larger number of cpDNA haplotypes, we reduced the data set to a representative subset of 15 accessions plus the outgroup (asterisked in Fig. 5), thereby also excluding similar or identical sequences likely resulting from reticulation (i.e., *S. flavus* subsp. *breviflorus*; and *S. rupestris* from Greece; see Discussion). Following this procedure none of the 105 comparisons made showed significant ($P \leq 0.05$) rate heterogeneity.

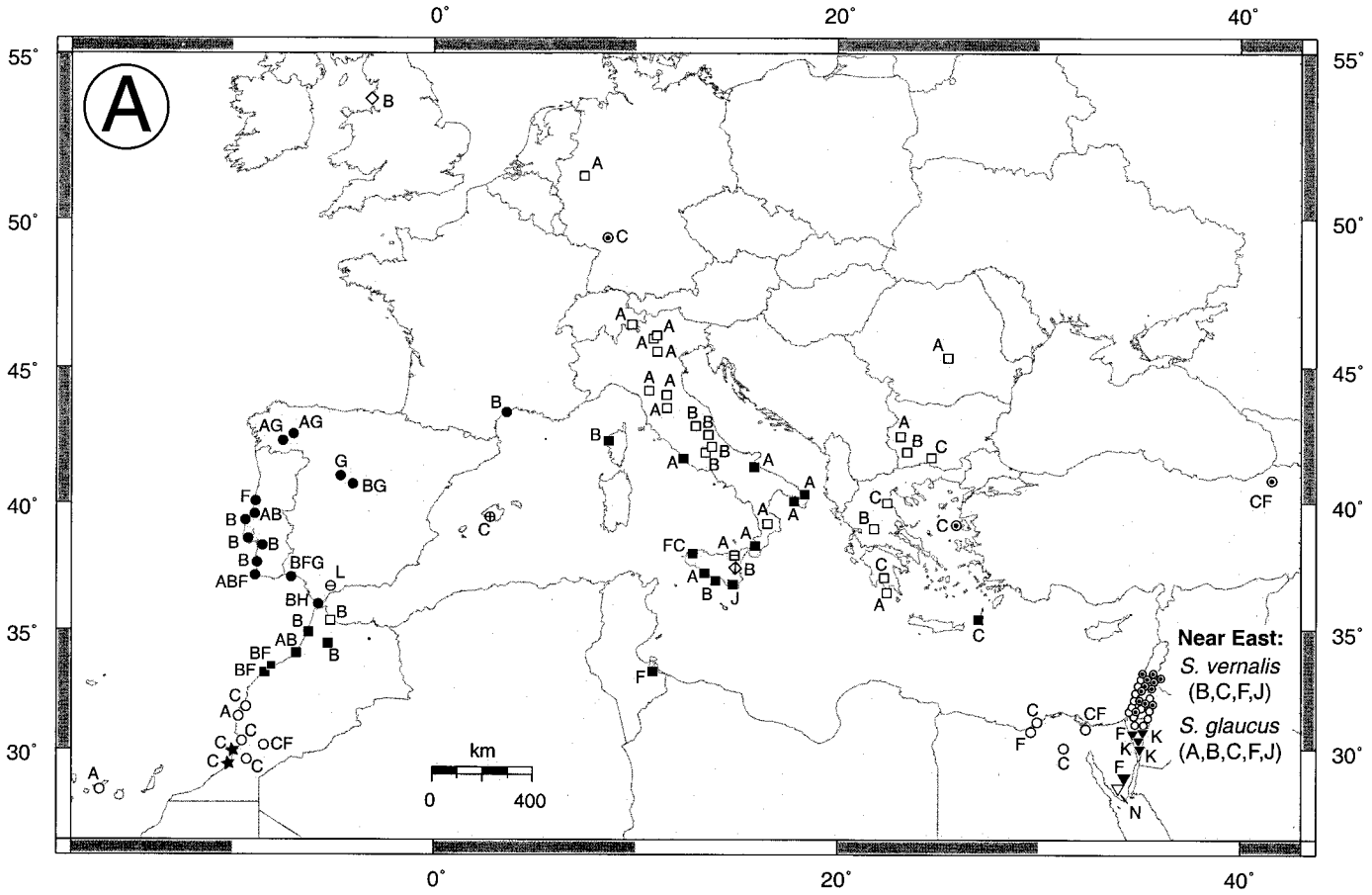

FIG. 3. (A) Geographical distribution of ten cpDNA haplotypes (*A, B, C, F, G, H, K, L, J, N*) found in eleven diploid species of *Senecio*, plus two subspecies (diploid, tetraploid) of *S. flavus*. Localities and taxon symbols are analogous to Figure 1. For *S. vernalis* and *S. glaucus* from the Near East, haplotypes found across populations are given in parentheses. (B) Distributions of clade I and clade II haplotypes according to Figure 2, and haplotypes *J* (star) and *N* (triangle). The size of the pie charts is proportional to the number of individuals analyzed. For *S. vernalis* and *S. glaucus* from the Near East, numbers next to symbols refer to the cumulative number of individuals showing that clade or haplotype.

Given the apparent clock-like evolution of ITS for the pruned subclades A and B, we approximated the date of origin of their stem-lineage (Fig. 5, node c), as well as their own diversification time (Fig. 5, nodes a and b) by using published estimates of ITS divergence rates calibrated for other Asteraceae/Senecioneae, and based on K2P distances. For species of *Robinsonia* from Masatierra and Masafuera (Juan Fernández Islands, Chile), Sang et al. (1995) estimated a relatively fast rate of 1.57% sequence divergence per million years (Myr). There are also two slower estimates for species pairs within *Dendroseris* (Sang et al. 1994), another endemic composite on the Juan Fernández Islands: 0.79% per million years (Myr) based on paleogeological information (assuming radiation shortly after formation of Masatierra), and 1.21% per Myr estimated from cpDNA restriction site mutations (assuming a later radiation). Based on these rates, the average pairwise K2P distance among accessions of subclades A and B, 3.77 ± 0.146% (estimate ±2 SE), corresponds to a date of origin of their stem-lineage (node c) ranging from 2.41 to 4.79 million years ago (MYA). Divergence times corresponding to the approximated 95% confidence interval (CI) for this estimate range from 2.31 to 4.97 MYA. Finally, the average pairwise distances within subclades A (0.69 ± 0.094%) and B (1.68 ± 0.68%) imply divergence times of their ancestral nodes ranging from 0.44 to 0.88 MYA (CI = 0.38–0.99), and 1.07 to 2.13 MYA (CI = 0.63–3.0), respectively.

DISCUSSION

ITS Variation, Times of Species Divergence, and cpDNA Polymorphisms

One of the most conspicuous, though perhaps not unexpected, conclusions to come from this survey is the frequent lack of differentiation among the majority of species of the Mediterranean *Senecio* complex in terms of cpDNA haplotype and, in the case of two species, also for ITS sequence variation. In this respect, the most problematic, and most challenging issues surround the annual and short-lived perennial species of subclade A of the ITS phylogeny (Figs. 4 and 5). This monophyletic clade, which is strongly supported in bootstrap replicates, contains numerous diploid species, mainly from the western and central part of the Mediterranean region, that were not clearly distinguishable in either ITS or

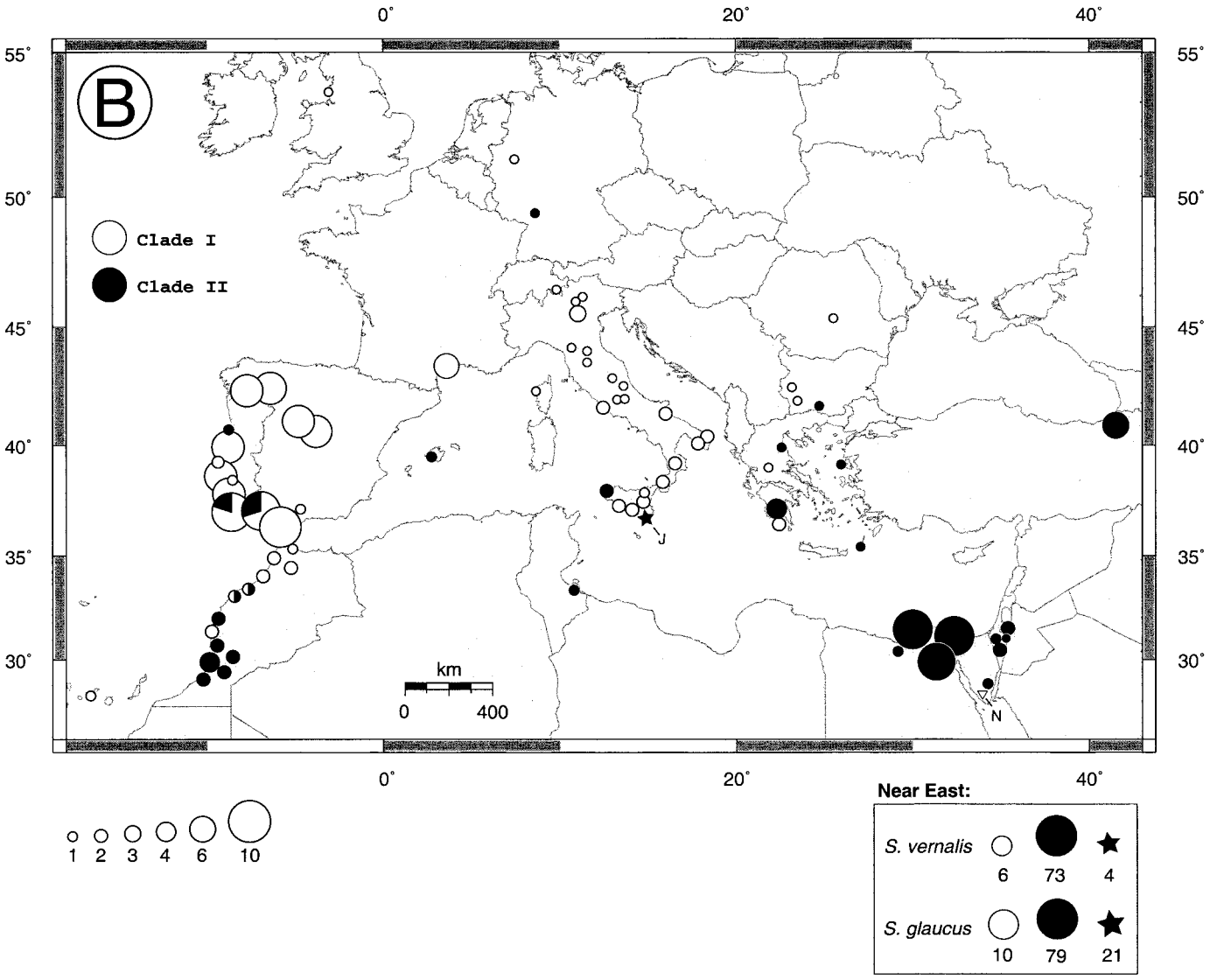

FIG. 3. Continued.

cpDNA. The average pairwise K2P distance within subclade A was very low (0.69 ± 0.094%), suggesting that the ten species involved (i.e., *S. aethnensis*, *S. chrysanthemifolius*, *S. gallicus*, *S. glaucus*, *S. hesperidium*, *S. leucanthemifolius*, *S. petraeus*, *S. rodriguezii*, *S. rupestris*, and *S. squalidus*) form a recently derived complex. In fact, even by considering the most conservative molecular rate calibration available for Asteraceae ITS sequences (0.79% per Myr; Sang et al. 1994), the upper confidence limit of this estimate corresponds to a time of about one million years ago. This suggests that many of these species have been primarily influenced by the dynamic Pleistocene history of the Mediterranean region (Suc 1984; Pons and Quézel 1985; Suc et al. 1995). Overall, we contend that the unresolved ITS phylogeny of subclade A lineages, with short terminal branches, reflects a real historical phenomenon of a near simultaneous and relatively recent diversification.

Regarding the cpDNA haplotype data, many species appear to be indistinguishable genetically by sharing the same range of cpDNA haplotypes (Table 3), and despite considerable taxonomic structure. Maximum parsimony identified two sets of related haplotypes (clades I, II; Fig. 2) and each set appeared to predominate in the west/central Mediterranean and North African/Near East region, respectively (Fig. 3B). Nonetheless, we can conclude that overall levels of intraspecific phylogeographic structure are generally low within the entire complex, and almost none of the haplotypes detected were species-specific or ''diagnostic.'' A notable exception involves *S. gallicus* from southwestern Europe, with a dramatic increase in the frequency of a unique haplotype (*G*) from Iberian coastal to ruderal inland populations (Fig. 3A; see also Comes and Abbott 1998). In addition, *S. petraeus*, known only from a small calcareous mountain range in southern Spain, harbors a unique tip haplotype, *L*, which is a mutational derivative of the ''*gallicus*-inland'' haplotype *G* (Fig. 2). Such clear genealogical relationships among cpDNA haplotypes provide strong evidence that the narrow endemic *S. petraeus* is a daughter species of the more wide-

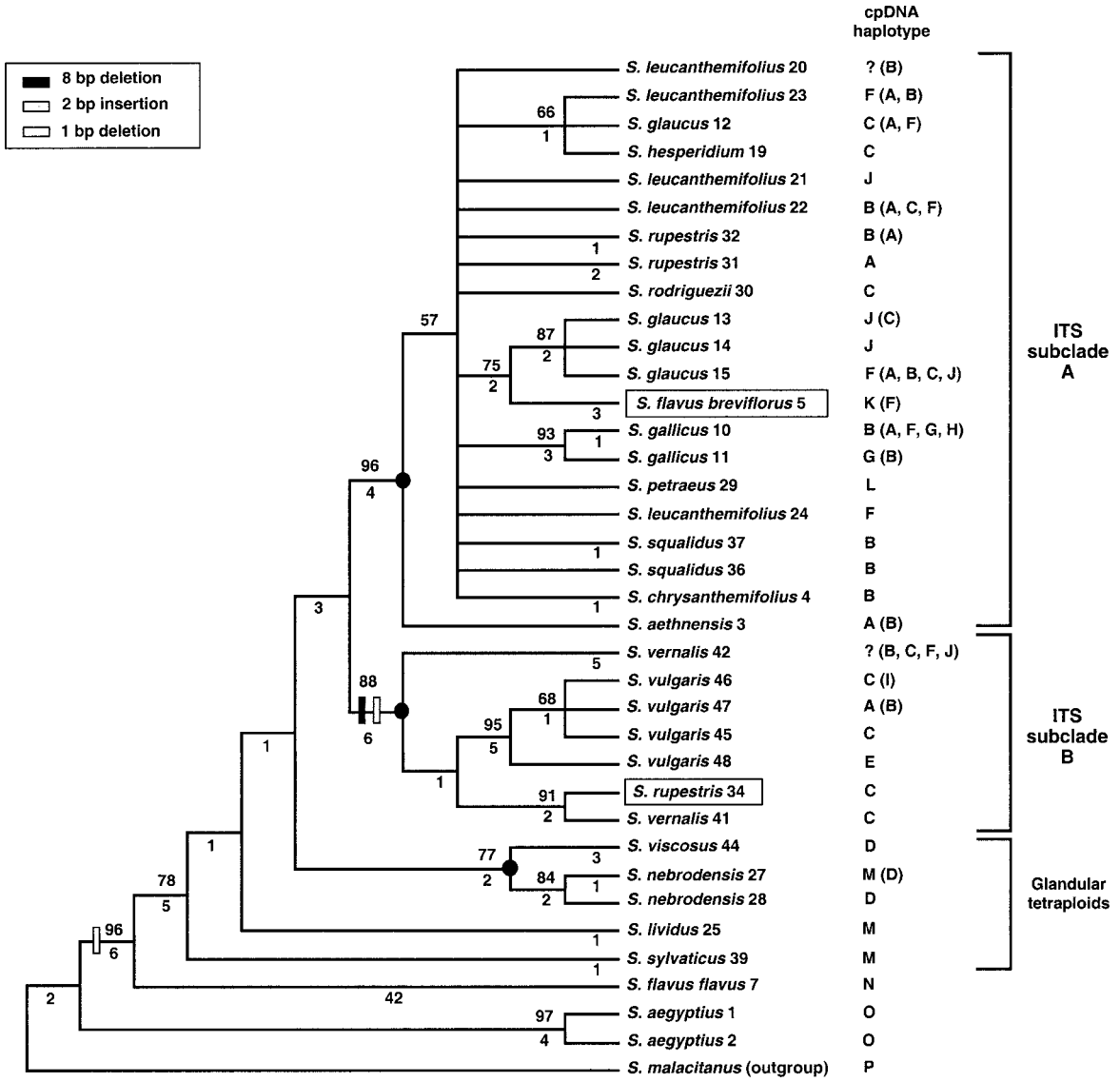

FIG. 4. One of two equally parsimonious trees of 37 accessions from 18 species of the Mediterranean *Senecio* complex obtained under Fitch parsimony using ITS sequence data (ITS-1 and ITS-2, excluding the 5.8S gene). Gaps (indels) were scored as missing data, with three informative indels (listed in the legend) later superimposed onto the cladogram. Numbers below branches refer to number of nucleotide changes along the branch, and numbers above branches indicate bootstrap proportions (>50%) from 100 replicates. Numbers following taxon names refer to populations identified in the Appendix I. Bullets indicate three subclades of *Senecio* accessions referred to in the text. The cpDNA haplotype which occurred within each sequenced individual is listed next to the tree, followed by haplotypes (in parentheses) detected in the same or adjacent populations. Boxes identify two major instances in which both the ITS tree and the cpDNA data contrast strongly with species taxonomy; see text for explanation.

spread *S. gallicus* (despite inconclusive evidence provided by the ITS data; Figs. 4, 5).

The pronounced sharing of cpDNA haplotypes among many species of the Mediterranean *Senecio* complex (and this is most evident for both subclade A *and* B lineages; Fig. 4, Table 3), might be explained by reticulation; or incomplete lineage sorting of chloroplast genomes from a polymorphic ancestor, possibly due to the recency of speciation (Neigel and Avise 1986; Pamilo and Nei 1988). Obviously, these alternative processes are not necessarily mutually exclusive and are notoriously difficult to disentangle. Nonetheless, for each process, we now focus on those cases within the complex, where our molecular data, together with ancillary information, suggest plausible interpretations. We recognize that it will not be possible to diagnose the relative impact of these processes for the entire complex or any single set of species, in part because we frequently have no clear data on their dominant phylogenetic relationships.

Two Major Instances of Reticulation

Our data provide evidence to support reticulation for two species pairs that show striking topological incongruencies in their ITS phylogeny (Figs. 4, 5) relative to taxonomic assignment: *S. flavus*/*S. glaucus* (as a result of past reticulation), and *S. rupestris*/*S. vernalis* (as a result of contemporary reticulation). These will be discussed in turn.

Senecio flavus (subsp. *flavus*) and *S. glaucus* are distributed in close geographic proximity across northern Africa and Sinai, with the former extending far into the Near East and southwestern Asia (Alexander 1979; Coleman et al. 2001). Nothing is known about the likely formation of fertile hybrids between these taxa in the wild or under experimental conditions, but there are known instances of both taxa coexisting at a single site (Tafraoute/Morocco, Dhahab/Sinai; R.J. Abbott, pers. obs.). Until recently, the self-compatible *S. flavus* has been reported to be a diploid like the self-incompatible *S. glaucus* (Alexander 1979), but late in the preparation of this paper, a new chromosome count of $2n = 40$ from *S. flavus* subsp. *breviflorus* from Israel became available (Coleman et al. 2001), warranting a new species description. In the light of these new findings, the most likely explanation of the presence of ''*glaucus*-like'' ITS sequences and cpDNA haplotypes (*F, K*) in *S. flavus* subsp. *breviflorus* is an allopolyploid origin of this taxon via hybridization between *S. flavus* subsp. *flavus* and *S. glaucus*. According to this, the latter species served as the maternal ''donor'' (thus the *S. glaucus*-like cpDNA) and interlocus concerted evolution of nrDNA loci occurred towards the *S. glaucus*-like ITS sequence (see also Wendel et al. 1995). The greatest problem concerning this model is that it does not account for the considerable morphological similarity of *S. flavus* subsp. *breviflorus* to subsp. *flavus* (Kadereit 1984b; Coleman et al. 2001). Neither does it account for our RAPD data which suggest that, apart from ITS, the nuclear genome of subspecies *breviflorus* is predominantly ''*flavus*-like'' (Fig. 6). This might lead one to favor an alternative scenario in which introgression of only a small part of the *S. glaucus* genome into *S. flavus* subsp. *breviflorus* occurred, independently of (before or after) autotetraploidization, and with only the ''*glaucus*-like'' ITS sequences persisting through subsequent backcrossing with *S. flavus* subsp. *flavus*.

Obviously, these two models are hard to distinguish but in either case reticulation is involved and thus could have contributed to the capacity of *S. flavus* to expand its range. Specifically, the ecogeographical differentiation of *S. flavus* subsp. *breviflorus*, as compared with the mainly North African subsp. *flavus*, has evidently occurred while colonizing much of the Near East and the entire Arabian Peninsula (see distribution map in Coleman et al. 2001). This raises the intriguing possibility that range expansion of *S. flavus* may have been triggered or facilitated through reticulation involving *S. glaucus*. Our view that this reticulation is ancient is supported by the observation that the majority of individuals of *S. flavus* subsp. *breviflorus* analyzed contain a chloroplast genome (*K*) that differs by one additional length mutation from the one originally captured from *S. glaucus* (see haplotype *F* in Table 3; and Fig. 2).

Based on a similar gene tree-organism tree discordance, we feel that a reasonable case can be made for contemporary hybridization and introgression involving two diploid and self-incompatible species belonging to ITS subclades A and B, respectively: *S. rupestris* and *S. vernalis*. Their assignment to these different lineages is strongly supported by a recent allozyme survey across eight species of subclade A and *S. vernalis* (81 populations; Comes and Abbott, unpubl. data), indicating that *S. rupestris* is firmly nested within the former group rather than being closely related to *S. vernalis*. The two species are also clearly distinguished by a number of morphological and life-history traits (e.g., *S. rupestris* is a short-lived perennial, whereas *S. vernalis* is a winter annual). Although these species have different ecogeographical requirements (*S. rupestris* occupies montane habitats above 1500 m, whereas *S. vernalis* is mainly restricted to lower altitudes; Kadereit 1991; Comes and Abbott, pers. obs.), they are often found in close geographic proximity throughout Greece, the Balkans, and the southeastern Carpathians (Meusel and Jäger 1992). In addition, hybridization studies indicate that reproductive isolating barriers have not fully evolved (King 1994).

Both our cpDNA and ITS data indicate that one accession of *S. rupestris* sampled from Mistras/Greece (no. 34) is indistinguishable from *S. vernalis* of German origin (no. 41), thereby differing from other ''conspecific'' ITS sequences and cpDNA haplotypes of *S. rupestris* (Fig. 4). Previous morphometric analyses (King 1994) have indicated that in overall phenotype material from the Mistras population not only overlaps that of German *S. vernalis* but also that of *S. rupestris* from Mt. Olympos (Greece), that is, what we consider genuine *S. rupestris* on the basis of allozyme evidence (Comes and Abbott, unpubl. data). Thus, an error in taxonomy is an unlikely explanation for the observed non-monophyly of *S. rupestris*. Instead, the most probable evolutionary process accounting for this fact is transfer (or capture) of both cpDNA *and* ITS molecules mediated by introgressive hybridization, with *S. vernalis* acting as the maternal or ''donor'' parent. Notably, and similar to the case of *S. flavus*/*S. glaucus*, homogenization among ITS repeats must have occurred in the direction of the maternal type.

Incomplete Lineage Sorting of cpDNA Haplotypes

To attribute the sharing of cpDNA haplotypes among *Senecio* taxa to incomplete lineage sorting rather than reticulation requires the maintenance of ancestral cpDNA polymorphisms through one or more speciation events (Neigel and Avise 1986; Pamilo and Nei 1988; Wendel and Doyle 1998). In particular for several widespread diploid species assigned to ITS subclade A (e.g., *S. gallicus*, *S. glaucus*, *S. leucanthemifolius*), with moderate to high levels of intraspecific polymorphism and several shared haplotypes (Table 3), this phenomenon is probable. Populations of these annual and mostly ''weedy'' species are primarily large, outbred, and possess pappus bearing fruits that facilitate long-distance dispersal of cpDNA (the inheritance of which is strictly maternal; Harris and Ingram 1992a). Such population characteristics may counterbalance the generally small effective population sizes of organellar genomes (Pamilo and Nei 1988; Hudson 1990; Maddison 1995), and hence their faster fixation or loss (i.e., faster coalescent times) compared to nuclear genes. In addition, coalescent times for cpDNA may exceed those for nuclear genes under some special scenarios invoking natural selection. Especially, as *Senecio* species have adapted to a diversity of new ecological situations, one may hypothesize that directional selection on the nuclear genome has promoted frequent selective sweeps and thereby shortened the coalescent time for many genes and adjacent

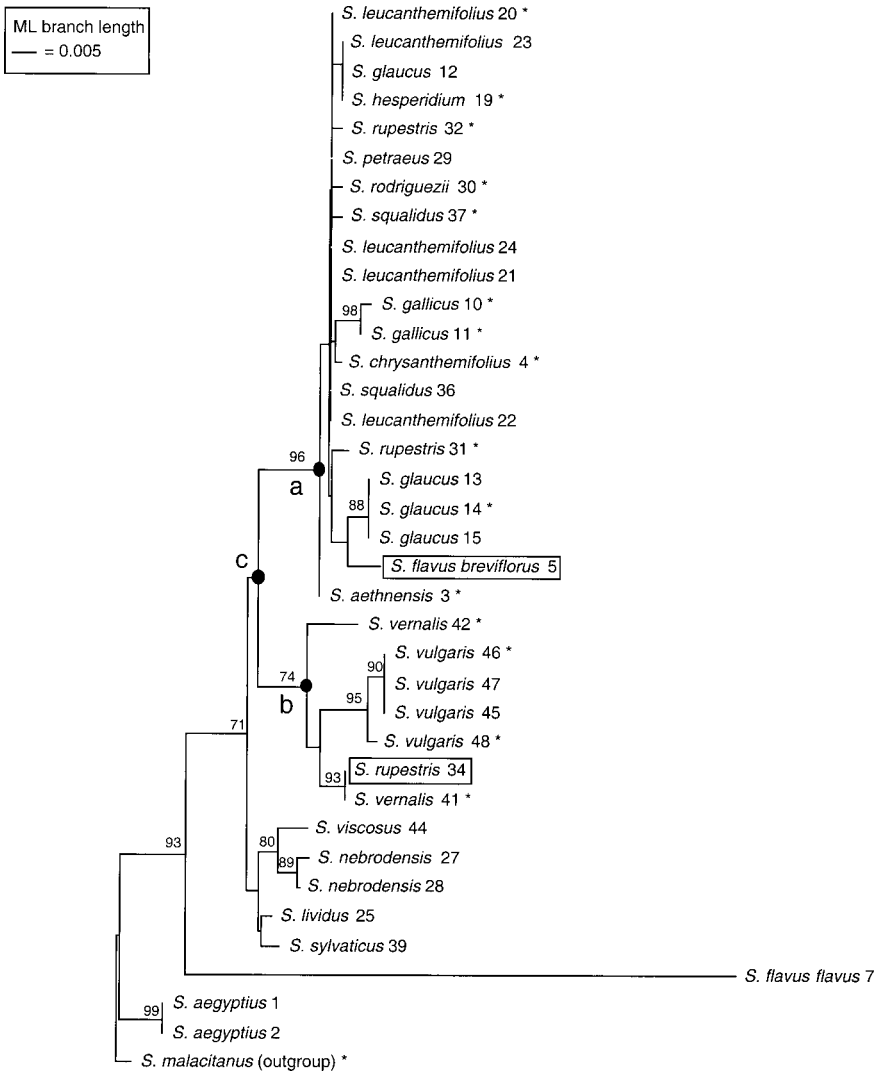

FIG. 5. Neighbor-joining tree of the relationships among *Senecio* accessions based on maximum-likelihood distances (under the TrNef + Γ model) calculated from combined ITS-1 and ITS-2 sequences. The maximum-likelihood estimate of the gamma shape parameter, α (=0.370) was calculated using the topology of the maximum-parsimony tree given in Figure 4. Branch lengths are in units of expected substitutions. Numbers above branches are percent support (when >50%) from bootstrap analysis based on 10,000 replicates. Accession numbers refer to populations given in Appendix I. Bullets and letters (a–c) indicate relevant branching events (nodes) discussed in the text. Accessions marked with an asterisk were chosen for a relative rate test.

linked sequences. In such a situation, trans-specific sharing of cpDNA haplotypes may be ascribed to incomplete lineage sorting, even in the presence of nuclear monophyly.

Perhaps the best example to illustrate this situation concerns *S. gallicus* from southwestern Europe. This species harbours three cpDNA haplotypes, *A, B* and *F*, that are shared in their entirety by two other annual and diploid species of subclade A that occur in northern Africa, that is, *S. glaucus* and *S. leucanthemifolius* (Table 3). However, apart from high interfertility with its putative derivative *S. petraeus* (see above), *S. gallicus* is a distinct species on the basis of both breeding experiments and morphological characters (cf. Alexander 1979). In addition, and unlike most other species of subclade A, there is clear evidence from nuclear allozyme data for the genetic cohesiveness of populations of *S. gallicus* from Iberia and southern France, and its likely sister group relationship to most (if not all) of the remaining members of this subclade (Linkohr 1998; Comes and Abbott, unpubl. data). There is no evidence of current sympatry or interbreeding with other diploid *Senecio* species that could be acting as sources of haplotypes *A, B,* and *F,* nor any evidence of introgression from the tetraploid *S. vulgaris*, Iberian populations which also possess haplotypes *A* and *B* (Table 3; see also Comes et al. 1997). It can not be ruled out, of course, that ancient reticulation has occurred in the past, maybe associated with latitudinal distributional changes across the Strait of Gibraltar associated with Pleistocene climatic changes, as the same haplotypes are also found in *S. glaucus* and *S. leucanthemifolius* from Morocco. Notwithstanding this potential complication, we feel that incomplete lineage sorting is a viable hypothesis for the presence of multiple, shared haplotypes in *S. gallicus*.

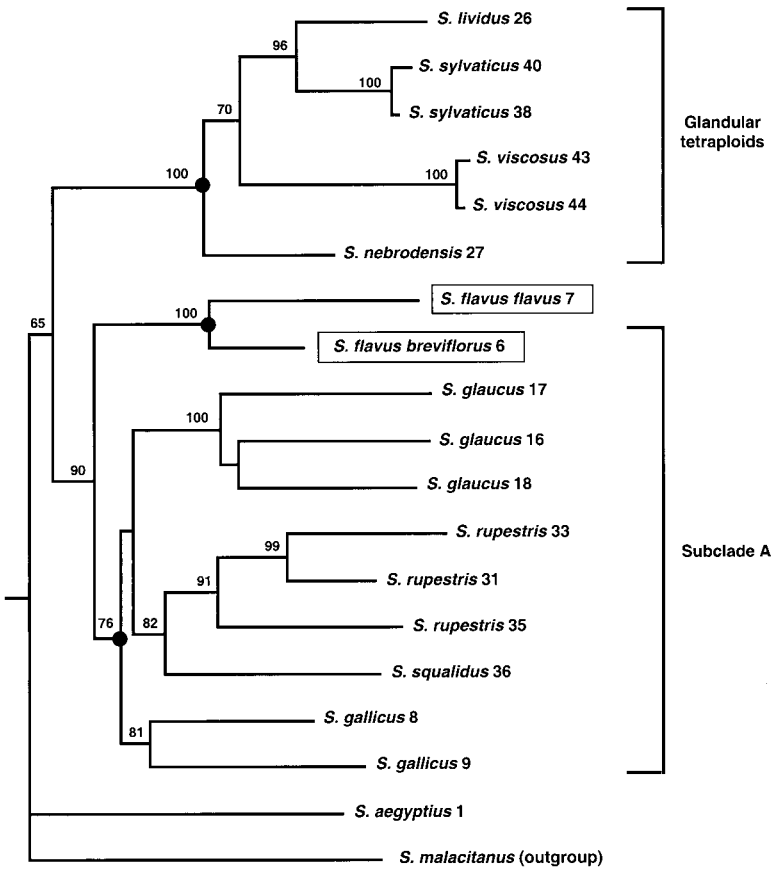

FIG. 6. Neighbor-joining tree of 18 accessions from 10 selected species of the Mediterranean *Senecio* complex based on mean character differences for 362 RAPD products obtained with 17 primers. Bootstrap values (>50%) based on 1000 replicates are shown above branches. Accession numbers refer to populations given in Appendix I. Boxes and/or bullets indicate groups of accessions referred to in the text.

It is tempting to utilize the inferred ITS phylogeny to infer the minimum coalescent times for cpDNA haplotypes shared between *S. gallicus* and its close relatives, that is, the minimum time for which these cpDNA polymorphisms would have to have survived multiple speciation events. This complementary approach, however, must be viewed with some caution. First, our average estimates of the time at which subclade A (and B) began to diverge based on ITS sequences have large standard errors (Hillis et al. 1996). Second, the ITS coalescent for these subclades, and hence their age, must be older than the time of divergence of the containing ''species'' lineages [to an extent determined by the effective population size and preexisting ITS lineage structure in the ancestral population(s)]. Finally, the potential effects of reticulation and concerted evolution on these ITS divergences and associated time measures are not known due to the lack of phylogenetic signal in those data. Nonetheless, if we assume for the moment that most subclade A species last shared a common ancestor more than 440,000 years ago (time estimate from above, conservatively using the fast ITS divergence rate, and assuming low levels of confounding effects), this would provide a first *minimum* estimate for the time scale over which cpDNA haplotypes may have survived. Given the likelihood of reciprocal monophyly after $4N_e$ generations following speciation (Neigel and Avise 1986), this proposition also requires the maintenance of *minimum* and long-term effective population sizes (N_e) of *Senecio* organellar genomes numbering in the tens of thousands (assuming a generation time of one or two years). However, for population demographic reasons discussed above (i.e., large effective population sizes, outbreeding, and a high seed dispersal rate), this possibility may not be discounted completely. Contrastingly, if we assume that subclade A and B species diverged more than 2.31 million years ago (time estimate from above, and using the fast ITS divergence rate), incomplete lineage sorting becomes an almost untenable hypothesis, particularly if one considers that N_e for cpDNA in these diploid *Senecio* species (as in other hermaphrodite plants) is about one-half of that for nuclear genes viz individuals. As a consequence, contemporary cytoplasmic introgression may need to be invoked in those cases where diploid subclade A and B species share numerous cpDNA haplotypes in areas of sympatry or secondary contact (e.g., *S. glaucus*/*S. vernalis* in the Near East; Comes and Abbott 1999).

The data presented in this paper do not allow us to further illuminate the much disputed origin of the cosmopolitan tetraploid *S. vulgaris*, or to diagnose the evolutionary processes responsible for its partial or complete sharing of cpDNA haplotypes with both *S. vernalis* (subclade B) and several species of subclade A (Fig. 4, Table 3). The ITS trees appear

to favor a hypothesized autopolyploid origin of *S. vulgaris* from *S. vernalis* (Kadereit 1984a) by firmly placing the two in the same subclade (Figs. 4, 5). However, given the possibility of homogenization of homoeologous nrDNA loci towards a single parental type in putative allopolyploids (Wendel al. 1995), we can not rule out that *S. vulgaris* arose following hybridization between *S. vernalis* and an unknown species of subclade A (see also Ashton and Abbott 1992a; Harris and Ingram 1992a).

Conclusions

According to Wendel and Doyle (1998), there are a plethora of evolutionary processes operating at the organismal as well as molecular levels that are likely to confound phylogenetic inference, including rapid diversification, gene tree-species tree discordance, reticulation, interlocus concerted evolution, and incomplete lineage sorting. The Mediterranean *Senecio* complex illustrates all these phenomena and demonstrates that an integration of phylogenetic and population-level studies is critical for understanding the evolutionary history of any single species under study. It should be noted that our combined cpDNA and ITS analysis focused on prominent instances of phylogenetic incongruence. However, more subtle modes of reticulation may have been missed due to a lack of molecular resolution.

As more datasets become available that uncover considerable intraspecific cpDNA variation in closely related angiosperms, the number of studies reporting the sharing of cpDNA polymorphisms across species boundaries increases as well (Asteraceae examples include Mason-Gamer 1995, 1999; Bain and Jansen 1997, 2000; this study). Our study suggests that (1) introgression need not be invoked to explain the sharing of cpDNA haplotypes between *S. gallicus* and its close relatives; rather, these chloroplast genomes may show evidence of incomplete lineage sorting even when the nuclear genome of the species does not; and (2) ancestral haplotypes may have been maintained to the present for at least 0.4 or even 1.0 million years, depending on molecular clock calibrations. Although these estimates have large variances, and are based on various assumptions, they nonetheless suggest that incomplete sorting of cpDNA lineages within species may be a more serious confounding effect in interspecific phylogenetic inferences than previously thought. This, in a general context, could apply to many recently evolved plant groups exhibiting population demographic characteristics similar to the ones described above for *Senecio*. However, to ascertain under which circumstances coalescent times for cpDNA lineages are typically too long relative to internodal times between speciations, more empirical studies are needed in conjunction with computer simulations. As a case in point, Hanson et al. (1996) simulated the retention of ancestral nuclear (*c1*) alleles shared among closely related (sub)species of maize by utilizing mutation rates and independently derived divergence times. An analogous approach focusing on cpDNA polymorphism remains a subject for further investigation.

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APPENDIX I

Collection and cpDNA haplotype information for *Senecio* individuals included in the ITS sequence and/or RAPD analysis. Specimens used for the latter analysis are marked by an asterisk. GenBank Accession numbers refer to the entire ITS1-5.8S-ITS2 region.

Senecio taxon and locality	cpDNA haplotype	ITS GenBank Accession no.	Collector/source[1]
S. aegyptius subsp. *aegyptius*			
* 1. unknown origin	*O*	AJ400777	?
S. aegyptius subsp. *thebanus*			
2. Egypt: Delta area, Damanhûr	*O*	AJ400778	RJA, HPC
S. aethnensis			
3. Italy: Sicily, Mt. Etna	*A*	AJ400779	JKJ
S. chrysanthemifolius			
4. Italy: Sicily, Mt. Etna	*B*	AJ400780	JKJ
S. flavus subsp. *breviflorus*			
5. Israel: Paran, 5 km W of Be'er Menuha, Rd. 13	*K*	AJ400781	RJA, HPC
* 6. Egypt: Sinai, Djebel Abbas	*F*		RJA
S. flavus subsp. *flavus*			
* 7. Egypt: Sinai, rd. from Sharm El-Sheikh to Dhahab	*N*	AJ400782	RJA
S. gallicus			
* 8. France: Hérault, Pioch Noir/Vic-La-Gardiole	*B*		JDT, CT
* 9. Portugal: Estremadura, Nazaré	*A*		RBG-E
10. Spain: Andalucia, c. 5 km SW of La Rabida	*B*	AJ400783	RJA, HPC
11. Spain: Castilla-León, Montuenga, Rd. N605	*G*	AJ400784	RJA, HPC
S. glaucus			
12. Morocco: Khmes ait Ouafka, nr. Tiffermit	*C*	AJ400785	RJA, AA
13. Israel: Judean Coast, Ashquelon	*J*	AJ400786	RJA, HPC
14. Israel: Judean Hills, Jerusalem	*J*	AJ400787	HPC
15. Israel: Negev Hills, below Mizpe Ramon	*F*	AJ400788	RJA, HPC
* 16. Israel: S Sharon Coast, Nof Yam	*J*		RJA, HPC
* 17. Israel: Coastal Plain, Tel Aviv	*J*		HPC
* 18. Egypt: Cairo	*C*		RJA
S. hesperidium			
19. Morocco: mouth River Massa, nr. Sidi Rbat	*C*	AJ400789	RJA, AA
S. leucanthemifolius			
20. France: Corsica, Calvi	?	AJ400790	RIM
21. Italy: Sicily, nr. Pozzallo[2]	*J*	AJ400791	RJA, HPC
22. Italy: Sicily, ca. 8 km E of Licata[2]	*B*	AJ400792	RJA, HPC
23. Morocco: Port de Jorf-Lasfar, nr. El-Jadida	*F*	AJ400793	RJA, AA
24. Tunesia: Medenine[3]	*F*	AJ400794	KK
S. lividus			
25. Spain: Galicia, Puente de Domingo Florez	*M*	AJ400795	RJA, HPC
* 26. unknown origin	*M*		BG-L
S. nebrodensis			
* 27. Spain: Cord. Cantabrica, Pto. de Leitariegos	*M*	AJ400796	JWK
28. Spain: Sa. Nevada, rd. from Capileira to Mt. Mulhacen	*D*	AJ400797	JWK
S. petraeus			
29. Spain: Andalucia, Sa. de las Nieves, nr. Ronda	*L*	AJ400798	ST, PGG
S. rodriguezii			
30. Spain: Majorca, Formentor	*C*	AJ400799	RJG
S. rupestris			
* 31. Germany: Bleiwäsche, nr. Bochum	*A*	AJ400800	FMK
32. Italy: Abruzzi, Sulmona	*B*	AJ400801	RIM
* 33. Italy: Trentino, Mt. Baldo	*B*		RJA, HPC
34. Greece: Peloponnisos, Mistras, nr. Sparta	*C*	AJ400802	AJR
* 35. Greece: Mt. Olympos	*C*		RIM
S. squalidus subsp. *squalidus*			
* 36. British Isles: England, Ainsdale	*B*	AJ400803	PAA
S. squalidus subsp. *araneosus*			
37. Morocco: Rif, Djebel Tazaote	*B*	AJ400804	PGG
S. sylvaticus			
* 38. France: Brittany, Merriene, nr. Quimper	*M*		RJA
39. Spain: Andalucia, E of Torre del Oro, Rd. N442	*M*	AJ400805	RJA, HPC
* 40. Spain: Andalucia, Huelva	*M*		PGG
S. vernalis			
41. Germany: Eppelheim, nr. Heidelberg	*C*	AJ400806	JWK
42. Israel: Golan Heights, nr. Zomet El Rom	?	AJ400807	RJA, HPC
S. viscosus			
* 43. British Isles: England, Wigan	*D*		PAA
* 44. Romania: S Carpathians, Sinaia	*D*	AJ400808	RIM

APPENDIX I. Continued.

Senecio taxon and locality	cpDNA haplotype	ITS GenBank Accession no.	Collector/source[1]
S. vulgaris var. *vulgaris*			
45. Spain: Andalucia, Matalascañas	*C*	AJ400809	RJA
46. Israel: Upper Jordan Valley, Bet Shean	*C*	AJ400810	RJA, HPC
S. vulgaris sp. *denticulatus*			
47. British Isles: Channel Islands, Jersey, Les Quennevais	*A*	AJ400811	HPC
48. Italy: Sicily, Monti Nebrodi, ca. 8 km after Cesaro	*E*	AJ400812	JWK
Outgroup			
S. malacitanus			
* 49. Morocco	*P*	AJ400813	PGG

[1] Collectors/sources: R. J. Abbott, A. Abbott, P. A. Ashton, H. P. Comes, P. G. Gibbs, J. K. James, K. Kubitzki, J. W. Kadereit, F. Martin-Kemper, R. I. Milne, A. J. Richards, S. Talavera, C. Thébaud, J. D. Thompson; Botanic Garden Liége, Royal Botanic Garden Edinburgh. Voucher specimens and seed samples of collections are stored at the St. Andrews (STA) herbarium.

[2] Populations from Pozzallo and Licata (Sicily), here designated as *S. leucanthemifolius*, are likely to harbor intermediates between the latter species and *S. glaucus* based on a preliminary allozyme survey (Comes and Abbott, unpubl. data).

[3] Material from Medenine (Tunisia) bears close resemblance to the Pozzallo/Licata material in the field (Abbott and Coleman, pers. obs.) and, therefore, was treated taxonomically the same.

APPENDIX II

States of 27 polymorphisms among 16 cpDNA haplotypes identified in the *Senecio* species studied. Character states are indicated as ancestral (0) or derived (1), based on comparison with *S. malacitanus* (haplotype *P*). Description of character states and polymorphisms are given in Table 2.

	Polymorphism number																										
cpDNA haplotype	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
A	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0
B	0	1	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0
C	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0	0
D	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
E	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	?	?	?	?	?	?	?
F	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0	0
G	0	1	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	?	?	?	?	1	0	1	0	0
H	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	0	0	?	?	?	?	1	0	1	0	0
I	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	?	?	1	0	1	0	0
J	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
K	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	?	0	0	?	1	0	1	0	0
L	0	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	?	?	1	0	1	0	0
M	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
N	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	0	0
O	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	1
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0