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Molecular systematics of *Solanum* section *Lycopersicum* (*Lycopersicon*) using the nuclear ITS rDNA region

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Abstract The phylogenetic relationships of all nine known tomato species of *Solanum* section *Lycopersicum*, together with other Solanum sections and species from several related genera, were investigated using parsimony analysis of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA). Most parsimonious reconstructions divided the section *Lycopersicum* into three clades, reflecting their mating behaviour and fruit colour. Data from sequencing studies were congruent with those from morphological and other molecular investigations, and provided detailed information concerning species relationships.

Keywords *Lycopersicon* · *Solanum* · ITS · rDNA · Phylogeny · Cladistic analysis

Introduction

Lycopersicon (tomato) was considered to be a small genus within the plant family Solanaceae. The genus as a whole displayed less morphological variation than many genera within the Solanaceae. In fact, it was only distinguished initially from the genus *Solanum* by the pollen-shedding characteristics of its anthers. Therefore, several authors have argued that, logically, it was inconsistent to maintain *Lycopersicon* at generic rank, whilst at the same time not dividing *Solanum* into several separate genera (D'Arcy 1991; Olmstead and Palmer 1992). The chloroplast restriction fragment length polymorphism (RFLP) study of Spooner et al. (1993), demonstrated that if the genus *Lycopersicon* was recognised it would be a sister to a paraphyletic *Solanum* or result in several other genera being recognised. Consequently, Spooner et al. (1993) transferred *Lycopersicon* into *Solanum* and created *Solanum* section *Lycopersicum*. The same authors also provided new combinations for many of the *Lycopersicum* species (Table 1). However, due to the number of historical references cited within this investigation, for the purpose of clarity the original *Lycopersicon* names are used in the following sections of the present paper.

Although *Lycopersicon* was a small genus compared to many others in *Solanum* (*e.g.* *Solanum* section *Geminata* with 82 species), it contains important horticultural crop species which are cultivated world-wide. The economic importance of these species has stimulated considerable scientific effort to characterise their genome organisation and evolutionary relationships (Bonierbale et al. 1988). Several species of *Lycopersicon* have been the subject of extensive hybridisation studies, both sexual and somatic, attempting to introduce agronomically useful traits and increased genetic diversity into cultivated species such as *Lycopersicon esculentum* (Rick 1978; Kinsara et al. 1986; San et al. 1990; Wijbrandi et al. 1990; Sakata et al. 1991; Brüggemann et al. 1995; Chen and Adachi 1998). Members of *Lycopersicon* are easily transformed and several species are used routinely as targets in plant genetic-manipulation experiments. *L. esculentum* is well characterised as a result, with detailed chromosome linkage maps based on both morphological (Sherman and Stack 1992) and molecular markers (Tanksley and Mutschler 1990; Tanksley et al. 1992). Since species of *Lycopersicon* have become important models for research into genome structure and function

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(Prince et al. 1993; Parokonny et al. 1997), a clear understanding of the evolutionary relationships between species will maximise the exploitation of these data. In addition, a comprehensive phylogeny will facilitate the development of conventional breeding strategies and, consequently, the management of wild genetic resources.

In an early taxonomic study of the genus, Miller (1768) included seven species in the genus *Lycopersicon*, three of which are now considered to be true Solanum species and one is an indistinguishable form of the common *L. esculentum*. Dunal (1813) credited six species to the genus, of which three have been reduced to synomy or subspecific rank with no new species being added to Miller's list. It was not until the work of Muller (1940) that further studies on the genus were carried out. He described six species which he divided into two subgenera based on fruit colour. Eulycopersicon contained the red-fruited species while Eroipersicon contained the green-fruited species. Many specimens of a given species in the genus *Lycopersicon* are morphologically dissimilar, resulting in the description of so-called indistinct species (Muller 1940; Luckwill 1943; Rick 1963).

Later intrageneric classifications divided the genus into two groups based on their breeding compatibility relationships (Rick 1979; Taylor 1986). The first was termed the "esculentum" group and contained the seven *Lycopersicon* species which can be crossed easily with cultivated tomato (*L. esculentum*), namely *Lycopersicon pimpinellifolium*, *Lycopersicon cheesmaniae*, *Lycopersicon chmielewskii*, *Lycopersicon parviflorum*, *Lycopersicon hirsutum* and *Lycopersicon pennellii*. The second "peruvianum" group is comprised of two species, namely *Lycopersicon chilense* and *Lycopersicon peruvianum*. Within these two groups, most sexual crosses are achieved without difficulty, although they are often possible only in one direction. Attempts at hybridisation amongst members of different groups often fail due to embryo degeneration (Lefrançois et al. 1993).

A subsequent study by Rick (1987) divided the nine accepted *Lycopersicon* species into four categories on the grounds of interspecific breeding barriers, these being: (1) autogamous (*L. esculentum*, *L. cheesmaniae*, *L. parviflorum*), (2) facultative (self compatible, SC; *L. pimpinellifolium*, *L. chmielewskii*), (3) facultative (SC and self incompatible, SI; *L. hirsutum*, *L. pennellii*, *L. peruvianum*), and (4) allogamous (SI; *L. chilense*).

Several investigations have been performed employing morphological and molecular assessments of the phylogenetic relationships within *Lycopersicon* (Rick and Fobes 1975; Palmer and Zamir 1982; McClean and Hanson 1987; Miller and Tanksley 1990; Bretó et al. 1993). Restriction-enzyme fragment analysis on chloroplast DNA (cpDNA) of seven species of *Lycopersicon* and three closely related species of *Solanum* (Palmer and Zamir 1982) indicated that *L. pimpinellifolium*, *L. esculentum* and *L. cheesmaniae* were closely related, with fruit colour providing an additional synapomorphy supporting the relationships. In contrast, work by McClean and Hanson (1987), using tomato mitochondrial DNA restriction fragment hybridisation, refuted these conclusions. In their later study, *L. hirsutum* was considered to be the closest relative of *L. esculentum*. Chloroplast restriction enzyme-fragment studies by Miller and Tanksley (1990), and allozyme electrophoresis data of Bretó et al. (1993), supported the conclusions of Palmer and Zamir (1982), grouping *L. esculentum* with *L. pimpinellifolium* and *L. cheesmaniae*, suggesting that both fruit colour and compatibility reactions are both true synapomorphies.

Studies have demonstrated that the genus *Solanum* is most-closely related to *Lycopersicon* (Rick 1979; Rick et al. 1986; Olmstead and Palmer 1992; Tanksley et al. 1992; Prince et al. 1993; Olmstead and Palmer 1997); four species of *Solanum* (*Solanum juglandifolium* Dunal, *Solanum lycopersicoides* Dunal, *Solanum ocranthum* Dunal and *Solanum rickii* Correll) display a close resemblance in chromosome number and morphology to species of *Lycopersicon* (Rick 1979; Rick et al. 1986). So marked are these similarities, that only comparatively recently was *Solanum pennellii* Correll transferred to *Lycopersicon* (Palmer and Zamir 1982). The ability of *S. lycopersicoides* to hybridise with *L. esculentum*, *L. cheesmaniae*, *L. pimpinellifolium* and *L. pennellii* led Rick (1979) and Rick et al. (1986) to propose *S. lycopersicoides* as being the species most-closely related to *Lycopersicon*.

This paper presents a detailed cladistic analysis of ITS nuclear rDNA sequences from all recognised species in *Lycopersicon* (=section *Lycopersicum sensu* Spooner et al. 1993), aimed to clarify relationships amongst the taxa.

Materials and methods

Plant material

Details of the material studied are given in Table 1. Vouchers are lodged in the Herbarium, Royal Botanic Gardens, Kew, Richmond, UK. Plants were cultivated in the glasshouse at the University of Nottingham under natural daylight, supplemented with a 16-h photoperiod provided by "Daylight" fluorescent illumination (180 µm m^{-2} s^{-1}).

DNA sequencing and parsimony analysis

DNA was extracted according to the CTAB procedure of Doyle and Doyle (1987). The ITS rDNA region was amplified in a thermal cycler (Perkin Elmer) using primers and a cycling protocol described by Baldwin (1992). The following primer sequences were used for the analysis (5′ to 3′) ITS4=TCCTCCGCTTATTGATATGC and (5′ to 3′) ITS5=GGAAGTAAAAGTCGTAACAAGG. DNA of the complete ITS region in each genomic DNA was amplified directly using the primers ITS5 and ITS4. These 100-µl reactions contained 72.5 µl of sterile water, 10 µl of 10× *Taq* polymerase reaction buffer (Boehringer Mannheim, Sussex, UK), 2 µl of 1 mM dNTPs in an equimolar ratio (Pharmacia Biotechnology, Buckinghamshire, UK), 0.5 µl of *Taq* DNA polymerase (5 $U.\mu l^{-1}$) (Boehringer Mannheim, Sussex, UK), 12 µl of 25 mM magnesium chloride (Boehringer Mannheim, Sussex, UK), 1 µl of 0.4% (w/v) bovine serum albumin, 1 µl of 100 $ng\mu l^{-1}$ primers ITS4 and ITS5, and 100 ng of genomic DNA. Reaction

Table 1 Plant material used in this analysis

Taxon name	Genbank number	Collection location	Taxon name *sensu* Spooner et al. (1993)	Voucher[a] no.
Cestrum parqii	AJ300217	Not known	–	Not collected[b]
Lycopersicon cheesmanii Riley	AJ300202	Bartolome, Galapagos Islands, Equador	*Solanum cheesmaniae* (Riley) Fosb.	LA0317
Lycopersicon chilense Dunal	AJ300203	Quebrada Taltal, Antofagasta, Chile	*Solanum chilense* (Dunal) Reiche	LA2930
Lycopersicon chmielewskii Rick, Kesickii, Forbes and Holle	AJ300206	Tambo, Ayacucho, Peru	*Solanum chmwielewski* (Rick, Kesickii, Forbes and Holle) Spooner, Anderson and Jansen	LA1306
Lycopersicon esculentum L. var. Ailsa Craig	AJ300201	Not known	*Solanum lycopersicum* L.	JM101
Lycopersicon esculentum var. *cerasiforme* Dunal	AJ300200	Not known	*Solanum lycopersicum* L. var. *cerasiforme* (Dunal)	JM100
Lycopersicon hirsutum Dunal	AJ300204	Not known	*Solanum agrimoniifolium*[c] (Dunal) J.F. Macbr.	LA1353
Lycopersicon parviflorum Rick, Kesickii, Forbes and Holle	AJ300207	Rio Pachachaca, Apurimac, Peru	*Solanum neorickii* Spooner, Anderson and Jansen	LA1326
Lycopersicon pennellii (Correll) D'Arcy	AJ300205	Not known	*Solanum penellii* Correll	JM105
Lycopersicon peruvianum (L.) Miller	AJ300210	Sobraya, Tarapacá, Chile	*Solanum peruvianum* L.	LA2744
Lycopersicon peruvianum (L.) Miller var. *humifusum*	AJ300208	Puente Muyuno, Cajamarca, Peru	*Solanum peruvianum* L.	LA2150
Lycopersicon peruvianum (L.) Miller ssp. *typicus* (Luckwill)	AJ300209	Not known	*Solanum peruvianum* L.	JM102
Lycopersicon pimpinellifolium (B. Juss.) Miller	AJ300196	Not known	*Solanum pimpinellifolium* B. Juss.	JM103
Nicotiana plumbaginifolia	AJ300214	Not known	–	Not collected[b]
Nicotiana tabacum	AJ300215	Not known	–	Not collected[b]
Nicotiana tomentosa	AJ300216	Not known	–	Not collected[b]
Petunia axillaris	AJ300213	Not known	–	Not collected[b]
Solanum lycopersicoides Dunal	AJ300212	Arica-Putre, Tarapacá, Chile	–	LA2407
Solanum nigrum L.	AJ300211	Not known		JM104

[a] Vouchers lodged in the Herbarium, Royal Botanic Gardens, Kew

[b] DNA sequence taken from database (Jodrell Laboratory, Kew)

[c] It should be noted that within the genus *Solanum*, subgenus *Potatoe*, section *Petota* series *Concibaccata*, there is a wild potato named *Solanum agrimonifolium* Rydb

mixtures were sealed with a drop of mineral oil to prevent evaporation during thermal cycling. Each reaction cycle was carried out at 97°C to denature the double-stranded template DNA, then at 48°C to anneal primers to the single-stranded template DNA and finally at 72°C to extend primers. To allow completion of the unfinished DNA strands, a 7-min 72°C extension time followed completion of the 40 thermal cycles. Reactions were monitored by the inclusion of positive and negative controls. The positive control was a known orchid DNA sample and the negative control was a water blank. Amplified DNA was purified using "Wizard" mini-columns (Promega, Southampton, UK) according the manufacturer's protocols. Purified amplification products were sequenced on an ABI 377 automated sequencer (Perkin Elmer) using standard fluorescent dye-terminator chemistry according to the manufacturer's protocols (Applied Biosystems Inc.). DNA sequences were submitted to the EMBL nucleotide sequence database (Genbank; Table 1).

DNA sequences were aligned by eye and using ClustalW for Power Macintosh (Thompson et al. 1995). Species from several related genera (*Solanum*, *Cestrum*, *Nicotiana* and *Petunia*) were included in the analysis, although only *Petunia* was designated as the outgroup taxon. All cladistic analyses were performed using PAUP version 3.1.1 (Swofford 1993). The data matrix was analysed using 1000 replicates of random taxon-addition order, tree bisection-reconnection branch swapping, MULPARS, and with all character transformations treated as equally likely (Fitch parsimony; Fitch 1971) under ACCTRAN optimisation. Tree and character manipulations were performed using MacClade version 3.06 (Maddison and Maddison 1992). Internal character support for various branching diagrams was assessed using 10000 bootstrap replicates (Felsenstein 1985) and by 10000 parsimony jackknife replicates (Farris et al. 1997). Alignment gaps were coded as additional binary characters using the "PaupGap" programme (Cox et al. 1997) and included with the nucleotide data.

Results

Alignment of all 19 ITS sequences yielded a consensus length of 726 characters, of which 190 (26%) were parsimony informative. Re-coded alignment gaps yielded 49 informative characters. Parsimony analysis of combined nucleotide and gap data yielded a single most-parsimonious (MP) tree of 812 steps (CI=0.71, RI=0.65) (Fig. 1).

As expected, the inclusion of re-coded gap characters increased resolution in the data matrix; analysis of nucleotide data produced only three MP trees. However, the strict consensus of these MP nucleotide-only trees is entirely congruent with the combined data topology. Only one node (uniting *L. pimpinellifolium* with *L. esculentum*) collapsed in the strict consensus of all MP nucleotide-only trees.

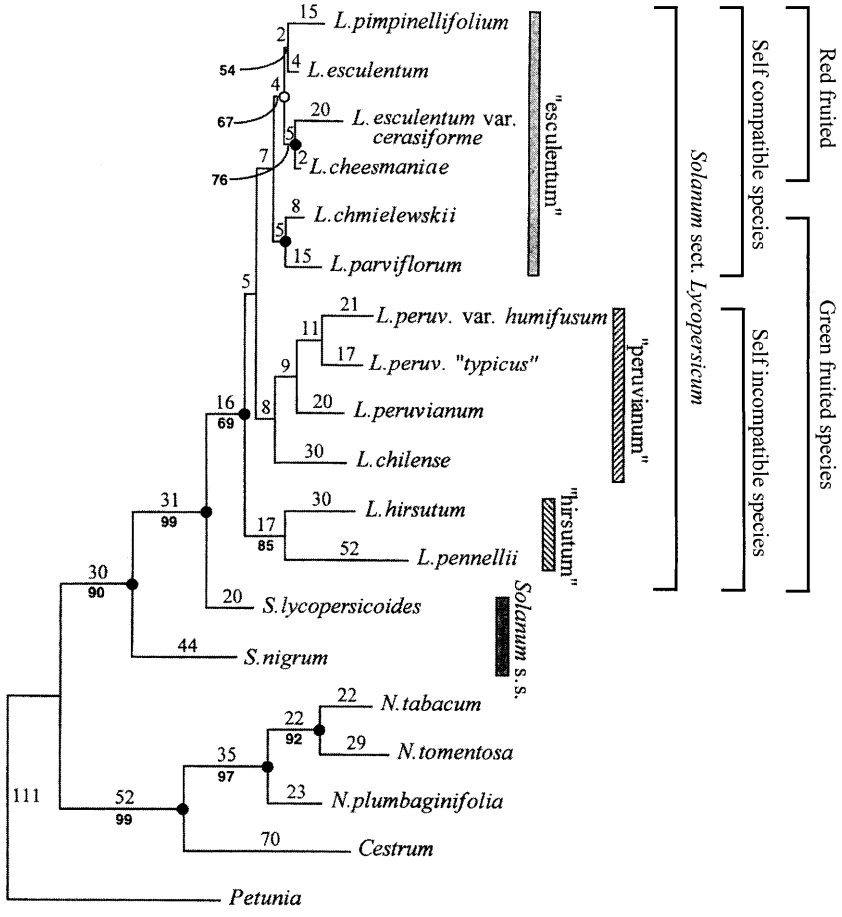

Fig. 1 A single most-parsimonious 812-step Fitch tree derived from the ITS sequence data from nine *Lycopersicon* species. *Numbers above branches* indicate numbers of nucleotide substitutions; *numbers below the branches* indicate levels of bootstrap support. *Closed circles* indicate nodes strongly supported by the jackknife test (63%); *open circles* indicate nodes receiving only weak support (50–62%)

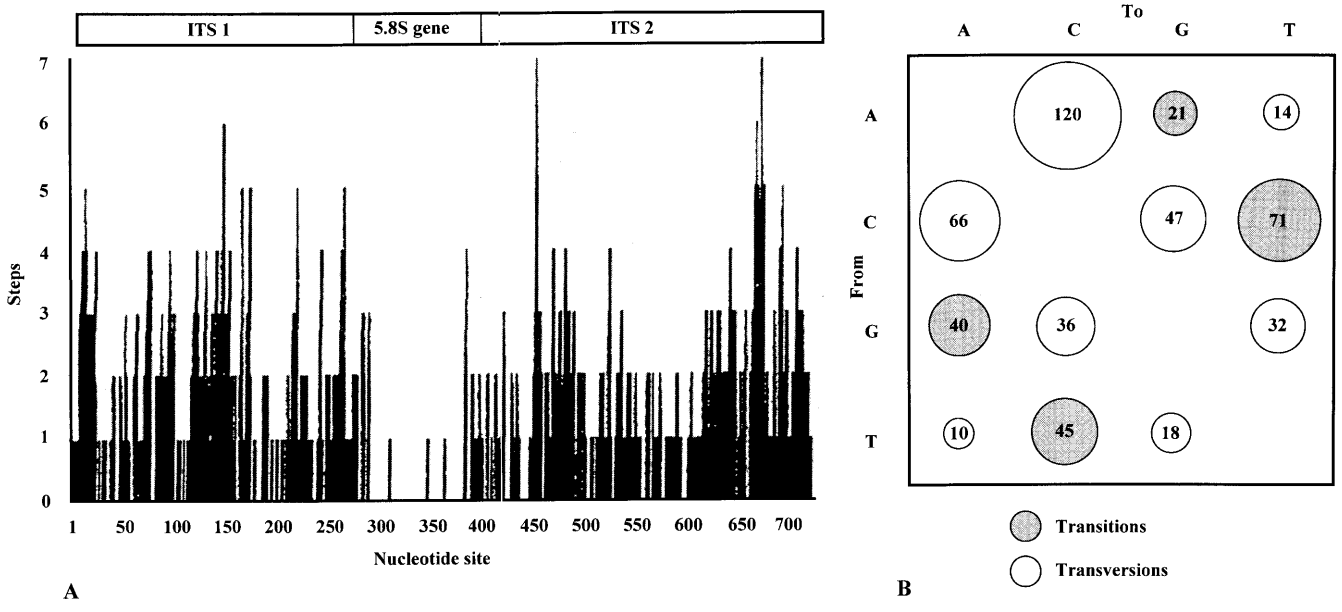

Fig. 2 **A** Nucleotide substitutions in the ITS and 5.8S gene sequences. **B** Number of transitions and transversions of the nucleotide sequence

As shown in Fig. 2A, the majority of inferred nucleotide substitutions occurred in the non-coding spacer regions ITS1 and ITS2 (Baldwin 1992; Hershkovitz and Zimmer 1996), whereas the 5.8S gene sequence displays a high degree of sequence conservation. Although ITS1 appears to be slightly more variable than ITS2 (Fig. 2A), several smaller regions in each appear to be “hotspots”

for nucleotide substitutions. This differential may reflect constraints imposed by the requirement of the ITS regions to form secondary structures necessary for correct removal of the non-coding regions during transcript maturation (Baldwin et al. 1995). As observed in other non-coding regions, there appears to be a marked asymmetry in nucleotide substitution patterns (Fig. 2B). It has been noted (Brown et al. 1972) that, in nucleotide sequence data, transversions occur more rarely than transitions. It is not clear whether this results from an initial bias in DNA composition or stems from bias in the mechanisms affecting base substitution.

Members (Fig. 1) of *Lycopersicon* are divided into three monophyletic groups which are hereafter referred to as the "esculentum", "peruvianum" and "hirsutum" clades. The "esculentum" clade contains six taxa, namely *L. pimpinellifolium, L. esculentum* var. *cerasiforme, L. cheesmaniae* and *L. esculentum*, which collectively are sister to a sub-clade comprised of *L. chmielewskii* and *L. parviflorum*. The "peruvianum" clade contains a monophyletic group comprising *L. peruvianum* and its subspecies, sister to *L. chilense*.

The remaining two species, *L. hirsutum* and *L. pennellii*, form a small clade sister to the "esculentum" and "peruvianum" clades (Fig. 1). The two *Solanum* species included in the analysis form a paraphyletic-grade sister to *Lycopersicon*, confirming the RFLP data of Spooner et al. (1993).

Although the MP topology is fully resolved, it lacks strong support from either the bootstrap or jackknife tests. The node uniting *S. lycopersicoides* and members of *Lycopersicon*, and the node uniting both *Solanum* and *Lycopersicon* (Fig. 1), receive significant support. In addition, nodes are strongly supported, which support the monophyly of *Nicotiana* and the sister group relationship of *Nicotiana* and *Cestrum*.

Discussion

The re-classification of *Lycopersicon* has rectified the major phylogenetic problem, namely that *Solanum* is now a natural (monophyletic) group. Since the study of Spooner et al. (1993) included only three species of *Lycopersicon*, these authors could not address questions concerning species relationships. The present ITS sequence-analysis has produced a pattern of species relationships that are congruent with much existing data, and which adds resolution that is not present in previous studies.

The major taxonomic characters which have been used to divide the genus are those of red-fruit colour and self-compatibility. In the phylogeny generated using the ITS sequences, branching separates the self-compatible species (*L. pimpinellifolium, L. esculentum, L. esculentum* var. *cerasiforme, L. cheesmaniae, L. chmielewskii* and *L. parviflorum*) from the self incompatible species (*L. peruvianum* var. *humifusum, L. peruvianum* ssp. *typicus, L. peruvianum, L. chilense, L. hirsutum* and *L. pennellii*). These divisions correspond to results obtained by molecular methods (Miller and Tanksley 1990) and by classical taxonomy (Muller 1940; Luckwill 1943). The next major branching occurs in the "esculentum" clade and separates the red-fruited species (*L. pimpinellifolium, L. esculentum, L. esculentum* var. *cerasiforme* and *L. cheesmaniae*) from the green-fruited species in the genus. These results agree with those of Miller and Tanksley (1990) and confirm the validity of these characters as taxonomic divisions. However, the data conflicts with the results obtained by McClean and Hanson (1987), who grouped the self-incompatible and green-fruited *L. hirsutum* with the self-compatible and red-fruited *L. esculentum* and *L. esculentum* var. *cerasiforme*. Traditionally, it was thought that self-incompatibility was an ancestral trait from which self-compatibility was derived (Whitehouse 1950). However, Bateman (1952) suggested that self-compatibility could be primitive in many groups. Indeed, recent phylogenetic studies of the distribution and evolution of self-incompatibility in angiosperms have shown that self-compatibility is ancestral in angiosperms. Consequently, self-incompatibility has evolved independently from self-compatibility in many clades in the angiosperms (Weller et al. 1995). Interestingly, alleles coding for self-incompatibility are present in some self compatible species in the Solanaceae, perhaps indicating that self-incompatibility may be ancestral in this plant family (Clark 1996). The *Solanum*, *Nicotiana*, *Cestrum* and *Petunia* samples incorporated in the outgroups in this study of *Lycopersicon* are self-compatible and thus the question of ancestry in relation to self-compatibility/self-incompatibility remains for the family Solanaceae. To this end, the search for self-incompatibility alleles in members of such outgroups will facilitate resolution of this issue.

The "esculentum" clade shows that *L. pimpinellifolium* is closely related to *L. esculentum* confirming results from previous studies (Palmer and Zamir 1982; Miller and Tanksley 1990). It is interesting to note that *L. cheesmaniae*, which is physically and genetically isolated on the Galapagos Islands, is more closely related to *L. esculentum* var. *cerasiforme* than *L. esculentum*, as *L. esculentum* var. *cerasiforme* was believed to be the wild version of the cultivated species (Muller 1940). However, it was suggested by Bretó et al. (1993) that *L. pimpinellifolium* would be a good candidate as the parent of *L. esculentum*, due to the close proximity of the cultigen and the likeness of *L. pimpinellifolium* in distribution, habit and inflorescense structure. The "esculentum" clade contains a subclade which divides the red-fruited species from the green-fruited species of the genus, the latter comprised *L. chmielewskii* and *L. parviflorum.* Both the latter are regarded as sibling species (Rick et al. 1976) and are found in close proximity on the west coast of Chile. They are autogamous and share many genes in common (Breto et al. 1993). Indeed, Rick et al. (1976) and Chmielewski and Rick (1962) noted that the green-fruited *L. chmielewskii* and *L. parviflorum* are closer to the "esculentum" complex than the "peruvianum" complex.

It is noticeable that within the "peruvianum" clade there are high levels of variation between the sub-species of *L. peruvianum*. In fact, there is greater variation within the *L. peruvianum* sub-species than that found within the "esculentum" clade. Miller and Tanksley (1990) proposed that a single accession of *L. peruvianum* would be 20-times more likely to add new allelles into a collection than a single accession of the cultivated tomato. In addition, work by Bretó et al. (1993) showed that *L. peruvianum* was one of the species which had the greatest genetic variability compared to that shown by *L. esculentum* and *L. cheesmaniae*, although it is not unexpected that species with self-incompatibility systems show the greatest genetic variabilty.

The accession *L. peruvianum* var. *humifusum*, from mountainous regions, has been shown to be morphologically distinct and have a high degree of cross-incompatibility with other *L. peruvianum* accessions (Rick 1963; Rick 1986). Our results demonstrate that *L. peruvianum* var. *humifusum* is incorporated within the "*peruvianum*" clade. This contradicts the results of Miller and Tanklsey (1990), who showed that *L. peruvianum* var. *humifusum* was closely related to *L. chmielewskii* and *L. parviflorum* and, as such, was felt by these authors to be sufficiently distinct from other accessions to qualify as a separate species.

Figure 1 shows that *L. peruvianum* and its subspecies are closely related to *L. chilense*, which confirms results obtained by Palmer and Zamir (1982) who observed that the variation within accessions of *L. peruvianum* encompassed the variation shown by *L. chilense* and so suggested relegation within the "peruvianum" complex to a subspecies. In the early taxonomic work of Muller (1940) and Luckwill (1943), *L. chilense* was treated as a synonym of *L. peruvianum* var. *denatum* and was not regarded as a separate species until 1955 by Rick and Lamm, who showed that *L. chilense* was morphologically, reproductively and geographically isolated from *L. peruvianum*.

The "hirsutum" clade closely links the very taxonomically stable species *L. hirsutum* (Luckwill 1943) with *L. pennelli*, a result supported by Palmer and Zamir (1982) and Miller and Tanksley (1990). Originally, *L. pennellii* was named *Solanum pennelli* (Correl 1958), but because of its morphological similarities and compatibilities with *Lycopersicon* species was transferred into the genus *Lycopersicon*.

The two Solanum species are paraphyletic to *Lycopersicon* confirming the results of Spooner et al. (1993). *S. lycopersicoides* is the closer of these two to the genus *Lycopersicon*, reflecting the ability of *S. lycopersicoides* to hybridise with species of *Lycopersicon* (Rick 1951).

It can be concluded, from the first phylogeny using rDNA cladistic analysis on the nine species of the genus *Lycopersicon* and a selection of species from the related *Solanum*, that the ITS sequence is a useful source of phylogenetic data in this group. Comparison of ITS phylogeny and existing classifications showed that self-compatibility and red fruits were true synapomorphs, confirming the validity of these characters as taxonomic divisions. Additionally, this work confirms the results of Spooner et al. (1993) that if *Lycopersicon* was recognised it would be sister to a paraphyletic *Solanum* or result in several other genera being recognised.

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