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SUBFAMILIAL RELATIONSHIPS WITHIN SOLANACEAE AS INFERRED FROM *atpβ-rbcL* INTERGENIC SPACER

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Abstract

A phylogenetic analysis of family Solanaceae was conducted using sequence data from the chloroplast intergenic *atp*β-*rbc*L spacer. Sequence data was generated from 17 species representing 09 out of 14 genera of Solanaceae from Pakistan. Cladogram was constructed using maximum parsimony method and results indicate that Solanaceae is mainly divided into two subfamilies; Solanoideae and Cestroideae. Four major clades within Solanoideae represent tribes; Physaleae, Capsiceae, Datureae and Solaneae are supported by high bootstrap value and the relationships among them are not corroborating with the previous studies. The findings established that subfamily Cestroideae comprised of three genera; *Cestrum, Lycium* and *Nicotiana* with high bootstrap support. Position of *Nicotiana* inferred with *atp*β-*rbc*L sequence is congruent with traditional classification, which placed the taxa in Cestroideae. In the current study *Lycium* unexpectedly nested with *Nicotiana* with 100% bootstrap support and identified as a member of tribe Nicotianeae. Expanded sampling of other genera from Pakistan could be valuable towards improving our understanding of intrafamilial relationships within Solanaceae.

Introduction

Chloroplast genome can be used as an excellent marker to establish phylogenetic relationships between and within plant genera and families (Dong *et al*., 2012; Shinwari *et al*., 1994). This is because of its structural evolution, conservation rate (Palmer, 1991; Wolfe *et al*., 1987), large but manageable size, very rare recombination, uniparental transmission and the easiness of amplification and sequencing of the genes (Dong *et al*., 2012; Hurst & Jiggins, 2005; Khan *et al*., 2013). Chloroplast genome contains many coding and noncoding regions including, *rbc*L, *atp*β, *mat*K, *ndh*F, *rpl*16, *atp*β-*rbc*L, *rps*4-*trn*S, *rps*16, *trn*H-*psb*A, *trn*L-F, *trn*S-G, etc. that have been used for phylogenetic re-establishment in different plant families (Gao *et al*., 2008; Hilu *et al*., 2008; Kim & Jansen, 1995; Li, 2008; Peterson *et al*., 2010; Shinwari *et al*., 1994a; Wilson, 2009). Noncoding regions (spacers) tend to evolve more rapidly than coding sequences in chloroplast genome (Wolfe & Sharp, 1988). The *atp*β-*rbc*L intergenic spacer separating the plastid genes *atp*β and *rbc*L was one of the first noncoding sequences used at various taxonomic levels for phylogenetic reconstruction (Chiang *et al*., 1998; Ehrendorfer *et al*., 1994; Hoot & Douglas, 1998; Hoot & Taylor, 2001; Janssensad *et al*., 2006; Manen & Natali, 1995; Manen *et al*., 1994; Walsh & Hoot, 2001).

Solanaceae is a cosmopolitan family and its members are mostly abundant and distributed in the tropical and warm temperate zones (D' Arcy, 1991; Hawkes, 1992). The family Solanaceae mostly contains agronomically, medicinally and economically important plant species (Edmonds & Chweya, 1997; Yousuf *et al*., 2006; Abbas *et al*., 2013; Halamova *et al*., 2013). The family composed of ca.96 genera and more than 3000 species worldwide (D' Arcy 1991) and only 14 genera and 52 species have been reported from Pakistan (Nasir, 1985).

The traditional classification of Solanaceae revised by D'Arcy (1991) and Hunziker (2001) based on different morphological and anatomical characteristics. This classification mainly recognized two subfamilies, Cestroideae and Solanoideae (D'Arcy, 1979, 1991; Hunziker, 1979, 2001). In the early 1990's, phylogenetic relationships within Solanaceae have been studied by chloroplast coding and noncoding DNA sequences and the findings challenged classical views of taxonomy and proposed some new subfamilies within Solanaceae (Martins & Barkman, 2005; Olmstead *et al*., 1999, 2008).

Intrafamilial relationships within Solanaceae have been reported based on separate or combined analysis of mapped chloroplast DNA restriction sites, sequences of *rbc*L and *ndh*F genes and some nuclear genes (Bohs & Olmstead, 1997; Martins & Barkman, 2005; Olmstead & Palmer, 1992; Olmstead *et al*., 1999). The *atp*β-*rbc*L spacer has been used to verify the monophyly of the genus *Capsicum* (Walsh & Hoot, 2001) and no other studies have been demonstrated with this spacer to infer any relationship within Solanaceae. In Pakistan, taxonomic status of medicinally important taxa of Solanaceae has been evaluated by SDS-PAGE analysis (Yousaf *et al*., 2006, 2008), however, no precise phylogenetic analysis has been established yet that encompasses broad representation of the family.

The focus of the present study is to infer subfamilial relationship in the family Solanaceae using molecular data from chloroplast noncoding *atp*β-*rbc*L intergenic spacer sequence. Such studies have already been successfully used to elucidate taxonomic relationships of difficult groups (Shinwari *et al*., 1994b; Shinwari, 1995).

Materials and Methods

Plant materials: Representative members of different genera belonging to the family Solanaceae were collected from their natural habitat including different regions of Karachi and interior Sindh (Gharo) during their flowering

season in the year 2011-2012. All plant specimens were morphologically identified with the help of Flora of Pakistan (Nasir, 1985), herbarium sheets of 17 identified species were prepared and deposited in the herbarium, University of Karachi and voucher numbers were recorded. Nucleotide sequences (*atp*β-*rbc*L) of two species including *Lycianthes rantonni* and *Nicandra physalodes* were retrieved from Genbank and all species names, locality, voucher number and GenBank accession numbers are listed in Table 1.

DNA Extraction: Total genomic DNA was isolated from fresh young leaves using modified CTAB extraction protocol (Doyle & Doyle, 1987), re-suspended in 1X Tris-EDTA buffer and stored at –20°C until further use.

Primer designing and PCR amplification: For primer designing, all available *atp*β-*rbc*L intergenic spacer sequences of family Solanaceae were retrieved from GenBank and aligned using ClustalW online software (Thompson *et al*., 1994). Primers were designed using consensus sequence of all genera of Solanaceae by using Primer3 version 0.4.0. (Rozen & Skaletsky, 1998). The sequences of primers are given in Table 2. The PCR amplification was carried out under the following conditions, each amplification reaction (20µl) comprised 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.6µM of each primer, 1 unit of DNA Taq Polymerase, 100ng DNA template and milli-Q water was added to make up the volume. Thermal cycler (Eppendorf, Germany) was programmed with the following parameters: Initial denaturation of the template DNA at 94°C for 4 minutes, 35 cycles each consisting of a denaturation step at 94°C for 30 seconds, annealing step 52°C for 35 seconds and extension step at 72°C for 1.5 minutes, followed by a final extension step of 72°C for 10 minutes. Amplified products were purified by using PCR purification kit (Bioneer, Korea) according to the manufacturer's protocol and sent to a commercial laboratory (Bioneer, Korea) for Sanger sequencing. Sequencing was performed with both forward and reverse primers in order to eliminate the chances of sequencing error.

Table 1. Description of collected and retrieved species of family Solanaceae from Pakistan.

Genus	Specific name	Locality	Voucher number	Accession number
Capsicum	*Capsicum frutescens L.*	Karachi	G. H. No. 86480	KF028643
	Capsicum annuum L.	Karachi	G. H. No. 86538	KF028647
Cestrum	*Cestrum diurnum* L.	Karachi	G. H. No.86532	KF028635
	Cestrum nocturnum L.	Karachi	G. H. No.86535	KF028636
Datura	*Datura innoxia* Miller	Karachi	G. H. No. 86478	KF028642
	Datura stramonium L	Karachi	G. H. No. 86475	KF028641
Lycium	*Lycium edgeworthii* Dunal	Karachi	G. H. No.86533	KF028645
Lycianthes	*Lycianthes rantonnei* Carr			AF397086
Physalis	*Physalis divaricata* D. Don	Karachi	G. H. No. 86474	KF028646
Nicandra	*Nicandra physalodes* (L.) Gaertn			AJ490882
Nicotiana	*Nicotiana tabacum* L.	Karachi	G. H. No. 86537	KF028648
Solanum	*Solanum esculentem* L.	Karachi	G. H. No. 86481	KF028644
	Solanum forskalii Dunal	Karachi	G. H. No.86534	KF028640
	Solanum incanum L.	Gharo	G. H. No.86531	KF028639
	Solanum melongena L.	Karachi	G. H. No. 86485	KF028638
	Solanum nigrum L.	Karachi	G. H. No. 86479	KF028637
Withania	*Withania coagulans* (Stocks) Dunal	Karachi	G. H. No. 86484	KF028633
	Withania somnifera (L.) Dunal	Karachi	G. H. No. 86476	KF028634

Table 2. Sequence of family specific *atp*β-*rbc*L primers of Solanaceae.

Primer name	Sequence	Tm
*atp*β-*rbc*L -F	5' AAATGTCCGCTAGCACGTC 3'	52°C
*atp*β-*rbc*L -R	5' AATTAAGAATTCTCACAACAACAAGG 3'	54°C

Phylogenetic analysis: Nucleotide sequence (*atp*β-*rbc*L) of *Nicandra physalodes* was used as an out group. BLASTn (Altschul *et al*., 1990) similarity searches were conducted for each sequence. Multiple sequence alignment of 18 species representing different genera of Solanaceae was performed using ClustalW (Thompson *et al*., 1994). The subfamilial relationship was inferred by maximum parsimony method in PAUP* version 4.0b10 (Swofford, 2002) using heuristics search with 1000 replicates, random stepwise addition of sequence and tree-bisection-reconnection (TBR) branch swapping algorithm. Robustness of clades was estimated using the 1000 bootstrap replicates (Felsenstain, 1985) with random sequence addition and TBR branch swapping. The consistency index (CI) (Kluge & Farris, 1969) and retention index (RI) (Farris, 1989) were calculated to estimate the level of homoplasy.

Results

Maximum parsimony analysis includes 17 species of Solanaceae belonging to different genera of Pakistan and *Nicandra physalodes* used as an outgroup. Of 562 total characters, 414 were constant, 55 were parsimony-uninformative and a total of 93 characters were phylogenetically informative. The 1000 replicate searches in the parsimony analysis using equal weight for all nucleotide positions generated two equally most parsimonious tree of length 198 with consistency index (CI) of 0.868, retention index (RI) of 0.8523 and rescaled consistency index (RC) of 0.7404. Results of the bootstrap analysis are presented as majority rule consensus tree in Figure 1.

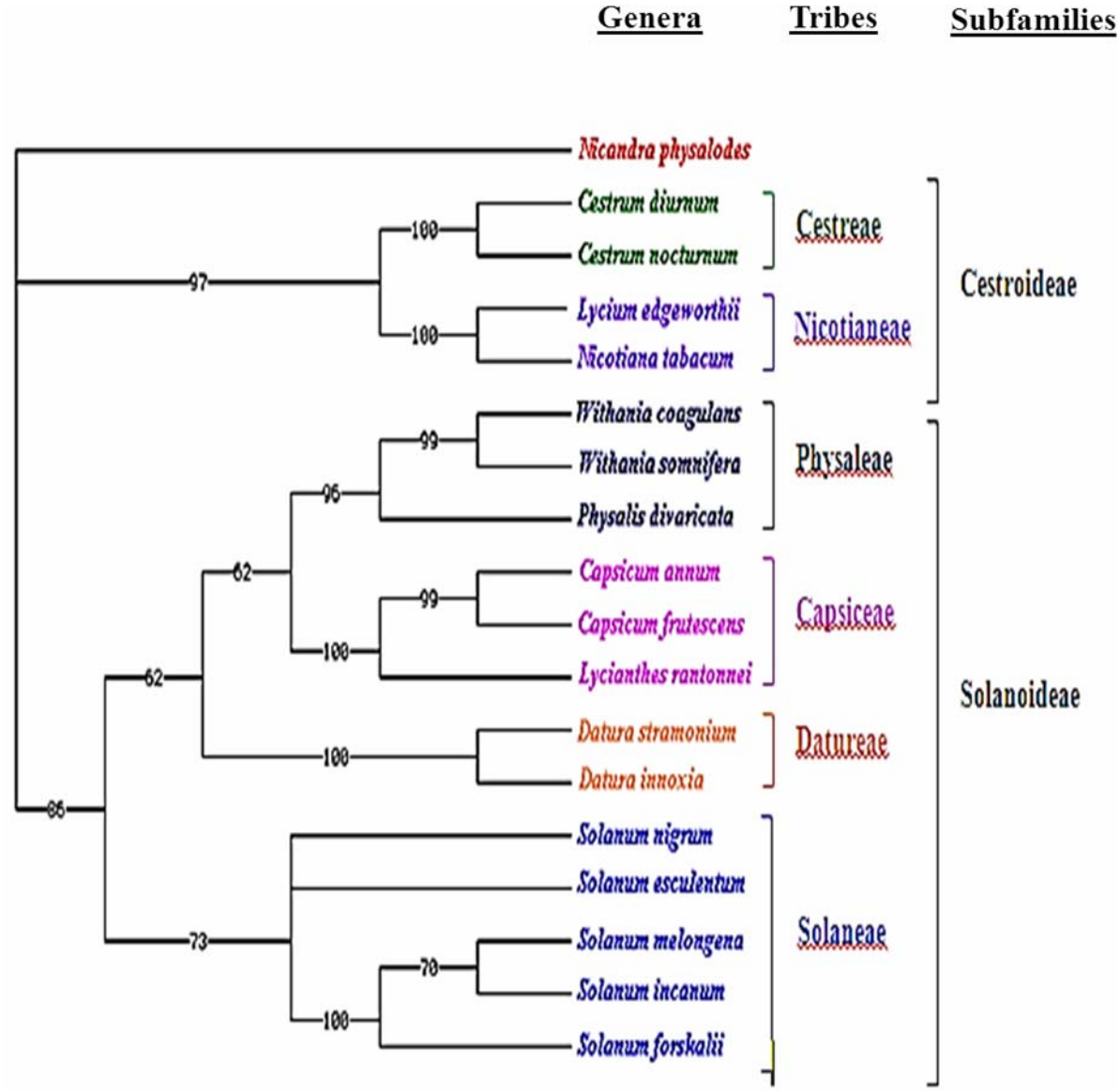

Fig. 1. Majority rule consensus tree of the two most parsimonious trees resulting from maximum parsimony analysis of *atp*β-*rbc*L intergenic spacer sequence for Solanaceae and outgroup taxa. Bootstrap supports in percentages based on 1000 replication analysis are shown in the nodes.

The topology of the tree was unresolved (data not shown) when an outgroup *Ipomea purpure,* a member of the sister family Convolvulaceae was used. A well-resolved monophyletic cladogram obtained with *Nicandra physalodes* as an outgroup, which divides the whole tree into two main branches; Solanoideae and Cesteriodeae. The consensus tree revealed moderate support (86% bootstrap) for the clade that represents Solanoideae, a subfamily of Solanaceae. Four major subclades can be recognized within Solanoideae and each indicates a distinct tribe: 1) Physaleae (*Withania* nested with *Physalis*) well supported by 96% bootstrap value; 2) Capsiceae (*Capsicum* nested with *Lycianthes*) strongly supported by 100% bootstrap; 3) Datureae (two species of *Datura*) 100% bootstrap and 4) Solaneae (five species of *Solanum*) moderately supported lineage with 73% bootstrapping. The other branch identified a subfamily Cesteroideae as monophyletic and the lineage is strongly supported by 97% bootstrap. Cesteroideae is further divided into two tribes with 100% bootstrap support; Cestreae (two species of *Cestrum*) and Nicotianeae (*Nicotiana* nested with *Lycium*).

Discussion

The present analysis used *Nicandra physalodes*as an outgroup as it does not belong to the genera of interest and also not represented in Pakistan. The current report focuses on the genera that are native to Pakistan and some of the species analyzed are first time included in molecular phylogenetic analysis of the family Solanaceae.

Molecular data based on *atp*β-*rbc*L sequence indicates that the family Solanaceae in Pakistan is divided into two subfamilies: Solanoideae and Cestroideae. The clade that moderately support the position of Subfamily Solanoideae is divided into four tribes; Physaleae, Capsiceae, Datureae and Solaneae and this relationship is in agreement with the studies (Martins & Barkman, 2005; Olmstead *et al*., 1999, 2008) in which they demonstrated the tribal relationship of Solanoideae and found Datureae as sister to other three tribes. However, in our analysis, Solaneae is found to have a sister relation to all other three tribes. The position of Datureae is also corroborates with the previously reported restriction site data (Olmstead & Palmer, 1992) where all currently investigated genera were found to be in a single tribe Solaneae and Datureae was a splitter within Solaneae.

According to the present study, Cestroideae is strongly supported (97% bootstrap) to be represented by three genera; *Cestrum*, *Nicotiana* and *Lycium*. Other molecular studies positioned *Nicotiana* into a separate subfamily Nicotianoideae (Martins & Barkman, 2005; Olmstead *et al*., 1999, 2008), however, our results are in congruence with traditional classification proposed by D`Arcy (1991) and Hunziker (2001) that was further confirmed by Olmstead & Palmer (1992) by using restriction site data in which they placed *Nicotiana* into subfamily Cestroideae.

The present research demonstrates that *Lycium* belongs to subfamily Cestroideae and found to be a sister taxa to *Nicotiana*. However, earlier reports (Martins & Barkman, 2005; Olmstead *et al*., 1999, 2008) showed that *Lycium* belongs to subfamily Solanoideae in the tribe Lyaceae. Previous studies based on chloroplast sequence data have shown poor resolution among some of the tribes within subfamily Solanoideae and in most cases *Lycium* is found near the base of Solanoideae (Olmstead & Palmer, 1992; Olmstead & Sweere, 1994; Olmstead *et al*., 1999), in addition to its position, the relationship with other Solanoideae members was found to be ambiguous and not well supported (42% bootstrap) (Martins & Barkman, 2005; Olmstead *et al*., 1999). Therefore, based on our analysis, it can be proposed that *Lycium* might be positioned within Cestroideae.

Within Cestroideae, two distinct clades of tribes are recognized; Cestreae and Nicotianeae. *Nicotiana* and *Lycium* emerged as sister taxa in tribe Nicotianeae with 100% bootstrap support. This relationship has not been suggested by previous studies. However, these two genera have been recognized in tribe Nicotianeae and Lycieae respectively (Martins & Barkman, 2005; Olmstead *et al*., 1999).

In the present study, incomplete genera were investigated; therefore, further sampling may provide a comprehensive description of taxa within Solanaceae.

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