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Complete plastome phylogeny and an update on *cox1* intron evolution of Hyoscyameae (Solanaceae)

C. L. Gandini[1] · V. N. Ibañez[1] · M. K. Zubko[3] · M. V. Sanchez-Puerta[1,2] (ID)

Abstract

Within the family Solanaceae, the tribe Hyoscyameae comprises eight genera distributed across Eurasia. Despite a few attempts to understand the relationships within this tribe, the affiliations among most genera remain unresolved. Recently, complete chloroplast genomes from several species of Hyoscyameae were published, enabling phylogenomic inferences. We sequenced the plastome of *Scopolia carniolica*, the second species of the genus to be reported. Genomic comparisons across the tribe revealed identical gene content and small differences in genome length. Phylogenetic analyses confirmed that *Atropa* is an early-diverging lineage sister to the rest of the tribe, resolved as two clades. One includes well-supported relationships between *Przewalskia*, *Physochlaina*, and *Scopolia*, and these three genera are sister to *Atropanthe*. This clade is sister to a second clade composed of *Hyoscyamus* and *Anisodus*. The strongly supported phylogenetic affiliations of *Atropanthe*, *Anisodus*, and *Hyoscyamus* represent the major advancements as previous studies were not able to resolve these relationships. Interestingly, the genus *Scopolia* is paraphyletic in respect to *Physochlaina*, based on ITS2 sequences. Finally, the evolution of the mitochondrial *cox1* intron is reinterpreted. Two independent horizontal acquisitions are inferred, one in the ancestor of *Przewalskia*, *Physochlaina*, and *S. japonica* and another in *Hyoscyamus*, with no intron losses.

Keywords cpDNA · *cox1* intron · Evolution · *Hyoscyamus* · Phylogenomics · *Scopolia*

Introduction

The tribe Hyoscyameae belongs to the family Solanaceae and includes eight genera distributed exclusively in Eurasia: *Archihyoscyamus*, *Anisodus*, *Atropa*, *Atropanthe*, *Hyoscyamus*, *Physochlaina*, *Przewalskia*, and *Scopolia* (Hoare & Knapp, 1997; Lu, 1997; Olmstead et al., 1999; Yuan et al., 2006). Two diversity centers can be identified in this tribe: the Mediterranean-Turanian region, where *Atropa* and *Archihyoscyamus* grow, and the Tibetan Plateau, where *Przewalskia*, *Anisodus*, and *Atropanthe* can be found (Tu et al., 2010). The genus *Hyoscyamus* has a wider distribution, with species growing in the Mediterranean Basin or Asia. *Physochlaina* spp. are found in Asia, and species of *Scopolia* are distributed disjointly in eastern Asia and south-eastern Europe (Tu et al., 2010). The main characteristic of this group is the presence of tropane alkaloids that were widely used by old civilizations and are still used today (Fatur, 2020; Hoare & Knapp, 1997; Ullrich et al., 2016; Xiao & He, 1983). Many of these substances are pharmaceutical products (Grynkiewicz & Gadzikowska, 2008). The anatomy, seed and pollen morphology, cytology, floral organogenesis, and embryology of members of the tribe Hyoscyameae have been studied (D'Arcy & Zhi-yun, 1992; Hoare & Knapp, 1997; Jang et al., 2011; Kaya et al., 2016; Lu, 1997; Tu et al., 2005; Yang, Zhang, Anming, et al., 2002a; Yang, Zhang, Lu, et al., 2002b; Zhang et al., 1994; Zhang et al., 2005). However, these features were not sufficient to identify the species belonging to this tribe and distinguish among genera and species. Molecular systematics of the tribe Hyoscyameae

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found strong support for the monophyly of the group, but not all relationships within the tribe were resolved with strong bootstrap support (Ghahremaninejad & Riahi, 2021; Olmstead et al., 1999; Sanchez-Puerta & Abbona, 2014; Särkinen et al., 2013; Tu et al., 2010). A study based on the gene "waxy" revealed the hybrid history of the polyploid *Atropa belladonna* and showed that it is sister to the rest of the tribe (Yuan et al., 2006). Recently, short reports described the complete chloroplast genomes (cpDNAs) of several members of the tribe Hyoscyameae (Jiang et al., 2019; Park & Lee, 2016; Tian et al., 2020; Tong et al., 2019; Zhang et al., 2017; Zhang & Chi, 2019) opening the possibility of a phylogenomic approach to understanding the relationships within the Hyoscyameae. A few of these studies included preliminary trees based on a subset of the taxa available, suggesting that chloroplast genome comparisons promise to resolve relationships among genera of the tribe (Jiang et al., 2019; Tian et al., 2020; Tong et al., 2019; Zhang & Chi, 2019). Up-to-date, complete chloroplast genomes of at least one species of each genus of the tribe Hyoscyameae (except for *Archihyoscyamus*) have been published: *Atropa belladonna* L. (Schmitz-Linneweber et al., 2002), *Anisodus acutangulus* C. Y. Wu & C. Chen (Tian et al., 2020), *Anisodus tanguticus* (Maxim.) Pascher (Zhang & Chi, 2019), *Atropanthe sinensis* (Hemsl.) Pascher (Jiang et al., 2019), *Hyoscyamus niger* L. (Sanchez-Puerta & Abbona, 2014), *Physochlaina orientalis* (Bieb.) G. Don (Gandini et al., 2019), *Physochlaina physaloides* (L.) G. Don (Tong et al., 2019), *Przewalskia tangutica* Maxim. (Zhang et al., 2017), and *Scopolia parviflora* (Dunn) Nakai (Park & Lee, 2016). In addition, a single nuclear marker, the internal transcribed spacer 2 (ITS2), has been reported for several species and genera of the tribe. Phylogenies based on this marker denote its usefulness to distinguish among species of Hyoscyameae (Chen et al., 2017; Hajrasouliha et al., 2014; Kim et al., 2003; Liu et al., 2017; Meiklejohn et al., 2019; Miller, 2002), but a comprehensive study of the phylogeny based on this nuclear marker is still missing.

An earlier evolutionary study of the tribe Hyoscyameae identified a highly mobile intron in the mitochondrial gene *cox1* (cytochrome oxidase subunit 1) of 14 species of the tribe and two other lineages of the family Solanaceae (Sanchez-Puerta et al., 2011). The number of independent intron acquisitions through horizontal gene transfer (HGT) events could not be determined given the unresolved phylogenetic relationships within the tribe Hyoscyameae (Sanchez-Puerta & Abbona, 2014; Sanchez-Puerta et al., 2011).

In the present study, we assembled the complete chloroplast genome sequence of *Scopolia carniolica* Jacq., the second species of the genus to be reported, and performed genomic comparisons and phylogenetic inferences of the tribe Hyoscyameae. The goals of this study were (1) to examine the phylogenetic relationships of the Hyoscyameae based on full-length chloroplast genome alignments and nuclear ITS2 sequences, (2) to identify highly variable intergenic regions that could be used as a source for future phylogenetic analyses of species within the tribe, and (3) to re-evaluate the evolution of the *cox1* intron within the Hyoscyameae.

Materials and methods

Plant material, DNA extraction, and sequencing

Plants of *Scopolia carniolica* from southeastern Europe were obtained from the Plantsman's Preference (Norfolk, England, https://www.plantpref.co.uk/). Total genomic DNA was extracted from fresh leaf tissue following Dellaporta et al. (1983) and sequenced at BGI Group (formerly Beijing Genomics Institute) using the Illumina HiSeq 4000 sequencing platform. A total of ~94.9 M clean paired-end reads, 150 bp in length, were generated from a 250 bp insert library.

Genome assembly

Genomic reads were assembled de novo using Velvet v1.2.10 with a k-mer value of 131 and ABySS v4.2.1 and SOAPdenovo v2.04 with a *k-mer* value of 127. Contigs were considered plastidial if they had BLAST hits against a database of chloroplast genomes from Solanaceae at the NCBI GenBank repository. Selected plastid contigs from the three assemblies were combined based on overlapping sequences to eliminate redundancy using the Geneious assembler v.11.1.5 (Kearse et al., 2012) with custom parameters (minimum overlap 300 bp, minimum overlap identity 100%). The five plastid contigs, ranging from 1151 to 92,284 bp, were extended individually to reveal possible repeats at contig ends using SSAKE v3.8.5. Finally, contigs were manually joined and curated using Consed v.29.0 (Gordon et al., 1998), reaching a final assembly of a circular molecule of 156,316 bp.

The mitochondrial gene *cox1* from *S. carniolica* was extracted from the Velvet assembly using all genomic reads and deposited in GenBank (accession number MW079308). Also, *S. carniolica*, *H. niger*, and *P. orientalis* nuclear internal transcribed spacer (ITS) sequences were assembled de novo with Velvet v1.2.10 assembler using a subset of the Illumina reads. The subset was generated by mapping each species read data (this study, SRX647921, and SRX5830318, respectively) over a custom ITS database using Bowtie2 local alignment with standard presets (Langmead & Salzberg, 2012). The three ITS sequences were deposited in GenBank (accession numbers MW079311, MW079309, and MW079310, respectively).

Genome annotation

The cpDNA of *S. carniolica* was annotated using Geneious R11 by transferring the annotations from the *P. orientalis* plastome (GenBank accession NC_044154.1). Annotations were manually curated (start and stop codons and exon/intron boundaries) by inspection of gene alignments. The *S. carniolica* cpDNA was deposited in GenBank under the accession number MT937171. The chloroplast genome map was plotted using OGDRAW.

Genome comparisons and phylogenetic analysis

The complete cpDNA of *S. carniolica* was compared to nine other Hyoscyameae genomes available at GenBank: *Anisodus acutangulus*, *Anisodus tanguticus*, *Atropa belladonna*, *Atropanthe sinensis*, *Hyoscyamus niger*, *Physochlaina orientalis*, *Physochlaina physaloides*, *Przewalskia tangutica*, and *Scopolia parviflora*. Pairwise comparisons and whole-genome identity between *S. carniolica* and the nine Hyoscyameae cpDNAs were performed with the mVISTA program (Frazer et al., 2004) in Shuffle-LAGAN and with a custom python script.

To accurately compare the IR junction regions between *S. carniolica* cpDNA and nine other Hyoscyameae species, we first re-annotated all the analyzed species as most genomes had missing annotations (Suppl. Data 1). In general, the LSC and SSC regions were not annotated, and the fragmented genes at the SSC-IRb and IRa-LSC junctions were mislabeled or missing. The newly annotated genomes were visually inspected using Geneious R11 (Kearse et al., 2012), and IR junctions were illustrated using Adobe Illustrator 2019.

To examine the evolutionary relationships among seven of the eight genera that compose the tribe Hyoscyameae, phylogenetic analyses based on the complete plastome sequences were performed. *Lycium chinense*, *Nicotiana tabacum*, and *Solanum tuberosum* were used as outgroups. Plastome sequences were aligned using MAFFT v. 1.3.7 (Katoh & Standley, 2013). The last inverted repeat region was removed to avoid sequence overrepresentation giving a total alignment length of 134,327 positions (Suppl. Data 2). Two short inversions were identified by inspecting the alignment and were removed from the alignment prior to the phylogenetic analyses. One inversion (*trnC-psbM*) had been described before (Sanchez-Puerta & Abbona, 2014). The other inversion (*rps16-trnK*) was examined here in detail. BLAST searches permitted inspecting the occurrence of the latter inversion in other plastomes. Mfold (Zuker, 2003) with default parameters was used to examine the secondary structure of the inversion and flanking sequences.

Maximum likelihood analyses were performed with RAxML v.8.0.0 (Stamatakis, 2014) with 1000 rapid bootstrapping replicates. Plastid and nuclear ITS sequences were analyzed independently. The best-fitting nucleotide substitution models (GTR+I+G and HKY85, respectively) were estimated by MODELTEST version 3.7 using the Akaike information criterion (Posada & Crandall, 1998). Besides, Maximum parsimony analyses were done using PAUP* (Swofford, 2002) with 1000 bootstrap replicates.

Results and discussion

General organization of *Scopolia carniolica* chloroplast genome

The cpDNA of *S. carniolica* was assembled into a single circular molecule of 156,316 bp in length. Like most land plant plastomes, it presents a quadripartite structure (Fig. 1). The long and small single-copy regions (LSC and SSC) are 86,479 bp and 18,031 bp long, separated by two inverted repeats (IRa and IRb) of 25,903 bp each. The overall GC content is 37.6% (IR, 42.9%; LSC, 35.7%; SSC, 31.9%), resembling other Hyoscyameae species (Gandini et al., 2019; Park & Lee, 2016; Sanchez-Puerta & Abbona, 2014; Zhang & Chi, 2019). The cpDNA presents 115 unique genes, including 80 protein-coding genes or conserved hypothetical chloroplast ORFs, four ribosomal RNAs, and 30 transfer RNAs (Table S1). Fourteen genes have one cis-spliced intron whereas three have two (*clpP*, *rps12*, *ycf3*). The *rps12* gene is trans-spliced, with one exon in the LSC region and the other two within the IR. Like in other Solanaceae, the gene *infA* is a pseudogene, and truncated copies of the *rps19* and *ycf1* genes are located over the IRa-LSC and IRb-SSC junctions, respectively. Seventeen genes and the two first exons of the *rps12* gene are included in the IR and, therefore, are duplicated (Table S1). Four genes present abnormal start codons: *ndhD* and *psbL* start with ACG and *rps19* and *ycf15* start with GTG. The non-canonical start codons ACG and GTG have been previously described in other plant species (Raubeson et al., 2007). The ACG start codons are likely converted to AUG by RNA editing (C to U), as shown for other Solanaceae species (Amiryousefi et al., 2018; Sasaki et al., 2003).

Genome-wide comparisons among the plastomes of the Hyoscyameae

We compared the plastome of *Scopolia carniolica* with nine other complete cpDNAs available at NCBI databases (Fig. 2). The Hyoscyameae chloroplast genomes are highly conserved, with identical gene content, gene order, and no variation in the start and stop codons (Suppl. Data 3). The majority of the genes show low divergence; the sequence identity is 99.5% on average for all genes across the tribe (Table S1, Fig. 2). The small plastid RNA gene (*sprA*) is the most divergent with identity of 95%. In addition, the $\psi rps19$ and $\psi ycf1$ also

Table 1 Mean identities for selected plastid regions and for complete chloroplast genomes across the tribe Hyoscyameae

Region	Global alignment coordinates		Average pairwise identity within Hyoscyameae [a]				Pairwise identity of the species within each genus									References
							Scopolia			Physochlaina			Anisodus			
	Start	End	Length (bp)	Including gaps (%)	Excluding gaps (%)	# nt diff	Including gaps (%)	Excluding gaps (%)	# nt diff	Including gaps (%)	Excluding gaps (%)	# nt diff	Including gaps (%)	Excluding gaps (%)	# nt diff	
trnH-psbA	81	571	490	94.3	97.7	11	96.7	99.8	1	95.1	99.2	4	99.4	99.8	1	Tu et al., 2010; Sanchez-Puerta and Abbona, 2014
ψinfA-rps8	85489	85609	120	93.8	97.8	3	91.7	99.2	1	99.2	99.2	1	100.0	100.0	0	This study
ndhE-ndhG	123981	124214	233	94.4	98.0	5	100.0	100.0	0	100.0	100.0	0	100.0	100.0	0	This study
ndhF-rpl32	118173	118998	825	92.6	98.2	15	99.4	99.4	5	99.8	99.9	1	63.0	99.4	5	Sanchez-Puerta and Abbona, 2014
trnK-rps16	4476	5258	782	91.8	98.6	11	92.6	99.2	6	99.4	99.7	2	99.9	99.9	1	Tu et al., 2010; Sanchez-Puerta and Abbona, 2014
sprA-trnL	119494	120165	671	87.8	98.6	9	99.9	99.9	1	100.0	100.0	0	99.9	100.0	0	This study
petA-psbJ	68125	68964	839	96.7	98.7	11	99.4	99.8	2	98.3	99.3	6	99.8	100.0	0	This study
trnE-trnT	33343	34242	899	92.7	98.8	11	99.4	99.4	5	99.9	99.9	1	99.8	99.8	2	This study
ycf3-rps4	47563	48894	1331	95.7	98.9	15	99.7	99.8	3	96.8	99.3	9	99.8	99.8	3	Sanchez-Puerta and Abbona, 2014
petD-rpoA	82742	83216	474	88.2	98.9	5	89.1	99.5	2	97.3	100.0	0	100.0	100.0	0	This study
trnC-psbM	29731	31705	1974	96.5	99.0	20	99.7	99.8	4	99.8	99.8	4	99.9	99.9	2	Tu et al., 2010; Sanchez-Puerta and Abbona, 2014
trnL-trnF	51282	51705	423	98.6	99.3	3	99.5	99.5	2	98.6	99.8	1	99.8	99.8	1	Olmstead et al., 2008; Sanchez-Puerta and Abbona, 2014
ndhF	115950	118172	2222	99.1	99.4	13	99.9	99.9	2	99.8	99.8	4	99.9	99.9	2	Tu et al., 2010; Sanchez-Puerta and Abbona, 2014
rps16-trnQ	6389	7739	1350	87.3	99.5	7	97.3	99.8	3	99.7	99.4	8	99.6	99.9	1	Sanchez-Puerta and Abbona, 2014
rbcL	59280	60725	1445	99.6	99.7	4	99.9	99.9	1	100.0	100.0	0	100.0	100.0	0	Tu et al., 2010; Sanchez-Puerta and Abbona, 2014
atpB	56937	58435	1498	99.7	99.8	3	100.0	100.0	0	99.9	100.0	0	99.9	99.9	1	Tu et al., 2010; Sanchez-Puerta and Abbona, 2014
cpDNA	1	134327	134327	98.2	99.4	806	99.5	99.8	269	99.6	99.9	134	99.6	99.9	134	This study

[a] The mean identity was calculated from pairwise comparisons of *Scopolia carniolica* and one of species of each genus of the tribe Hyoscyameae
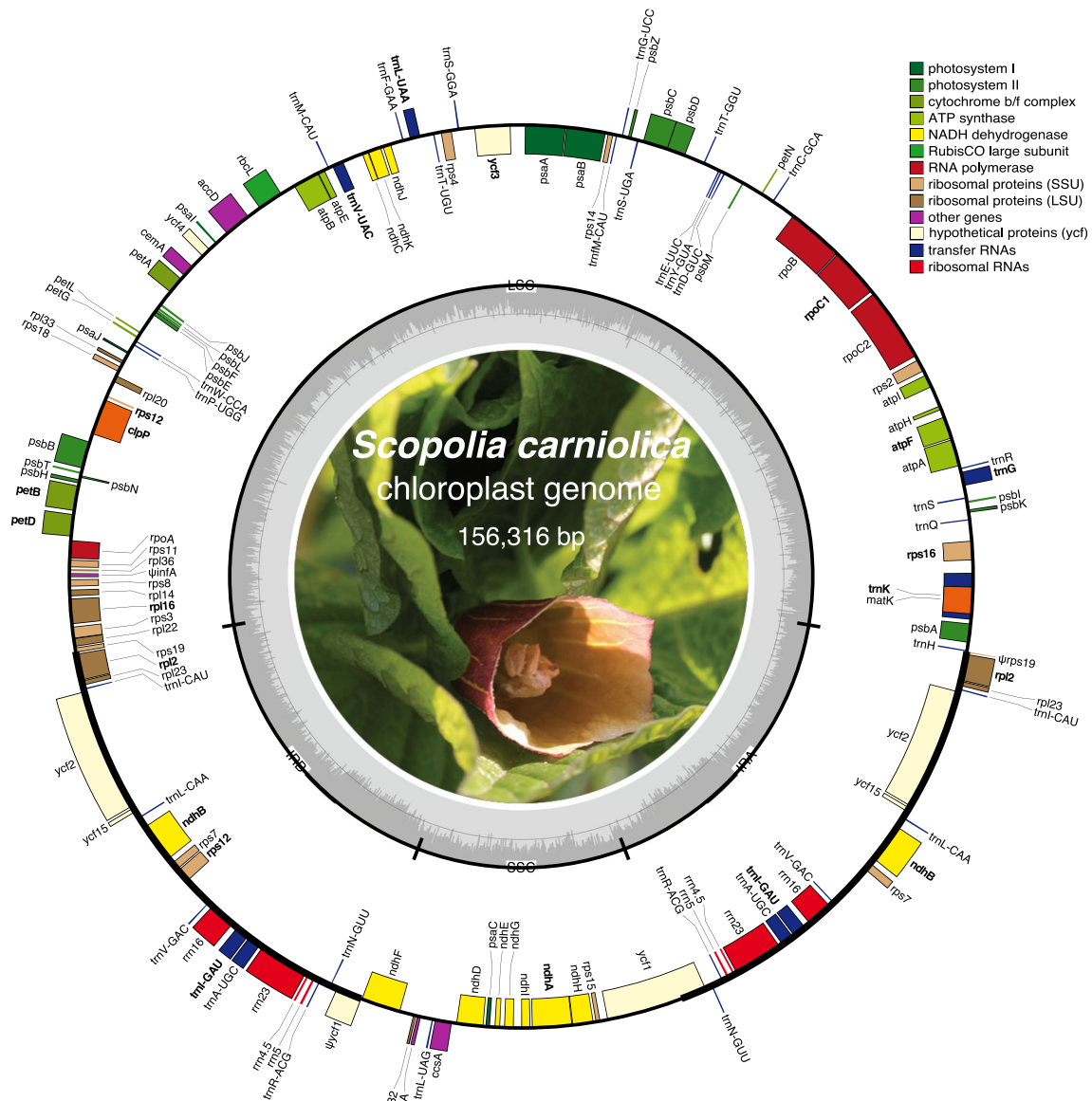
Fig. 1 The chloroplast genome of *Scopolia carniolica*. Large and small single-copy regions (LSC and SSC, respectively) and inverted repeats (IR) are indicated. Genes inside and outside the circle indicate clockwise and counterclockwise transcription directions, respectively. Gene positions are color-coded according to functional categories of genes. The GC content across the cpDNA is shown in the internal circle, and the 50% GC content is depicted with a thin line. Genes with introns are in boldface. The photograph shows the individual of *S. carniolica* studied here (courtesy of Tim Fuller)

present lower identities of 96.6% and 93.6%, respectively. This is expected for pseudogenes with no selection pressure. Chloroplast genome sizes within the tribe range from 155,570 bp in *Przewalskia tangutica* (Zhang et al., 2017) to 156,687 bp in *Atropa belladonna* (Schmitz-Linneweber et al., 2002). This length variation can be attributed to differences in non-coding regions, as all genes are almost identical in size, and to the contraction/expansion of the inverted repeats. The mean pairwise identity of *S. carniolica* cpDNA and the rest of the genera of the tribe is 98% (Fig. 2). Non-coding regions harbor higher levels of divergence, with a mean identity of

97.4%. The overall identity was much higher (99.5%) between the two species of *Scopolia* (Fig. 2).

Expansion and contraction of IR regions within Hyoscyameae

Contractions and expansions at the IR regions' borders represent the main reasons for the size variation of chloroplast genomes (Kim & Lee, 2004) and may reflect the phylogenetic history. IR lengths vary slightly within Hyoscyameae, from 25,282 bp in *P. tangutica* to 25,906 bp in *A. acutangulus*. Comparisons of the boundaries among the
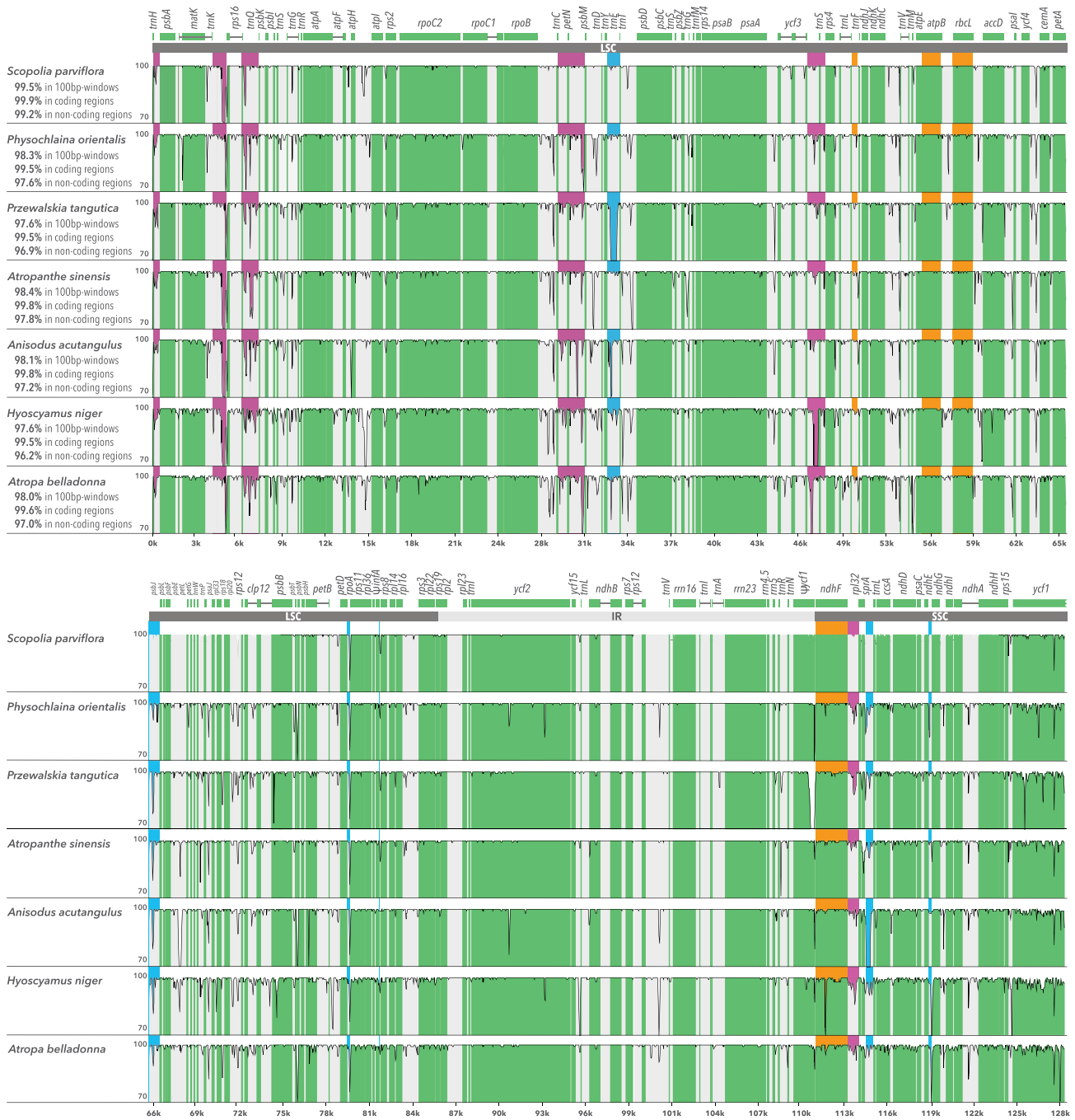
Fig. 2 Similarity plot comparing *Scopolia carniolica* chloroplast genome to other members of the tribe Hyoscyameae. The Y-axis represents the sequence identity (within the range 70–100%) from pairwise comparisons between the chloroplast genomes of *Scopolia carniolica* and other species of the tribe using the VISTA program. Coding and non-coding regions are in green and gray colors, respectively. The percent identities across the whole genome, including all coding and all non-coding regions of *S. carniolica*, and each species of the tribe are shown below species' names. Purple and orange bars indicate previously reported variable and more conserved plastid regions, respectively. Novel variable regions described in this study are in blue

four chloroplast regions (LSC, IRb, SSC, IRa) between the tribe Hyoscyameae and *N. tabacum* show that all species, except *P. tangutica*, exhibit a larger IR region as reported for other Solanoideae (Amiryousefi et al., 2018) (Figure S1). The larger IR includes 59 to 75 bp of the *rps19* gene creating a

truncated ψ*rps19* copy at the IRa-LSC junction. In contrast, the IR region in *P. tangutica* was slightly shorter to expand the LSC region over the *rpl2* gene creating instead a truncated ψ*rpl2* at the IRa-LSC junction. On the other end of the inverted repeat, and in agreement with most angiosperm

chloroplasts, a complete copy of the *ycf1* gene spans over the SSC-IRa junction duplicating the 5'end at the IRb-SSC junction. In nine of the ten Hyoscyameae analyzed species, the IR also spanned over a greater region (~450 bp) of the *ycf1* gene creating a larger $\psi ycf1$ than *N. tabacum* and other Solanoideae. In contrast, a contraction in the *P. tangutica* plastome resulted in a shorter $\psi ycf1$. No obvious differences in the length of the IR have been observed between species of the same genus (Fig. 2). Nonetheless, a study of the IRa-LSC region in 13 *Nicotiana* species showed that the IR region suffered contractions and expansions during the evolution of the genus but found IR to be identical in four cultivars of *Nicotiana tabacum* (Goulding et al., 1996).

Short inversions in the plastomes of Hyoscyameae

Small inversions are common among chloroplast genomes (Kelchner & Wendel, 1996; Kim & Lee, 2005). In general, they are located downstream the 3'ends of genes, they present inverted repeat sequences at their ends, and they form stem-loop hairpin structures that are thought to stabilize the corresponding mRNA (Kim & Lee, 2005). A previously described 10-nt inversion in the *trnC-psbM* spacer (Sanchez-Puerta & Abbona, 2014) was observed in the two species of *Anisodus* and in *P. tangutica*. During alignment screening, we also detected a 46-nt polymorphic stretch in the *Scopolia carniolica* cpDNA, which was the result of an inversion in the *trnK-rps16* intergenic region (Figure S2). This inversion is not present in *Scopolia parviflora*. By analyzing the surrounding sequence with Mfold (Zuker, 2003), we detected a stem-loop hairpin structure with identical inverted repeats of 15 bp flanking the inversion and relatively high free energy (Figure S2). This inversion is located within the plastid spacer *rps16-trnK*, 27 bp downstream the 3'end of the *rps16* gene. A BLASTn search in the nucleotide NCBI databases revealed that this inversion is also present in other Solanaceae, such as *Markea huilensis*, *M. lopezii*, *M. epifita*, *Trianaea neovisae*, and *T. speciosa* from the tribe Juanulloeae (Figure S2). A study of the phylogeny of the Juanulloeae using this molecular marker recognized this homoplasious inversion and excluded it from the data set (Orejuela et al., 2017). The absence of this inversion in all other Hyoscyameae species and the variable presence in species of *Markea* and *Trianaea* indicate that this short inversion can be generated within a short time by intramolecular recombination between the inverted flanking sequences, as reported for other inversions in plant chloroplast genomes (Kim & Lee, 2005).

Phylogenetic relationships within the Hyoscyameae based on complete plastomes

We examined the phylogenetic relationships among seven of the eight genera that compose the tribe based on complete chloroplast genome alignments. Three other Solanaceae species, *Lycium chinense*, *Nicotiana tabacum*, and *Solanum tuberosum* were included as outgroups. The identified short inversions in the cpDNA disrupted the site-wise homology and were excluded from the alignment before phylogenetic inference. The maximum likelihood (ML) and maximum parsimony (MP) phylogenetic analyses found a highly resolved tree in which all branches showed high bootstrap support values (Fig. 3A). *S. carniolica* is closely related to *S. parviflora* and to a clade formed by the two species of *Physochlaina* and *Przewalskia tangutica*. This relationship is supported by seed characters, plastid markers, and a nuclear gene (Olmstead et al., 2008; Sanchez-Puerta & Abbona, 2014; Tu et al., 2010; Zhang et al., 2005), and it differs slightly from trees based on complete cpDNAs of fewer taxa (Tian et al., 2020; Tong et al., 2019). This clade is placed as a sister group to *Atropanthe sinensis* with strong support (97–100%). The second clade included *Anisodus tanguticus* and *Anisodus acutangulus* as sister to *Hyoscyamus niger* (Fig. 3A). The strongly supported phylogenetic affiliations of *Atropanthe*, *Anisodus*, and *Hyoscyamus* represent the major contributions of this work as previous studies based on less than ten plastid markers (Olmstead et al., 2008; Sanchez-Puerta & Abbona, 2014; Tu et al., 2010; Zhang et al., 2005) or a nuclear gene (Yuan et al., 2006) were not able to resolve these relationships. Finally, *Atropa belladonna* is sister to the rest of the tribe, as clearly demonstrated analyzing a retrotransposon inserted in the waxy gene (Yuan et al., 2006).

Plastid markers at the species level in Hyoscyameae

Despite a much clearer picture of the relationships within the tribe Hyoscyameae, the number and delimitation of species within the multispecific genera of the tribe and their phylogenetic relationships remain poorly understood given very limited molecular data from species of the tribe. Here, we examined variable plastid regions as potential molecular markers for the identification and distinguishing species of Hyoscyameae. Within the tribe, the most variable regions larger than 100 bp include *trnH-psbA*, *trnK-rps16*, *rps16-trnQ*, *trnC-psbM*, *trnE-trnT*, *ycf3-rps4*, *petA-psbJ*, *petD-rpoA*, $\psi infA$-*rps8*, *ndhF-rpl32*, *sprA-trnL*, and *ndhE-ndhG* (Fig. 2, Table 1). No variable loci were located within the IR regions consistent with the reduced rate of synonymous substitutions that characterize these regions (Mower & Vickrey, 2018; Zhu et al., 2015). Some of the variable regions, along with other more conserved ones, have been previously used for taxonomic purposes in the family Solanaceae, particularly for the tribe Hyoscyameae (Table 1, Fig. 2). However, phylogenetic trees of the Hyoscyameae based on those markers were not fully resolved. In particular, the phylogenetic affiliations of *Atropanthe*, *Anisodus*, and *Hyoscyamus* were not strongly supported (Olmstead et al.,
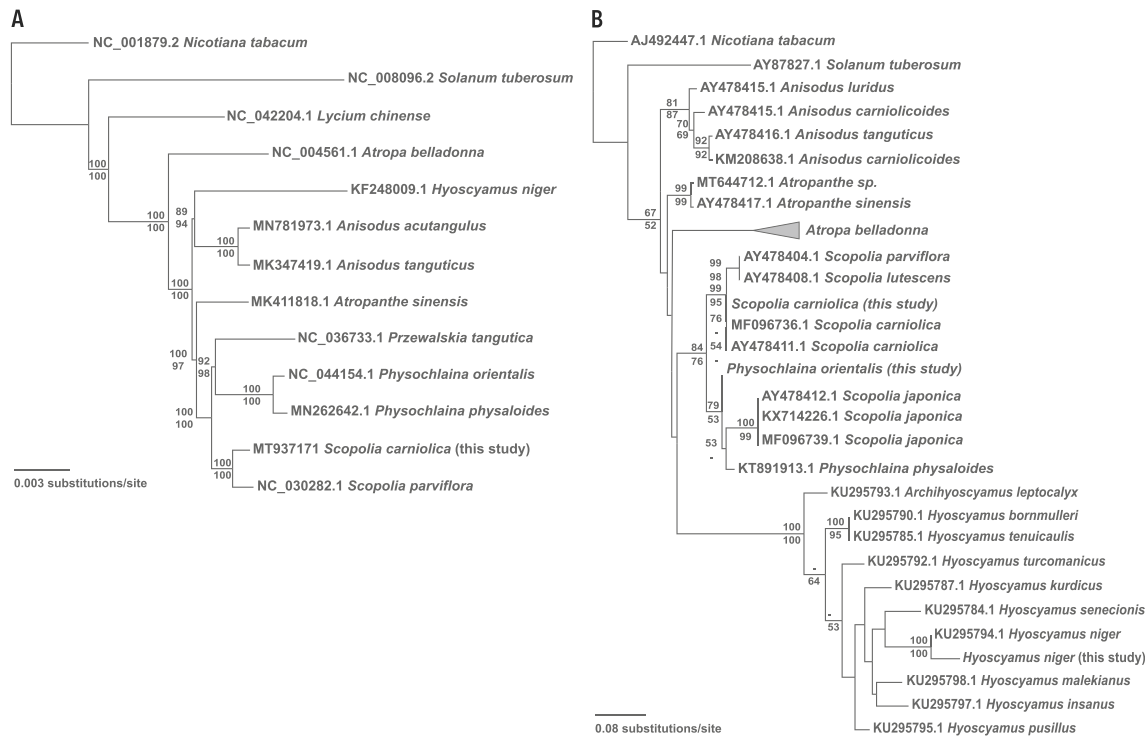
Fig. 3 Maximum likelihood phylogenetic trees of the tribe Hyoscyameae. **A** Tree based on the complete chloroplast genomes. The last inverted repeat region was removed to avoid sequence overrepresentation. The alignment length is 134,327 nt. **B** Tree based on nuclear ITS2 sequences. The seven individuals of *Atropa* *belladonna* (KX674979, MF096061, MF348980, MG217536, MK895642, KX166233, AY028147) were identical and are represented with a triangle to simplify the display of the tree. Numbers above and below the branches represent support values based on 1000 bootstrap replicates of ML and MP analyses, respectively

2008; Sanchez-Puerta & Abbona, 2014; Tu et al., 2010), and the monophyly of the genus *Scopolia* was questioned (Olmstead et al., 2008). We compared variable regions' identity between species of the same genus to evaluate their potential value as taxonomic tools at the species level (Table 1). The number of polymorphisms between the same genus species was very low indicating that these regions are not sufficiently informative to assess phylogenetic relationships among species within the tribe Hyoscyameae. Even when the whole cpDNA is considered, a total of 201 polymorphic sites on average differentiate species of the same genus (Table 1). We conclude that only full-length chloroplast genomes may be useful for phylogenetic inference at the species level. Alternatively, a more promising approach would be developing nuclear molecular markers, which are more variable and thus may be more informative for species of the tribe Hyoscyameae.

Phylogenetic inference based on nuclear ITS sequences

Sequences from a single nuclear marker (i.e., ITS2) with broad taxon sampling for meaningful phylogenetic analyses of the tribe Hyoscyameae are available in public databases. Highly variable regions such as ITS can help to compare species and closely related genera

(Baldwin et al., 1995). We performed phylogenetic analyses based on ITS2 sequences from all genera of the tribe Hyoscyameae (except for *Przewalskia*) along with that of the individual of *S. carniolica* studied here. The ITS2 sequence of the individual of *S. carniolica* under study is highly similar to that of other individuals of this species (99.8%) confirming its taxonomic identification. Even though the backbone of the ITS2 tree is poorly resolved in general (Fig. 3B), it recovers all genera as monophyletic, except for *Scopolia*. The single species of *Archihyoscyamus* is sister to the monophyletic genus *Hyoscyamus*, in agreement with previous studies that support its segregation based on its unique habitat (Lu, 1997), seed morphology (Zhang et al., 2005), and recent molecular analysis (Ghahremaninejad & Riahi, 2021).

The ITS2 sequences from all three samples of *S. japonica* cluster with *Physochlaina* with moderate bootstrap support (79%) in the ML tree, while those of *S. carniolica*, *S. parviflora*, and *S. lutescens* form a monophyletic group sister to the clade formed by *Physochlaina* spp. and *S. japonica* (Fig. 3B). The paraphyly of the genus *Scopolia*, specifically due to the unexpected position of *S. japonica*, had been previously noticed based on plastid markers (Olmstead et al., 2008). However, another study using plastid markers

but with different sampled individuals suggested that *Scopolia* was monophyletic (Tu et al., 2010). In this study, we analyzed ITS2 sequences from three individuals of *S. japonica* generated independently by different groups (Chen et al., 2017; Kim et al., 2003; Liu et al., 2017). Unfortunately, no ITS data are available for the individuals of *S. japonica* analyzed by Olmstead et al. (2008) or Tu et al. (2010) to assess the discrepancies.

The number and delimitation of species within the genus *Scopolia* remain unclear (http://www.theplantlist.org; Jang et al., 2011, Festi, 1996). *S. carniolica* is a type of species that has a wide distribution across central and southeast Europe (Festi, 1996). Two species grow in Korea, *S. parviflora* and *S. lutescens*, while *S. japonica* is found in Japan. The individuals of *S. parviflora* and *S. lutescens* have been included within the species *S. japonica* due to the lack of distinct morphological differences (Festi, 1996; Jang et al., 2011). However, molecular comparisons of ITS sequences from these four species distinguished *S. carniolica* and *S. japonica* from the Korean species, which had identical ITS sequences (Kim et al., 2003). The lack of diagnostic features and identical ITS2 sequences indicates that *S. parviflora* and *S. lutescens* are synonyms (Kim et al., 2003). The sample of *S. japonica* studied by Tu et al. (2010) was obtained from South Korea, while the sample analyzed by Olmstead et al. (2008) comes from a Japanese collection. The genus *Scopolia* may be paraphyletic due to the odd phylogenetic relationships of Japanese samples of *S. japonica* observed with plastid (Olmstead et al., 2008) and ITS2 (Fig. 3B) sequences, while Korean samples are closely related to *S. carniolica*. Detailed and comparative studies based on cytoplasmic and nuclear markers of different samples of *Scopolia* obtained from Japan, Korea, and Europe are needed to test this hypothesis further and to determine the number of species of the genus.

Multiple acquisitions of the *cox1* intron within the tribe Hyoscyameae

The *cox1* intron is known as highly mobile among plant mitochondria, with more than 80 described horizontal transfers during angiosperm evolution (Cho et al., 1998; Sanchez-Puerta et al., 2008). A signature sequence generally accompanies intron acquisition, termed co-conversion tract (CCT), which replaces a short region of the second exon's 5' end. Within the family Solanaceae, three horizontal transfers of the *cox1* intron (in *Brunfelsia*, *Mandragora*, and the tribe Hyoscyameae) were described, while 426 species of Solanaceae that belong to 70 genera lack this intron (Sanchez-Puerta et al., 2011). *Brunfelsia jamaicensis* obtained its *cox1* intron in an independent event and from a different

source than the other two intron-containing Solanaceae lineages. On the other hand, intrafamilial transfers between *Mandragora* spp. and the tribe Hyoscyameae explain highly similar introns in these two unrelated lineages of Solanoideae (Sanchez-Puerta et al., 2011). Within the Hyoscyameae, four genera have the *cox1* intron (and the CCT): *Hyoscyamus* (nine species), *Physochlaina* (three species), *Przewalskia* (one species, *P. tangutica*), and *Scopolia* (one species, *S. japonica*; Sanchez-Puerta & Abbona, 2014 made a mistake in Fig. 3 stating that *S. carniolica* [instead of *S. japonica*] has the *cox1* intron). In contrast, the other three genera of the tribe (*Anisodus*, *Atropa*, and *Atropanthe*) lack the intron and the CCT, indicating that they never had the *cox1* intron (Sanchez-Puerta & Abbona, 2014; Sanchez-Puerta et al., 2011). The partially resolved phylogeny of the tribe based on 10 plastid markers leads Sanchez-Puerta and Abbona (2014) to propose two alternative evolutionary scenarios explaining the distribution of the *cox1* intron (and the CCT) in Hyoscyameae. One hypothesis suggested a single *cox1* intron acquisition in the ancestor of a clade formed by *Hyoscyamus*, *Physochlaina*, *Przewalskia*, and *Scopolia*, and the second hypothesis inferred two independent intron gains: one in *Hyoscyamus* and a separate one for the other three genera.

We assembled the *cox1* gene of *Scopolia carniolica* and found that it lacks the intron and the co-conversion tract (Suppl. Data 4). This result was somewhat surprising because *S. japonica* presents the *cox1* intron and the CCT (Sanchez-Puerta et al., 2011). Noticeably, the sample of *S. japonica* with the *cox1* intron studied by Sanchez-Puerta et al. (2011) was closely related to *Przewalskia* and not to *S. carniolica* based on two plastid markers (Olmstead et al., 2008). Again, it is unfortunate that only fragmentary data are available and that the *cox1* intron status of other species of *Scopolia* and in particular of those samples of *Scopolia* studied by Tu et al. (2010) is unknown.

According to the plastid (Olmstead et al., 2008) and ITS2 (Fig. 3B) phylogenies of the Hyoscyameae, the genus *Scopolia* may be paraphyletic in respect to *Physochlaina* and *Przewalskia*. Under this evolutionary scenario of the tribe Hyoscyameae, two intron acquisitions could explain the presence of the *cox1* intron in distinct lineages of the tribe (Fig. 4), as suggested by Sanchez-Puerta and Abbona (2014). However, the timing of the *cox1* intron gain is different here, as it is proposed to have taken place in the ancestor of *Physochlaina*, *Przewalskia*, and *S. japonica*, while all other species of *Scopolia* may not present the intron nor the CCT (Fig. 4). Overall, two and four horizontal transfer events of the *cox1* intron (and no intron losses) took place during the evolution of the tribe
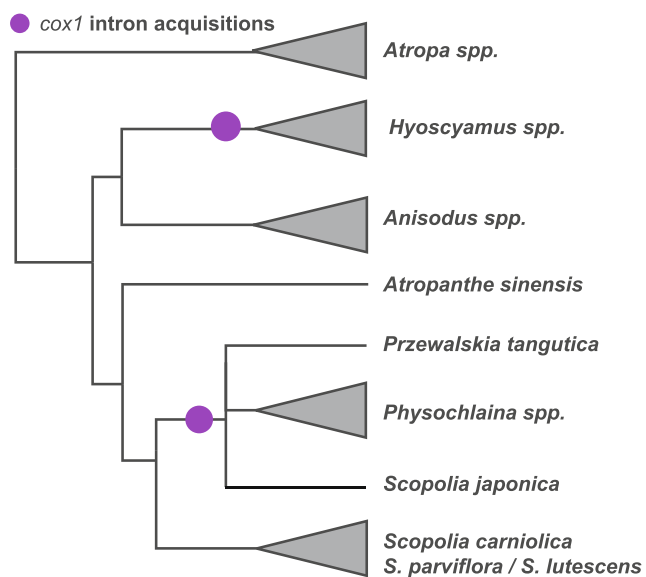
Fig. 4 An evolutionary scenario for the *cox1* intron acquisitions in the tribe Hyoscyameae. Purple dots indicate mitochondrial *cox1* intron acquisitions by horizontal gene transfer. The paraphyly of the genus *Scopolia* is supported by plastid and nuclear markers (see text and Fig. 3)

Hyoscyameae and the family Solanaceae, respectively. Three of them were intrafamilial HGT events and include intron transfers between *Mandragora*, *Hyoscyamus*, and the ancestor of *Physochlaina*, *Przewalskia*, and *S. japonica*. This agrees with the observation that intrafamilial transfers are frequent in other angiosperm lineages (Sanchez-Puerta et al., 2008).

Declarations

Ethics approval Not applicable.

Consent to participate All authors have read and approved this manuscript.

Consent for publication All authors have read and approved this manuscript.

Competing interests The authors declare no competing interests.

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