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DNA Barcodes for certain endangered Indian Tylophora species

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Abstract

$tRNA^{Leu}$ (UAA) (trnL) intron sequences have been used as a molecular marker for differentiating plant species and to assess phylogenetic relationships among three closely related Tylophora species. Mutation events were calculated based on the sequences generated. Nucleotide substitutions were 31% and indels, 69%. Transversions of G↔C were dominant as compared to A↔C and A↔G. AT repeats of variable length were observed in all the three species. Further three repeat clusters with a p value of 1.58x10-5 were observed in all the three species. cpDNA polymorphism can provide significant insight into the evolution of the species and its genetic structure.

Key words: Tylophora, cpDNA, tRNALeuUAA (trnL) intron

Introduction

A DNA BARCODE uses nucleotide sequences from nuclear, mitochondrial and chloroplast genes to identify a species in a sufficiently conserved variable region of the genome in order to design primers that can amplify short regions (Taberlet et al., 2006).

The maternally inherited chloroplast, a circular molecule in angiosperms which lacks recombination capacity has a more stable genome than the mitochondrial genome. It is highly conserved in size and structure (Shinozaki et al., 1986), and evolves faster than mitochondria and shows a considerable mutation rate (Taberlet et al., 1991; Clegg, 1993;). Notwithstanding its conserved nature, the highly variable segments of the noncoding regions are suitable for generating barcodes, through designing universal primers for genetic analysis, phylogeography studies, molecular systematics (Muir and Filatov, 2007), population genetics, and in species identification (Gielly and Taberlet, 1994; Brouat et al., 2001). Besides there are many copies of cpDNA per cell as compared to nuclear DNA sequences, the amplification is efficient and rapid. Therefore, it is a more attractive method than traditional RFLP and RAPD for genetic marker identification and to differentiate closely related species (Collins et al., 2003).

DNA barcodes can be generated in the highly variable trnL-F part of the chloroplast genome. This region consists of tRNA genes for trnT (UGU), trnL (UAA) and trnF (GAA) (Fig. 1). These are arranged in tandem and separated by noncoding spacer regions. This segment is located in the large single copy region approximately 8kb downstream of rbcL. The trnL gene contains a group I intron positioned between the U and the A of the UAA anticodon loop. The position of this intron, interrupting the anticodon of the tRNALeu (UAA) gene (U-intron-AA), is conserved from cyanobacteria to plant chloroplast (Kuhsel et al., 1990). The conserved status renders the intron in the tRNALeu (UAA) gene a suitable candidate for developing barcodes. The trnL intron was the first group I intron described in chloroplast DNA and also the first one described to interrupt a tRNA gene (Bonnard et al., 1985; Simon et al., 2003). Group I introns are self splicing RNA enzymes (ribozymes) from pre-RNA. It also encodes conserved primary and secondary structures required for autocatalysis. Therefore, this intron has catalytic ability as well as a capacity to store information.

In plants, the trnL intron usually shows sequence conservation in the regions flanking both trnL exons [i.e, trnL (UAA) 5’ exon and trnL (UAA) 3’ exon], whereas the central part is highly variable (i.e trnL intron). This implies that trnL and trnF are probably co-transcribed (Bonnard et al., 1985). The structure has complementary regions that form nine stem-loop structures (P1-P9) (Taberlet et al., 2006; Hermandez-Maqueda et al., 2008). There are three gaps in the trnL introns of which two belong to the P8 stem and loop, and one is in the P9 element. No secondary structural elements have been found within the intergenic spacer between trnL (UAA) 3’exon and trnF (GAA) exon that could serve as splicing points. Analyzing the secondary structure in the spacer region and the trnL intron P8 loop are useful in studying phylogenetic relationships. However, in order to establish phylogenetic relationships at a generic level more variable regions in cpDNA, such as rps4, trnT and trnF, should be explored further (Taberlet et al., 2006).

The tRNALeu (trnL) intron has many drawbacks. It has a low resolution compared with

several other noncoding chloroplast regions. It has a very low discriminating capacity for closely related species which makes it inept for phylogenetic studies. The low resolution of tRNALeu (trnL) intron is therefore linked to a lower intraspecific variation and compared with other noncoding regions of chloroplast DNA (Shaw et al., 2005).

In spite of the above drawbacks it has many advantages. tRNALeu (trnL) intron is used for phylogenetic studies among closely related genera and species (Gielly and Taberlet, 1996) because universal primers are available for this region (Taberlet et al., 1991). The evolution of tRNALeu (trnL) intron has been analyzed in detail and is well understood (Quandt and Stech, 2005). The number of tRNALeu (trnL) intron sequences available in databases is the maximum available non coding chloroplast DNA sequences in the database. Furthermore, this region is the only group I intron in chloroplast DNA (Palmer, 1991) and has a conserved secondary structure with alteration of conserved and variable regions (Quandt et al., 2004). Therefore, robust primers can be designed for amplifying short variable region in between the tRNALeu (trnL) intron. Such short regions are the P6 loop of the 10-143bp region and the P8 loop present within the trnL region (Won and Renner, 2005). Primers developed for this region are highly conserved from Bryophytes to Angiosperms. Studies show that the amplification of the entire tRNALeu (trnL) intron and the P6 loop allows a much greater identification of species in comparison to amplification based on psbB–psbH, rpoB–trnC (GCA), rps16 intron, trnD (GUC)–trnT (GGU), trnH (GUG)–prbA and trnS (UGA)–trnM (CAU) primers. Moreover the tRNA-Leu (trnL) intron does not represent the most variable non-coding region of chloroplast DNA and shows polymorphic phylogeny in various Tylophora species (Liede et al., 2002). These factors make the trnL intron an apt choice as a barcode in Tylophora species.

Many controversies exist over the value of DNA barcoding. Taxonomists consider that the traditional morphology based identification of a plant species would diminish and result in incorrect species identification as cpDNA relies solely on genetic divergence (Kress et al., 2005). Moreover, taxonomy of science is based on a detailed understanding of morphology, physiology and behavioural attributes (Balakrishna, 1999; Ebach and Holdrege, 2005; Arvind et al., 2007; Pandey, 2007) and barcoding generates information, not knowledge. Moritz and Cicero (Moritz and Cicero, 2004) suggested that species identification should be based on multigene phenotype rather than a single gene sequence. Though DNA barcoding provides rapid species identification, its accuracy relies on PCR technology by using a standardized DNA region as a tag (Hebert and Gregory, 2005). This technology itself has been cited as producing artefacts and inconsistent data.

Three species of genus Tylophora (family Asclepiadaceae), Tylophora indica Burm.f. Merrill, Tylophora rotundifolia Ham.ex Wight and Tylophora fasciculata Ham.ex Wight are important indigenous medicinal plants found in restricted localities in Gujarat, India

and are facing threats of different proportions (Shah, 1978; D'Cruz, 2003). Though Tylophora indica has a spatially distributed population and is found in the plains, hilly slopes and the outskirts of the forests of Gujarat, the other two species do not have a scattered population and are listed as endangered species. Tylophora rotundifolia and Tylophora fasciculata are over exploited to such an extent that of late they can be found in restricted regions in the inner recesses of the Shoolpaneshwer sanctuary in South Gujarat (D'Cruz, 2003).

Though various regions can be used to develop a barcode, trnLLeu (UAA) intron region (about 550pb sequence) was analysed in the present study to develop barcode for the three Tylophora species. Moreover, the objective of generating a trnL intron based barcoding was to document and compare the patterns of cpDNA diversity within Tylophora species and to assess the competence of cpDNA to quantitatively differentiate species and populations. Further, this information can be used for identifying germplasm for conservation programmes.

Materials and Methods

Population sampling

Tylophora indica, Tylophora rotundifolia and Tylophora fasciculata were collected exclusively from central and south Gujarat during the monsoon season. Fresh and tender leaves were used for DNA extraction using the Nucleon PhytoPure DNA extraction kit (Nucleon Extraction and Purification Protocols, GE Healthcare). In order to develop a tRNA-Leu (trnL) intron marker, the purified DNA was amplified with specific trnL primers, (Sense primer: 5'-CGAAATCGGTAGACGCTACG-3'; Antisense primer: 5'-GGGGATAGAGGGACTTGAAC-3' Taberlet et al., 2006). The 25µl PCR master mix contained 10x Taq buffer, 2mM MgCl2, 0.4mM dNTP mix, and 2 units of proof reading Taq DNA Polymerase. This was spun briefly and 1µl each of the forward and reverse primers (10pmoles/µl) were added. Then 2µl (50ng) of plant genomic DNA and 21µl of nuclease free water was added to make a 50µl final volume. Amplification was carried out with the following programme of initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 1 minute at 94°C for denaturation, 1 minute at 50°C for annealing, and 1 minute at 72°C for extension. This was followed by a final extension at 72°C for 5 minutes.

The amplification product was loaded on a 2% agarose gel and electrophoresed using a modified 1X TAE buffer (40mM Tris-Acetate, 0.1mM Na2EDTA pH 8.0,) at 50V. A 100bp ladder was also loaded on either side of the PCR product. The amplification produced a single band of 550bp. This PCR product of 550bp was sliced from the gel and extracted as described using DNA Gel Extraction kit (Millipore Corporation, Bedford) and sequenced.

Sequence Analysis

Homology searches were performed within GenBank's nonredundant database using BLASTN 2.2.20 (Basic Local Alignment Search Tool) algorithm at http://www.ncbi.nlm.nih.gov/BLAST/ of the National Centre for Biotechnology Information (NCBI). The sequences of tRNALeu (trnL) introns were aligned to similar data sets at NCBI for 519-521bp lengths using Bioedit. Twenty published trnL intron sequences from Tylophorinae clade were used for phylogenetic analysis as an ingroup and two outgroups (Cynanchum acutum and Asclepias longifolia) were included in the analysis. The different mutational events (substitutions, as wells as indels that are derived from either deletion or duplications of sequences) were coded manually. The following formula was used to calculate the proportions of observed mutation for each pair of species. Proportion of mutational events = [(TS + TV + ID)/L] X 100, where TS = number of observed transitions, TV = number of observed transversions, ID = number of observed insertions/ deletions (multibase length differences were scored as 1), and L = sequence length (TS + TV + ID + number of sites showing the same nucleotide) (O'Donnell, 1992). Phylogenetic distance was measured using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The repeated sequences were identified using REPFIND.

Results

The amplified cpDNA produced a single band of about 550bp on a 2% agarose gel. The sequencing resulted in an unambiguous 550bp for Tylophora indica (GenBank, accession number: FJ890345), 551bp for Tylophora rotundifolia (GenBank, accession number: GU060630), and 554bp for Tylophora fasciculata (GenBank, accession number: GU060631). The percentage distributions of the bases are given in table 1.

The proportion of mutation events (substitutions and indels) was calculated based on the alignment of tRNALeu (trnL) intron accessed from NCBI data bank (Table 2). Deletions were observed consistently at five positions for Tylophora indica (16, 17, 25, 26, and 76) and insertions at positions 28 and 197. No deletions were observed in Tylophora rotundifolia, but two insertions were observed at positions, 16 and 76. Tylophora fasciculata had three deletions at positions, 16, 28 and 76. Nucleotide substitutions were 31% and indels, 69% in Tylophora species. Transversions of G↔C were dominant as compared to A↔C and A↔G (Table 3). The AT rich region of variable length (position 306 – 420) was observed in all the three species studied.

Further, the tRNALeu (UAA) intron and the significant BLAST result were aligned using Bioedit. The repeated sequences identified using REPFIND showed that the most significant clusters were between 174 to 203 with three repeat sequences of GTTGG, GTTGGTA, TTGGTAG with a p value of 1.58x10-5 in all the three species.

Discussion

Molecular barcoding based on cpDNA is a useful technique for species identification and assessing genetic diversity. Closely related species can be distinguished based on information about deletions, insertions, and substitutions of nucleotide bases in the trnL region (Bakker et al., 2000). The process and pattern of these events are important for estimating species divergence and are phylogenetically informative. Clegg (Clegg, 1993) observed that the process of nucleotide substitutions between purines (A-G) or pyrimidines (C-T) is generally considered biased, and indels occur twice as many as substitutions (Bakker et al., 2000). Therefore, the purpose of sequence analysis was to compare and compute the level of variation detected in Tylophora indica, Tylophora rotundifolia, and Tylophora fasciculata with the existing data base at NCBI.

The low sequence variations observed in tRNALeu (UAA) intron are deletions of a single base. Moreover, the subspecies of Tylophora studied can be easily distinguished by five nucleotide substitutions identified at various positions. The observed result is in accordance with earlier studies which show that minor substitutions and deletions can occur in spite of the secondary structure and catalytic property making this intron less polymorphic (Kuhsel et al., 1990).

62% A+T content of Tylophora indica, Tylophora rotundifolia, and Tylophora fasciculata in the sequenced tRNALeu (trnL) intron region was similar to other Tylophora tRNALeu (trnL) intron database information available in the GenBank. This high A+T content in angiosperms is assumed to be due to large proportion of transversions. This is in accordance with the observations of Bakker et al. (Bakker et al., 2000) that nucleotide substitutions are biased towards transversions rather than transitions. The AT rich regions are highly prone to replication slippage due to local intra-helical denaturation and displacement in replicating strands (Cummings et al., 1994). In spite of a high A+T content in the tRNALeu (trnL) intron region, A↔T transversion was found to be occurring less frequently than G↔C transversion in angiosperms (Yang, 1994; Bakker et al., 2000). This earlier observation corroborates the present BLAST results of Tylophora indica tRNALeu (trnL) intron. Reduced frequency of transversion is possibly due to fidelity in DNA replication.

The relative frequencies of nucleotide substitutions and indels in the noncoding chloroplast DNA sequences have raised conflicting hypothesis. Clegg (Clegg, 1993) proposed that, indels may occur more frequently than nucleotide substitutions, whereas Gielly and Taberlet suggested that indels occur with nearly the same frequency as nucleotide substitutions (Gielly and Taberlet, 1994). The present result agrees with the hypothesis of Clegg. Nucleotide substitutions were 31.6% and 68.4% were indels in Tylophora species.

Understanding the process and pattern of nucleotide substitution is important. It helps to estimate the number of substitution events between DNA sequences since their divergence. The process of nucleotide substitution is generally considered biased towards transitions/substitutions [between purines (A-G) or pyrimidines (C-T)], rather than transversions (between purines and pyrimidines) although twice as many transversions are possible. Moreover at low DNA sequence distances, this transition bias results in a substitution pattern that is characterized by transition/transversion (ti/tv) ratios typically ranging between 2 and 10 (Bakker et al., 2000). In Tylophora species this ratio was two.

A+T and A+G repeats generated by slippage during replication as observed in earlier studies on Gnetales (Won and Renner, 2005) were not observed in the present work on Tylophora indica, Tylophora rotundifolia, and Tylophora fasciculata.

The tRNALeu (trnL) intron is well suited for inferring plant phylogenies between closely related species for various reasons. Primarily, double stranded cpDNA can easily be amplified for a wide taxonomic range of plant species using the universal primers. Secondly, this noncoding region is small and can be sequenced by using only the amplification primers. Such regions of cpDNA can be used to resolve phylogenetic relationships at the intra-generic level. Furthermore, the primers are universal enough to work on a wide taxonomic range (Gielly and Taberlet, 1994). The cpDNA noncoding intron region exhibits lower level of variation than do nuclear base loci. Yet it provides more phylogenetic information characters as indels and substitutions in the 550 bases sequenced. A possible explanation for the low molecular divergence seen by us is that divergence in Tylophora complex occurred recently (Liede, 2001) so that neutral markers like tRNALeu (UAA) intron would not have had sufficient time to be fixed in different populations. The multiple alignment of the three species showed that, Tylophora indica and Tylophora rotundifolia are probably closer to each other than to Tylophora fasciculata (Fig. 2). The phylogenetic tree constructed (Fig. 3) based on the neighbour joining method for the 25 samples, grouped all Tylophora species close enough to affirm a lack of deviation. A close relationship between various Tylophora species and the low levels of cpDNA divergence also suggest the possibility of low gene flow in these three species of Tylophora.

Conclusion

The analysis of cpDNA sequences in the Tylophora species opens up a novel interpretation of relationships among the Tylophora species. This allows for more useful taxonomies to be generated. It emphasises the complexity of relationships between morphological and molecular markers. The low level of cpDNA divergence suggests the possibility of diminished gene flow.

In conclusion we have shown that DNA profiling is a powerful tool for identification of the original species of Tylophora. Sequences between 174 to 203 with three repeat sequences and the AT rich variable length can lead to authentic identification of Tylophora. The polymorphic insertions exclusively found at positions of 16 and 76 in the trnL intron of Tylophora rotundifolia suggest that this insertion could have been polymorphic in the common ancestor. In order to clarify theses points, further analysis of cpDNA variation need to be done. The trnL intron sequences thus helps to infer phylogenetic relationships in the genus Tylophora and is of help to understand the evolution of this species.

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Table. 1. The percentage distributions of the bases in the three Tylophora species.

Species	% A	% T	% G	% C
T. indica	35.45%	26.54%	20%	18%
T. rotundifolia	35.3%	26.1%	20.1%	18.3%
T. fasciculata	35.1%	26.1%	20.5%	18%

Table. 2. Proportion of mutation events analysed based on tRNALeu (UAA) trnL intron in certain Tylophora species accessed from NCBI.

Species	Accession number	TS/TV	ID	L	% variability
Tylophora yunnanensis	AJ320441	0	3	519	0.58
Tylophora villosa	AJ320435	0	3	519	0.58
Tylophora hirsute	AJ320420	0	3	519	0.58
Bionida henryi	AJ410191	1	3	519	0.77
Vincetoxicum stocksii	AJ410278	0	3	519	0.58
Vincetoxicum hirundinaria	AJ410275	1	3	519	0.77
Vincetoxicum atratum	AJ410269	1	3	519	0.77
Tylophora flexuosa	AJ320414	1	3	519	0.77
Tylophora apiculata	AJ410254	1	3	520	0.77
Tylophora anomala	AJ410251	2	3	519	0.96
Pentatropis madagascariensis	AJ410236	2	3	519	0.96
Tylophora heterophylla	AJ410260	1	3	519	0.77
Tylophora flanaganii	AJ410257	2	3	519	0.96
Tylophora tenuis	AJ320432	2	3	519	0.96
Tylophora coriacea	AJ320408	2	3	519	0.96
Tylophora biglandulosa	AJ320402	2	3	519	0.96
Tylophora sylvatica	AJ410266	1	3	519	0.77
Pleurostelma cernuum	AJ410242	2	3	519	0.96
Pentatropis nivalis	AJ410239	3	3	519	1.15
Blyttia fruticulosum	AJ410194	3	3	519	1.15

Table. 3. Substitution types in regions of trnL intron (UAA) in certain Tylophora species.

Species	Substitute type	% of substitution
Tylophora villosa	T↔G	0.19
Tylophora flexuosa	G↔C	0.19
Tylophora apiculata	G↔C	0.19

Tylophora anomala	G↔A, G↔C	0.38
Tylophora heterophylla	G↔C	0.19
Tylophora flanaganii	G↔A, G↔C	0.38
Tylophora tenuis	G↔C, A↔C	0.38
Tylophora coriacea	G↔A, G↔C	0.38
Tylophora biglandulosa	G↔C, A↔C	0.38
Tylophora sylvatica	G↔C	0.19
Tylophora flexuosa	G↔C, A↔T, T↔C	0.57
Tylophora parviflora	A↔C, G↔C, G↔A, T↔A	0.77
Tylophora tenuipedunculata	A↔C, G↔A, G↔C, G↔C	0.76

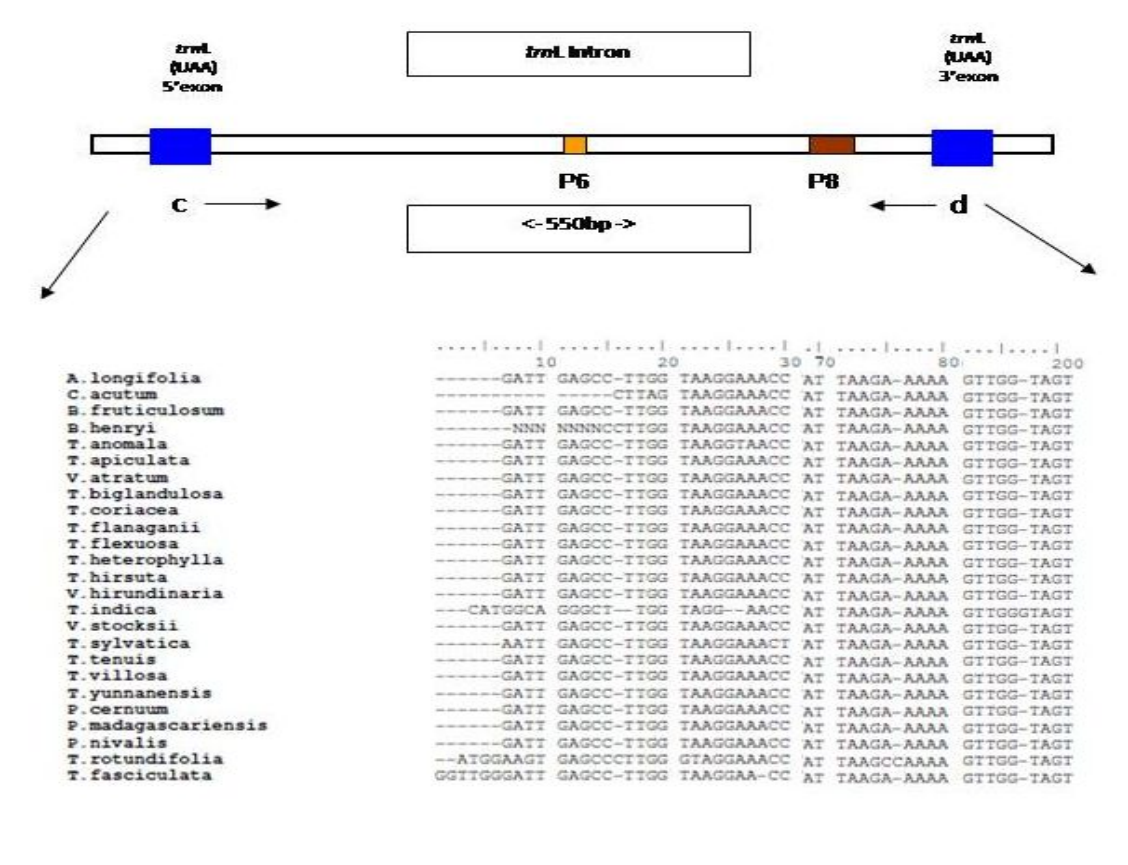

Figure 1. Sebastian et al. 2009

Figure 1. Positions and directions of universal primers (c and d) used to amplify the chloroplast trnL (UAA) intron region (Taberlet et al., 2006). c = Forward primer, d = Reverse primer.

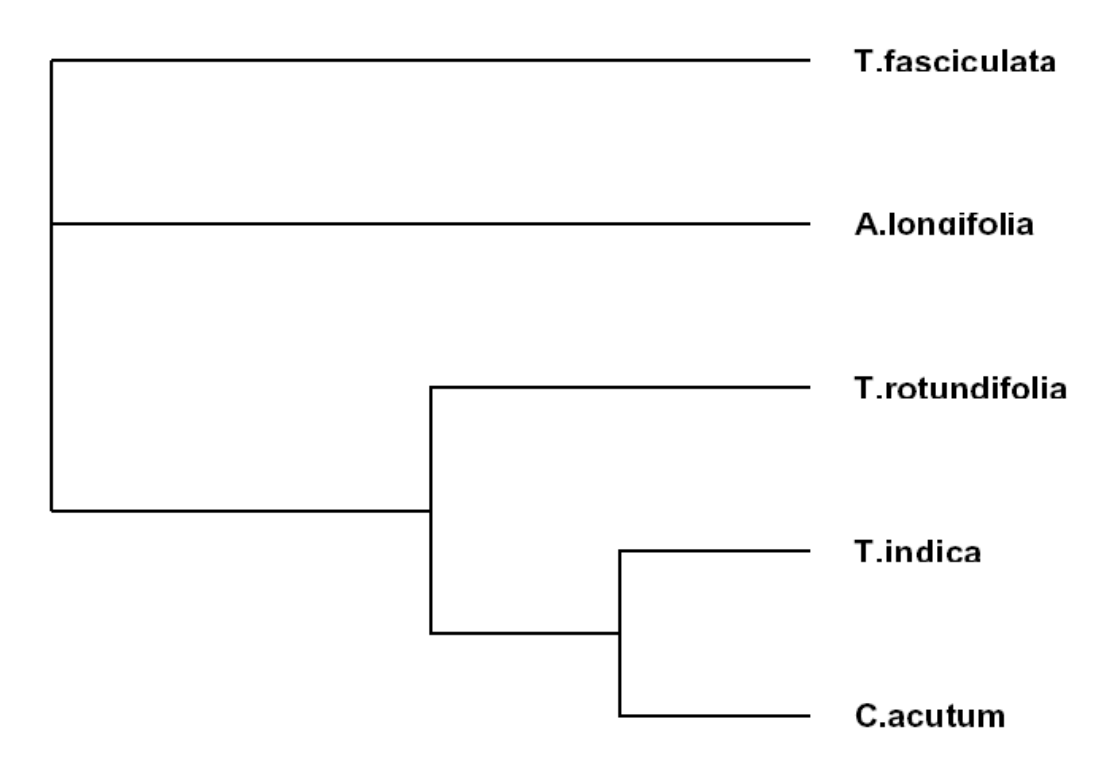

Figure 2. Sebastian et al. 2009

Figure 2. The maximum likelihood tree based on CLUSTAL analysis for the three species of Tylophoras. The outgroups are A. longifolia and C. acutum.

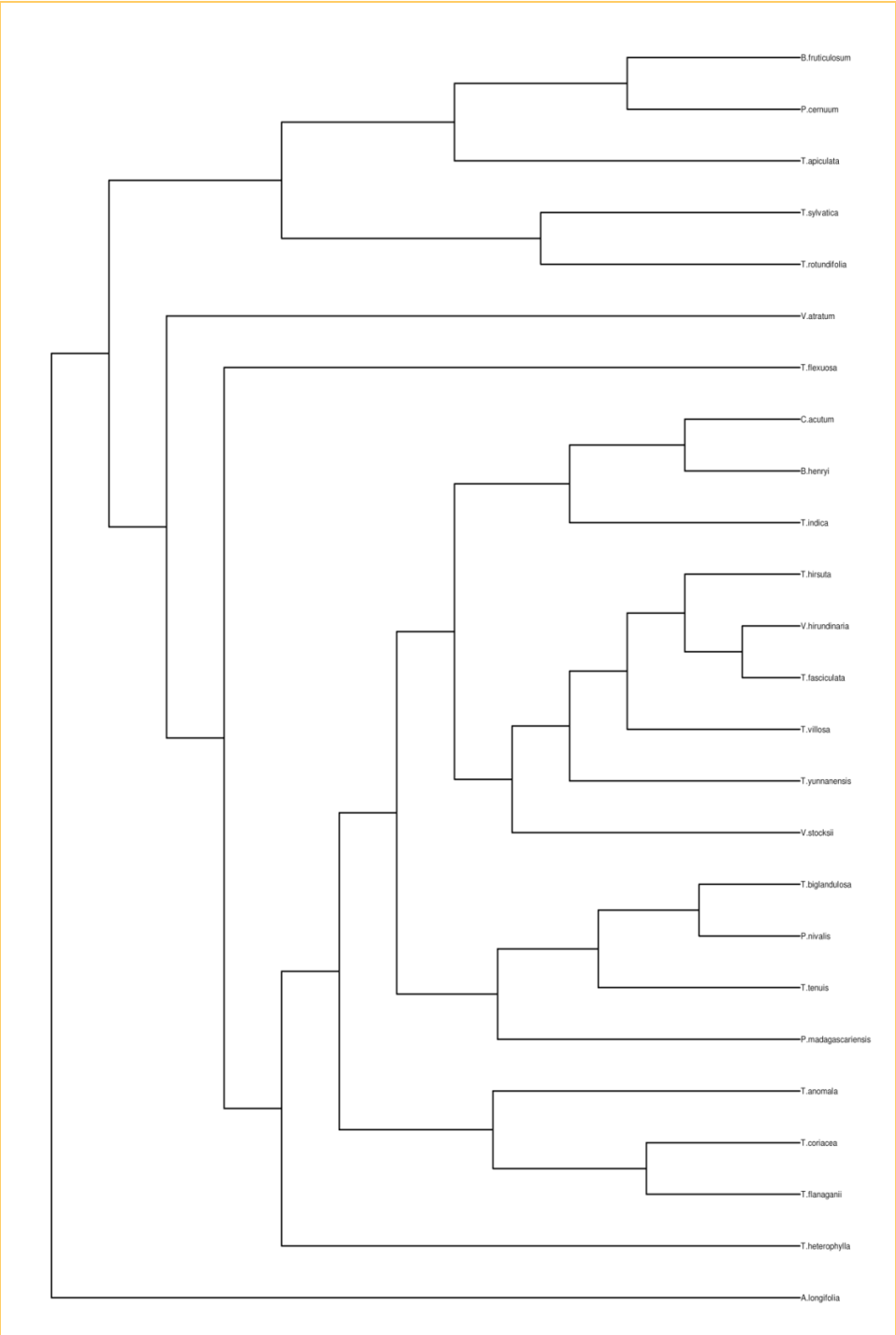

Figure 3. Sebastian et al. 2009

Figure 3. Phenogram showing the relation among 25 Tylophorinae species based on CLUSTAL analysis.