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A molecular phylogeny of the subfamily Plusiinae (Lepidoptera: Noctuidae) in India inferred from mitochondrial and nuclear ribosomal DNA sequences

Twinkle Sinha
Indian Agricultural Research Institute
P. R. Shashank (spathour@gmail.com)
Indian Agricultural Research Institute https://orcid.org/0000-0002-8177-6091

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Abstract

Background

The subfamily Plusiinae is an economically important moth pest group under species rich family Noctuidae (Lepidoptera). The evolutionary history of this important subfamily has not been resolved completely. Particularly in India, the genus is represented by a species complex, but the taxonomic delineation between them is still unclear.

Methods and results

In present study, a comprehensive phylogenetic relationship among Indian species of this subfamily has been inferred for the first time based on mitochondrial marker, Cytochrome Oxidase I (COI), and nuclear gene marker, Ribosomal Protein S5 (RPS5), emphasizing tribal level classification. Here, we analyzed 125 plusiinae taxa from eight biogeographical zones of India comprising 2 molecular markers: mitochondrial marker, Cytochrome Oxidase I (COI), and nuclear gene marker, Ribosomal Protein S5 (RPS5). The results revealed that Plusiinae tribes were monophyletic and considered sister groups that shared many derived characteristics. The ML/MP cladogram based on the barcoding gene successfully separates all the species, but not all tribes. While the nuclear gene marker, RPS5 separated all the species according to their tribes. The combined analysis of both genes showed tribe resolution into distinct clades.

Conclusions

This is the first comprehensive study on phylogenetic studies of 25 species of plusiinae from India which gives a clear information about species position and arrangement within taxa.

Introduction

The family Noctuidae is one of the diverse group of moths with approximately 11,772 described species under 1,089 genera throughtout the world [1] This is the most controversial family because of the constant changes in its phylogeny [2, 3]. Plusiinae is the smallest and type subfamily of Noctuidae, has a cosmopolitan range distribution, comprises approximately 500 species in 3 tribes and 36 genera worldwide [4]. They are a small group of moths, having different structural diversity in their morphology. They are fast flyers and their caterpillars, known as semiloopers, feed on garden vegetable, field crops, medicinal and ornamental plants [5, 6]. Most of the species belongs to this group are important crop pests, such as the cabbage looper (*Trichoplusia ni*), soybean looper (*Chrysodeixis includens*), alfalfa looper (*Autographa californica*), celery looper (*Anagrapha falcifera*), bean leaf skeletonizer (*Autoplusia egena*) etc. They cause major destruction through leaf feeding. The degree of damage caused by this subfamily from mild to severe depending on the genera. Till date, a checklist of total 59 species reported from India [7]. Some commonly found plusiines belong to genus *Thysanoplusia*, *Chrysodeixis*, and *Ctenoplusia*, while few species belonging to *Dactyloplusia*, *Cornutiplusia*, and *Plusiopalpa* which has its limited distribution. Many authors have contributed to describing Plusiinae from India including pest

species with isolated publications, relying only on morphological characters for species delimitation [8, 9]. Recently, few studies conducted by including morphological and DNA barcoding data on Plusiinae from India [10, 11, 12, 13, 14].

In last decade, molecular approach such as RAPD-PCR, AFLP markers, multiplex PCR which produce species-specific banding patterns, microsatellite/ ISSR markers results being interpreted and used to understand insect phylogenetics. We have DNA barcoded twenty five species in our previous work [11], however the phylogenetic analsysis was lacking to understand interrelationship among different taxa from India or elsewhere. Along with available COI gene data we have added nuclear gene RPS5 data to infer phylogenetic relationship within the Indian species and also discussed tribal level relationship.

Material And Methods

Taxon sampling and specimen acquisition

The Plusiinae specimens herein studied were collected using a light trap and white cotton screen. The collected specimen was further processed including pinning, wing spreading, drying, labeling and species were identified morphologically using available literature [4, 15]. All studied specimens were deposited in Lepidoptera laboratory, National Pusa Collection, Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi.

DNA extraction, amplification and sequencing

The total genomic DNA was extracted from the hind legs (preserved in 70% ethanol) of specimens using DNeasy Blood and Tissue Kit (Qiagen). The fragment of RPS5 gene was amplified by polymerase Chain Reaction (PCR) using universal pair of primers RpS5 F (5’- ATG GCN GAR GAR AAY TGG AAY GA -3’) RPS5 R (5’- CGG TTR GAY TTR GCA ACA CG -3’) [16].The concluding volume of the PCR mixture was 25 µl consisting of 12.5 µl of Thermo Scientific maxima hot start PCR master mix, 8.5 µl of molecular grade water, 1 µl each forward primer and reverse primer, and 2 µl of genomic DNA. PCR amplification was run under the followingcondition: initial denaturation for 5 min at 94°C followed by 35 cycles of denaturing for 30 sec at 94°C, annealing for 40s at 52°C and extension time of 40 s at 72°C, with a final extension for 5 min at 72°C .The amplified products were resolved in 1% agarose gel, stained by ethidium bromide, after a passage of 45 minutes at 80 V and visualized in a gel documentation system (DNr, Bio-Imaging systems, MiniLumi). With our expected band size on the gel 617 bp for RPS5 gene, the desired products were sent for sequencing at AgriGenome Lab, Cochin, Kerala.

DNA sequence dataset preparation

Sequences of all the specimen were checked for insertion, deletion and stop codons to avoidthe amplification of pseudogenes. The similarity check of generated sequences were performed through BLASTn in NCBI database. DNA sequences have a length 617bp for RpS5 gene. All the generated sequences from this study have been deposited in GenBank for accession no. (Table 1). All the

sequences were aligned by using Bioedit software version 7.2 and further analysed in MEGA (Molecular Evolutionary Genetics Analysis) version 11.0 [17].

Table 1
Plusiinae species with details of generated sequence accession Number

S. No.	Species	Accession no. COX I Gene (Retrieved from our previous paper)	Accession no. RPS5 Gene (This study)
1.	*Trichoplusia ni*	MN036490,MN036513 MN036470,MN036481 MN036485	MK861929 MW509640
2.	*Thysanoplusia intermixta*	MN036495	-
3.	*Thysanoplusia reticulata*	MN036517	MK861924 MW509627
4.	*Thysanoplusia orichalcea*	MN036492,MN036482 MK783022,MK783023 MK783024,MK783025	MK861923 MN122103
5.	*Thysanoplusia daubei*	MN036484, MN036485	-
6.	*Dactyloplusia impulsa*	MN036509, MN036510	MW509629 MK861925
7.	*Ctenoplusia albostriata*	MN036502, MN036469 MN036470, MN036471	MK861926 MW509631
8.	*Ctenoplusia furcifera*	MN033321, MN033322 MN033323	MK861927 MW509638
9.	*Ctenoplusia limbirena*	MN036468, MN036491 MN036489	MK000724 MW509632
10.	*Ctenoplusia placida*	MN036465, MN036466	MK840846 MW509630
11.	*Ctenoplusia mutans*	MN036493, MN036488	MK840845 MW509634

S. No.	Species	Accession no. COX I Gene (Retrieved from our previous paper)	Accession no. RPS5 Gene (This study)
12.	*Ctenoplusia tarassota*	MN036473, MN036507 MN036463, MN036499 MN036464, MN036487	MK861922 MW509633
13.	*Ctenoplusia kosemponesis*	MN036498	MW509639 MK783022
14.	*Plusiopalpa adrasta*	MN036465, MN036422	MN036485 MW509638
15.	*Argyrogramma signata*	MN036510, MN036518	MN036510 MW509663
16.	*Zonoplusia orcheata*	MN036474, MN036516 MN033324, MN033325 MN033326	MK861928, MW509635
17.	*Chrysodeixis acuta*	MN036512, MN036494 MN036500, MN036479	MK861930 MW509662
18.	*Chrysodeixis chalcites*	MN36277, MN36278	MK861931 MW509661
19.	*Chrysodeixis eriosoma*	MN036467, MN036477 MN036462, MN036475 MN036519, MN036466 MN036505, MN036478	MW509660 MF140488
20.	*Scriptoplusia nigriluna*	MG648733, MN036520	MW509639 MW509640
21.	*Anadevedia peponis*	MN036496, MN036497 MN036483, MN036480	MW465946 MW465946
22.	*Macdunnoughia tetragona*	MN036521, MN036501 MN036508	MW509628 MW509628
23.	*Sclerogenia jessica*	MN036486, MN036504	-

S. No.	Species	Accession no. COX I Gene (Retrieved from our previous paper)	Accession no. RPS5 Gene (This study)
24.	*Antoculeora ornatissima*	KY886404, KY886405, KY886406, KY886407 KY886408	-
25.	*Autographa nigrisigna*	MN036476, MN036472	MW509627
		MN036514, MN036461	MW509629

Phylogenetic analysis

The COI gene sequences of Plusiinae, 81 sequences we used from our published paper [11] (Table 1) and downloaded 48 sequences from Barcode of Life Datasystem (BOLD)/ National Centre of Biological Information (NCBI) database were prepared for phylogenetic analysis and comparison between sequences of Indian Plusiinae with rest of the world population. A total 129 sequences were aligned with Clustal W [18] and analyzed using Maximum Likelihood (ML) and Maximum Parsimony (MP) tree based on Kimura-2-Parameter [19] distances with MEGA (Molecular Evolutionary Genetics Analysis) version 11 [17]. The best-fitting model of nucleotide substitution was GTR + I + G for the genes, the composition and the K2P distance between and within species were calculated by averaging pairwise comparison of sequence difference across all individuals and imported as a Microsoft Excel file for our analysis. The ML tree was displayed by MEGA 11.0 and to verify the robustness of the internal node of the ML tree, bootstrap analysis [20] was carried out using 2000 pseudoreplication.

Results

Characterization of DNA sequences

We have given all the details of the genetic region with their sequence variable sites and parsimony informative sites which were observed (Table 2). The base composition of the sequences showed a strong bias of A + T which is commonly found in insect mitochondrial genomes [21, 22].

Table 2
Number of taxa and characters for the three gene regions explained.

Gene region	Number of sequences	Alignment length	A (%)	T (%)	C (%)	G (%)	Variable sites	Parsimony informative sites
COX I	81	658	40.2	15.5	29.7	14.6	239	215
RPS5	44	617	23.9	26.1	23.8	26.1	143	108
Combined	42	1275	27.05	32.39	20.48	20.06	317	191

Nuclear gene RPS5 (Ribosomal Protein S5)

Ribosomal protein S5 is a protein-coding nuclear gene that is used for the identification of species up to family, subfamily, and tribe level showing variation in mutation speed and phylogenetic depth We have used this gene to differentiates between the two tribes having the highest number of variable sites observed. For deeper phylogeny (Family/tribe level) COX 1 is not useful because it tends to reach saturation and is not informative enough (especially the third codon position). In this case slow evolving genes from nuclear genome can help. We have also generated RPS5 sequence data (21 species) of Plusiinae and submitted it to the NCBI database. All the sequences of this gene belonging to 21 species (42 sequences) are novel from India and submitted for the first time from India and around the world.

Phylogenetic analysis

The pair-wise genetic distance among species of tribe the Argyrogrammatini ranges from 1.1–11.2%. According to the pairwise gene distances, the shortest and largest distance was obtained between *C. eriosoma* and *C. acuta* (1.1%); *A. peponis* and *A. signata* (11.2%) respectively. The maximum intraspecific divergence ranged from 0–2.9%. Similarly, the pair-wise genetic distance among species of the second tribe Plusiini ranges from 0.075 to 0.099. ML and MP tree of Plusiines represent 25 major clades for 25 different species (Fig. 1). According to the pairwise gene distances the intra-generic genetic divergence between genus *Sclerogenia* and *Antoculeora* is 10.2% and the lowest between *Antoculeora* and *Macdunnoughia* 7.5%. The molecular diversity analysis revealed negligible intraspecific variation for *A. nigrisigna* population of India and Pakistan and *A. ornatissima* from China and India with pairwise genetic distance ranging from 0.2–3.1%. Interspecific genetic divergence between species belonging to 14 different genera with 25 species was calculated by Kimura 2-parameter model (K2P) represented in Table 3.

Table 3
Interspecific divergence of species of tribe Argyrogrammatini and Plusiini based on mitochondrial cytochrome oxidase I (COI)

S.No.	Species	Interspecific genetic divergence	Tribe
1.	*Chrysodeixis chalcites*	0.112	**Argyrogrammatini**
2.	*Anadevidia peponis*	0.112	
3.	*Argyrogramma signata*	0.055	
4.	*Chrysodeixis acuta*	0.039	
5.	*Chrysodeixis eriosoma*	0.011	
6.	*Thysanoplusia orichalcea*	0.067	
7.	*Trichoplusia ni*	0.050	
8.	*Ctenoplusia tarassota*	0.057	
9.	*Scriptoplusia nigriluna*	0.054	
10.	*Ctenoplusia limbirena*	0.055	
11.	*Dactyloplusia impulsa*	0.054	
12.	*Plusiopalpa adrasta*	0.073	
13.	*Zonoplusia ochreata*	0.058	
14.	*Ctenoplusia mutans*	0.073	
15.	*Ctenoplusia albostriata*	0.054	
16.	*Thysanoplusia daubei*	0.046	
17.	*Thysanoplusia reticulata*	0.055	
18.	*Ctenoplusia placida*	0.059	
19.	*Ctenoplusia furcifera*	0.045	
20.	*Thysanoplusia intermixta*	0.037	
21.	*Ctenoplusia kosemponesis*	0.072	
22.	*Autographa nigrisigna*	0.106	**Plusiini**
23.	*Sclerogenia jessica*	0.099	
24.	*Antoculeora ornatissima*	0.102	
25.	*Macdunnoughia tetragona*	0.075	

We have sequenced 21 species (4 species data not being successfully generated so we have removed those species) data of Plusiinae based on RPS5 gene and phylogenetic analysis was separately done for this gene. The intraspecific distance of all species is given (Table 4). The maximum intraspecific variation was observed between *Anadevidia peponis* and *Macdunnoughia tetragona*i.e 12.1% and minimum intraspecific distance was calculated between *Chrysodeixis eriosoma* and *Chrysodeixis chalcites* is 0.5%. The phylogenetic tree depicts the two tribes Argyrogrammatini and Plusiini cluster separately (Fig. 2) and also segregate all the species. For phylogenetic analysis best fit model GTR + G + I with bootstrap value 2000 replicates were used in the present evolutionary tree with MEGA 11.0 software.

Table 4
Interspecific divergence of species of tribe Argyrogrammatini and Plusiini based on RPS5 gene sequences

Species	Interspecific genetic divergence
Dactyloplusia impulsa	0.037
Ctenoplusia placida	0.037
Ctenoplusia albostriata	0.027
Ctenoplusia tarassota	0.027
Ctenoplusia limbirena	0.015
Ctenoplusia furcifera	0.032
Ctenoplusia kosemponesis	0.020
Ctenoplusia mutans	0.053
Zonoplusia ochreata	0.010
Thysanoplusia reticulata	0.022
Thysanoplusia orichalcea	0.030
Trichoplusia ni	0.030
Plusiopalpa adrasta	0.035
Scriptoplusia nigriluna	0.032
Chrysodeixis eriosoma	0.017
Chrysodeixis chalcites	0.005
Chrysodeixis acuta	0.022
Argyrogramma signata	0.035
Anadevidia peponis	0.101
Macdunnoughia tetragona	0.121
Autographa nigrisigna	0.055

Combined data analysis of COI and RPS5 gene

Maximum likelihood (ML) analysis of combined gene region (COI, RPS5) was constructed in MEGA 11.0 (Fig. 3) using the best-fit substitution model shown with a bootstrap value of 2000 replicates. This combined dataset phylogenetic tree is also informative and constructed to compare the result with other single gene COI and RPS5 gene. The tree of combined dataset analysis is almost similar to COI and RPS5

gene tree and shows the similarity with the species arrangement and tribe placement except very few species displacements.

Discussion

This is the first initiative of evolutionary studies of 25 species of subfamily Plusiinae from India. Starting from COI sequences with two different phylogenetic analysis methods: Maximum Parsimony (MP) and Maximum Likelihood (ML), the topology of these methods was similar but reshuffle some species position within the tribe. The pair-wise genetic distance is same, and the result indicates ML tree is most suitable. **T**he species also shows the monophyly for two tribes (considered as sister groups), which shared many derived characteristics. The pairwise gene distances based on COI gene/the shortest distance was obtained between *C. eriosoma* and *C. acuta* (1.1%) that showed lower divergence, no insertion/deletion of nucleotides, and the largest genetic distance was obtained between *A.peponis* and *A.signata* (11.2%). In Lepidoptera, COI sequence divergence is > 2% for species discrimination [23]. In the case of *C. eriosoma* and *C. acuta* lower interspecific divergence is observed and these species are also considered as sister species because most of the morphological characters are very similar and they are very confusing in their appearance and shared most of the similar characters. The results for the intergeneric divergence, the maximum intergenic difference was observed between *Anadevedia* and *Argyrogramma* which is 12.3% and lowest between *Chrysodeixis* and *Thysanoplusia* i.e 5.9%. In the tree topology, the species belonging to the genus *Ctenoplusia, Dactyloplusia, Thysanoplusia*, *Argyrogramma* always make their clades next to each other. Remaining *Trichoplusia, Scriptoplusia, Plusiopalpa, Zonoplusia*, and *Chrysodeixis* are also nearby clades, and *Anadevidia* is always present at the base of tree representing, Argyrogrammatini is getting diversed. Similarly, genus *Macdunnoughia, Autographa, Antoculeora* and *Sclerogenia* present at the base of the tree making their own cluster.

While based on RPS5 gene the maximum intraspecific variation was observed between *Anadevidia peponis* and *Macdunnoughia tetragona* i.e 12.1% and minimum intraspecific distance was observed between *C. eriosoma* and *C. chalcites* is 0.5%. The COI tree differentiates among species but doesn't differentiate between tribe as well as RPS5 cluster two tribes separately. The topology of the species is almost similar the species belonging to genus *Ctenoplusia, Dactyloplusia, Thysanoplusia*, *Argyrogramma* always make their clades next to each other. The remaining *Trichoplusia, Scriptoplusia, Plusiopalpa, Zonoplusia* and *Chrysodeixis* are also nearby clades, and *Anadevidia* always present at the base of tree representing, Argyrogrammatini is getting diversed. Similarly, genus *Macdunnoughia* and *Autographa* presnt at the base of the tree. This tree depict two separate tribe cluster. The tree of combined dataset analysis is also similar with COI and RPS5 gene tree in species arrangement, but does not separates two tribes like our RPS5. We concluded that for species level species differentiation COI gene is useful and for tribe level differentiation RPS5 is very useful. We will add more genes in our upcoming studies for a detailed analysis of this group of moths. Our main goal is to discover the phylogenetic relationships within the species based on different gene markers and it is clearly represented with the single genes and the combined gene analysis. The present dataset is also will be the first effort toward the molecular

studies and evolutionary analysis of Plusiinae species and to compare the species diversity. To date, all these studies are not known in Plusiinae and these are new additions for this group of moths.

Declarations

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Compliance with Ethical Standards

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Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Informed consent

Not applicable.

Author contributions

TS and SPR collected the specimen, conceptualized and designed the study. TS and SPR performed the experiments and data analysis. TS and SPR wrote the manuscript. SPR recived funding for this study.

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Figures

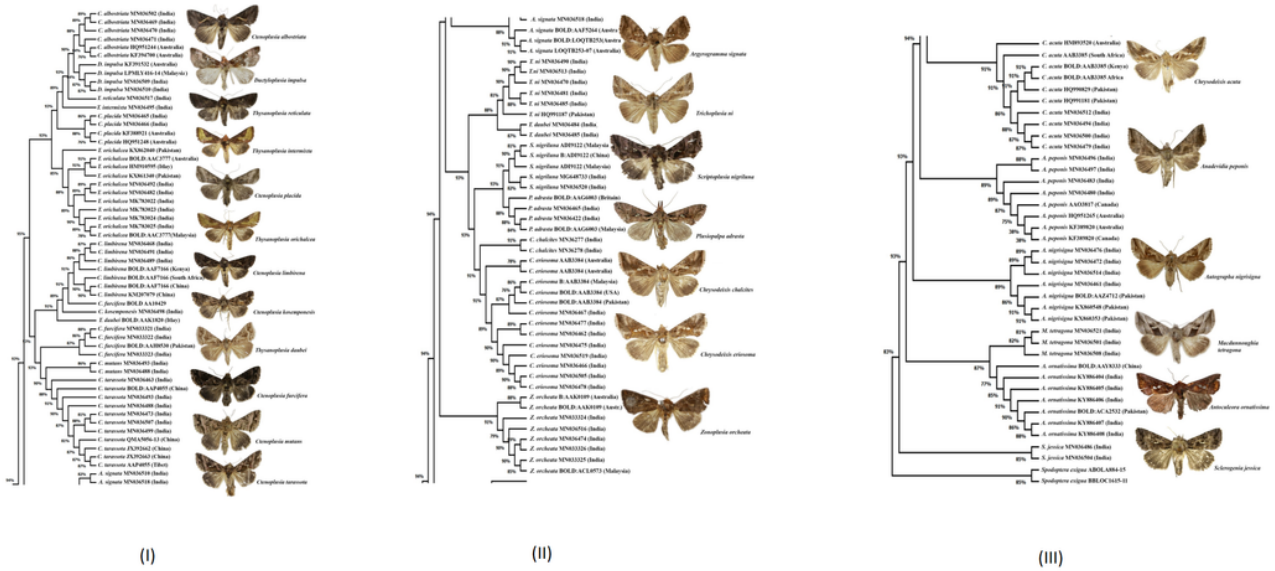

Figure 1

Maximum Likelihood (ML) tree based on the COI sequence dataset of the Plusiinae species

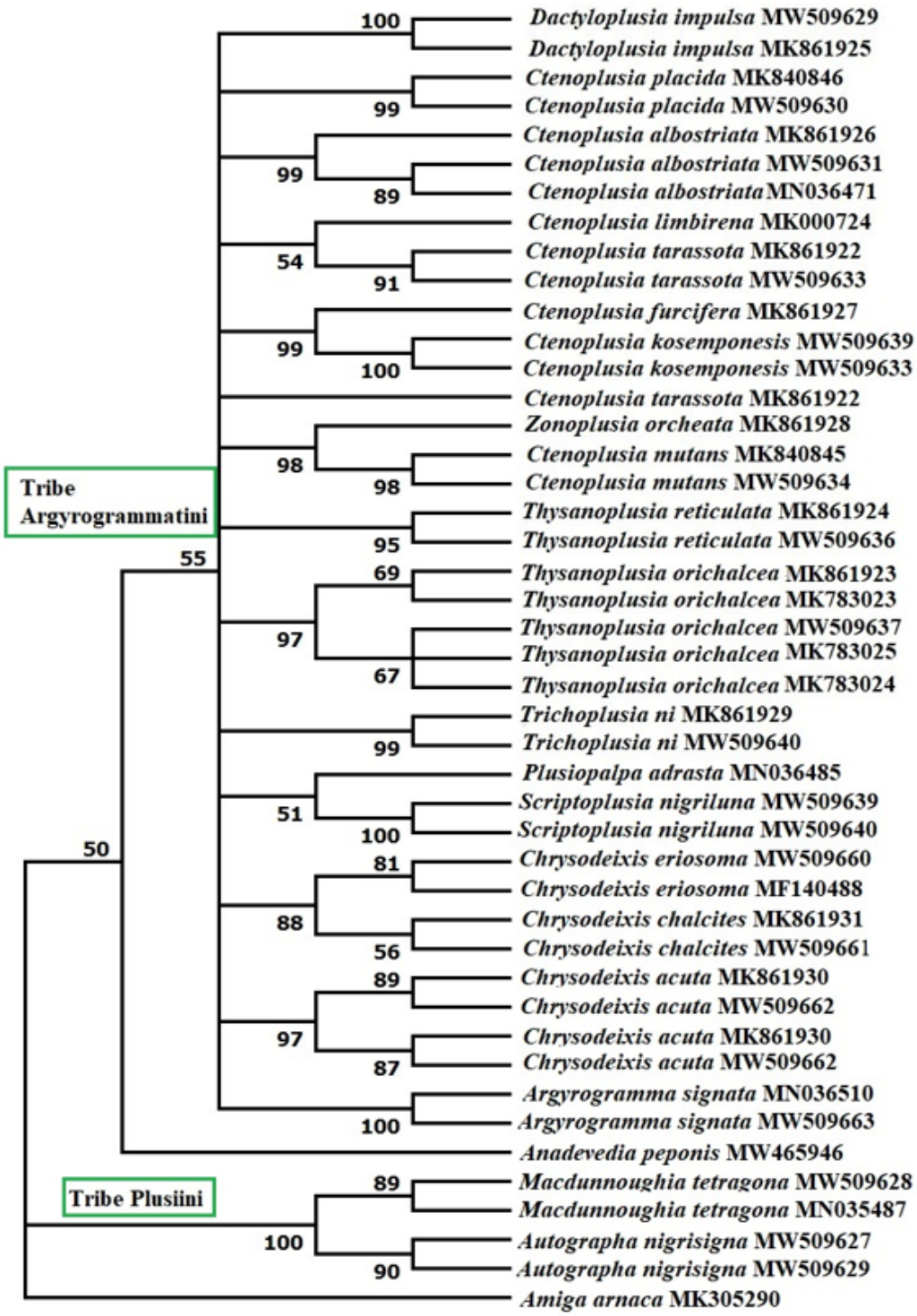

Figure 2

Neighbor joining tree of Tribe Argyrogrammatini and Tribe Plusiini based on RPS5 gene sequences

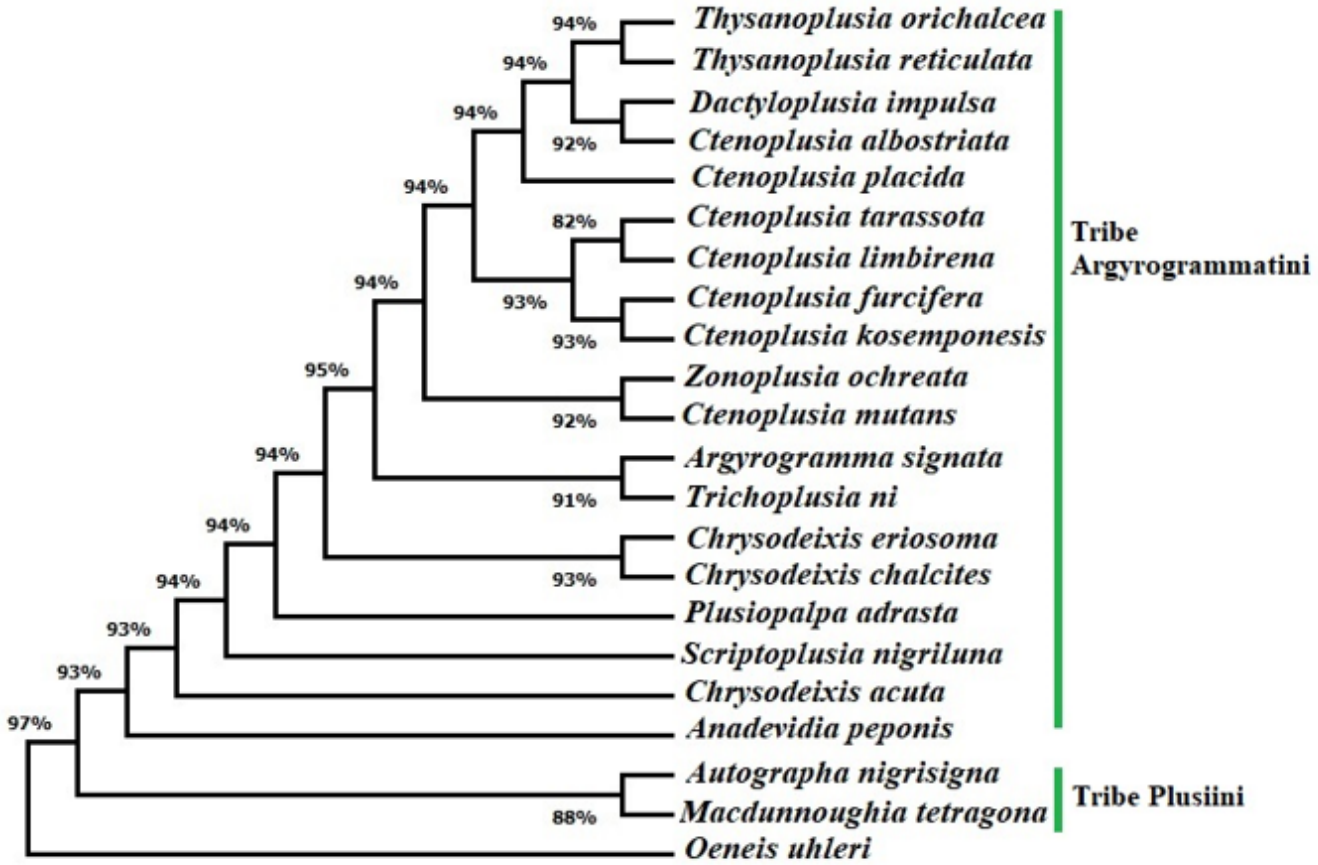

Figure 3

Maximum-likelihood tree estimated from combined dataset (Cox1 and Rps5 gene) of Tribe Argyrogrammatini and Plusiini in this study