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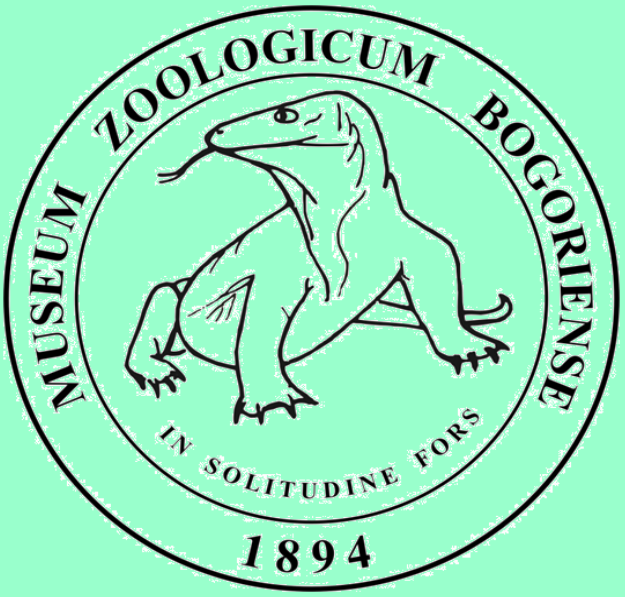

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Editor's Note

Another yearly volume of Treubia is published. I have only recently become involved in the publication of this journal and I can say that the research in this issue is increasingly interesting. I hope to remain actively involved in the publication of this journal and that we can continue to reach a larger audience as time goes on.

This volume of TREUBIA contains 5 papers of vertebrates and invertebrates. The contents of these papers vary widely from vocalizations of frogs to tropical forest spider communities. I can only hope in the future that we continue to receive interesting submissions from all areas of zoology of the Indo-Australian Archipelago.

Also this year two esteemed colleagues from LIPI retired from the service of science, Dr. Mas Noerdjito who studied the ecology of birds and Dr. Agustinus Suyanto who dedicated his life to the study of mammals.

Finally I would like to thank all of the co-editors, referees, computing assistants, secretaries and administrative assistants for their collaborative work without which this journal could not be published. I also acknowledge financial support from the Director of Research Center for Biology, LIPI to publish this essential journal.

Cibinong, December 2011

Chief Editor

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MOLECULAR PHYLOGENY OF INDONESIAN AGANAINE MOTHS (Lepidoptera: Noctuidae) BASED ON CO I GENE

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ABSTRACT

Systematic of Aganaine moths has been long in dispute since they show both noctuids and arctiids morphological characteristics. Even the relationship among genera within Indonesian Aganaines is still unclear, and their phylogenetic relationships need to be reexamined since the morphological hypothesis proposed previously was not able to show the relationship among them. In order to clarify the phylogenetic relationship among five genera of Indonesian Aganaines, I used sequence of mitochondrial CO I gene (610-bp) to reconstruct their phylogenetic relationship using MP and NJ tree building methods. The results showed that the phylogenetic relationship proposed in this study contradicts the previous hypothesis. The monophyly of subfamily Aganainae has a strong bootstrap support at any tree building methods (88-95%). *Neochera* was divided into two clades and branched off first and then was followed by *Euplocia*, *Peridrome*, *Agape*, and *Asota*. The similarity between the previous hypothesis and this study is only on the sister-group relationship between *Euplocia* and *Peridrome* and the division of *Neochera* into two clades. The synapomorphy of *Euplocia* + *Peridrome* is a large androconial patch on the forewing upperside at the costal base. This study also showed that all internal nodes gained least supports. It indicates that the relationships among internal nodes proposed here were poorly supported due to the limited number of species and only a short fragment of one mitochondrial gene included in the analysis. Further studies are needed to be done by including more other species, other nuclear genes, and genitalia characters in order to test the validity of the relationships proposed here.

Key words: Aganaine, *Asota*, *Agape*, *Euplocia*, *Peridrome*, *Neochera*, phylogeny.

INTRODUCTION

Aganainae, a small subfamily of Noctuidae, include about 100 species placed in 11 genera: *Psephea* Billberg, *Asota* Hübner, *Euimata* Billberg, *Euplocia* Hübner, *Hypsa* Hübner, *Lacides* Walker, *Neochera*

Hübner, *Peridrome* Walker, *Philona* Walker, *Agape* Felder, *Digama* Moore (Munroe 1982, Kitching & Rawlins 1998). They distribute throughout the tropical and subtropical areas of the Old World. In Indonesia, at least five genera are recorded and preserved in the Museum Zoologicum Bogoriense Collection: *Asota*, *Euplocia*, *Neochera*, *Peridrome* and *Agape*. Holloway (1988) listed also the same genera based on Bornean fauna.

The adults are generally brightly coloured. Most of them are nocturnal and are readily attracted to light (Common 1990). Two genera, *Peridrome* and *Euplocia*, exhibit extreme sexual dimorphism, males having large androconial patches on the forewing upperside at the costal base. The larvae are sometimes aposematic, being patterned variously in black and white, especially in species that oviposit egg masses, which develop into groups of gregarious larvae. Other species oviposit singly and their larvae are usually cryptic. Larvae appear hairy because primary setae are very long and arise from chalazae (Holloway 1988). Moraceae, Apocynaceae and Asclepiadaceae have been reported as the host-plants of the larvae (Kitching & Rawlins 1998, Holloway 1988, Common 1990).

The status of Aganainae has been long disputed since the moths in this subfamily show both noctuid and arctiid characteristics. Aganaines share three character states with Arctiidae: a bar-like retinaculum in males, a prespiracular tympanal hood, and loss of the adenosma (ventral prothoracic gland) in the larvae. However, Aganainae lack the two derived character states of Arctiidae (the metathoracic microtymbal organs and dorsal eversible pheromone glands associated with the anal papillae in females) used by Kitching & Rawlins (1998) to define Arctiidae. However, absence of these two arctiid synapomorphies does not exclude the possibility that Aganainae are the sister group of Arctiidae, with the association supported by the three character states listed above. On the other hand, based on molecular studies Mitchell *et al*. (2006) stated that sister-group relationship between Aganainae and Herminiinae has strong (>90%) bootstrap support, but this association is not supported by morphological characters. Therefore, the association of Aganainae with Herminiinae must await confirmation by other data sources, such as molecular studies using more nuclear genes, longer gene sequences, and more taxa (only two genera of aganaines and two herminiines were included in Mitchell *et al*. (2006)). Other studies have shown that Aganainae have a prespiracular tympanal hood, a character state shared with Herminiinae (and Arctiidae), but the bar-like retinaculum in males, loss of the adenosma, and fully quadrifine hindwing venation all suggest a closer association of Aganainae with Arctiidae rather than with Herminiinae (Fibiger & Lafontaine 2005, *Lafontaine & Fibiger 2006*).

Not only its relationship with the other subfamilies within Noctuidae, but the relationship among genera within this subfamily itself also is still disputed. Based on Bornean fauna, Holloway (1988) presented a clear hypothesis for the relationship among genera within this subfamily based on morphological characters, with the exception of the the position of the genus *Agape* which remained unclear (Fig. 1). Obviously, the phylogeny of subfamily Aganainae needs to be reexamined using more explicit procedures, such as molecular evolution studies. *Cytochrome Oxidase subunit I* (CO I) has been reported to be useful to reconstruct the relationship among genera within Tribe Lamiini (Coleoptera: Cerambycidae) (Toki & Kubota 2010). Moreover, CO I is relatively conserved compared to other mitochondrial genes such as CO II (Sutrisno *et al*. 2006, Sutrisno 2006, Hebert *et al*. 2010).

In the present study, I used nucleotide sequences of the mitochondrial protein coding gene CO I to reexamine the relationship among genera within subfamily Aganainae of the Indonesia fauna. The other goal of this research is also to populate the genetic information of each species of Indonesian Aganaines and to clarify the identity of each species distributed in Indonesia. Those identities are very important to justify any invasive species of this subfamily from other countries that may enter Indonesia. Moreover, available sequencing data of Indonesia fauna will lead to the next step in clarifying the whole phylogenetic relationship of this subfamily and even in establishing its classification.

MATERIAL AND METHODS

Moth specimens

A total of 16 species/subspecies of four genera were collected from several localities in Indonesia (Table 1). No fresh materials of the genus *Peridrome* were available in our collections therefore we included sequence of *P. subfascia* from the CBOL database with sample ID No. YB-KHC1728 in our analysis. The voucher specimen was deposited in Smithsonian Tropical Research Institute Center for Tropical Forest. Sequence of *Neochera mormorea* from CBOL with sample ID No. YB-KHC3639 also was included. The voucher specimen was deposited in Smithsonian Tropical Research Institute Center for Tropical Forest as well. Adult moths were collected by using light traps and were preserved in absolute ethanol.

For the outgroups, I chose three species of Arctiidae with their accession numbers: *Spilosoma lubricipedum* GU654842; *S. urticae*: HQ565498; and *Creatonotos transiens* HQ006198.

Species identification

Species identifications was conducted based on external and internal characters. The genitalia slides were prepared by the customary method of boiling in 10% potasium hydroxde for about 10-11 minutes. Disection of Genitalia was performed under a binocular steroscopic microscope.

DNA extraxtion and sequencing CO I gene.

For DNA extraction from each individual, a thorax was ground in a 1.5 ml microcentrifuge tube containing 600 ul CTAB buffer with 4% olyvinyl pyrrolidone and incubated at 55 °C for 2 hours. The thorax was used as DNA sources since almost all specimens were not preserve within absolute ethanol (using a single leg, probably produce a small number of DNA). We preserved all the genitalia into slides. This solution was extracted several times using phenol saturated with TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA); firstly with one volume of phenol: Chloroform: iso-amyl alcohol (25: 24: 1). The solution was again extracted for a second time with chloroform: iso-amyl alcohol. The aqueous phase was transferred to a new tube, and then 1,5 volume of isopropanol was added to precipitate DNA and left at -20 °C for more than 1 hour. The DNA precipitate was pelleted by centrifugation at 15.3 g for 20 minutes. The DNA pellet was washed with 70% ethanol, air-dried and dissolved in 50 ul of TE buffer.

The complete sequences of the primers used are: LepF1: 5' ATT CAA CCA ATC ATA AAG ATA TTG G 3', and LepR1: 5' TAA ACT TCT GGA TGT CCA AAA AATCA 3' (Hebert *et al*. 2010). The amplification was conducted in the following PCR conditions: one cycle of denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 92 °C for 30 sec., annealing at 47 °C for 30 sec, and extension at 72°C for 1 min. 30 sec. These cycles were completed by a final extension at 72°C for 10 min (Sutrisno 2008).

The PCR products were purified using Qiaquick PCR purification Kit (Qiagen Inc., USA). Sequencing was performed using ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit (Perkin-Elmer Inc., USA) on ABI PRISM model 310 Genetic analyzer (Applied Biosystems Inc; USA). The sequence were alignment using BioEdit sequence align-ment Editor (Hall 1999).

Base composition analysis.

I used the base frequencies option in PAUP* version 4.0b. 10 for 32-bit Microsoft Windows to evaluate the base composition of each

sequence and the homogenity of the base frequency across taxa. For the sequence divergence I selected K2P distance model since we assume that substitution of transition occurs twice than transversion within this gene.

Phylogenetic analysis.

Phyloegenetic analyses were performed with PAUP* version 4.0b. 10 for 32-bit Microsoft Windows based on CO I gene sequences by using Maximum Parsimony (MP) and Neighbor Joining (NJ) approaches (Swofford 2001). The statistical confidence of the two methods were evaluated using bootstrap test with 1000 replicates.

Results

Eighteen sequences of 18 species/subspecies of five genera of Aganaines and three species outgroups (*Spilosoma lubricipidum* and *S.urticae* and *C. transiens*) were aligned with no evidence of insertion and deletion. Conserved region was found at position between 447-460 (14-bp: TTTGATCAAATACC) and between 552-562 (11-bp: CGAAATTTAAA). Aligned sequences have been submitted to the Gen-bank with accession numbers presented on the Table 1.

Over the entire 631-bp region of both the ingroup and outgroup sequences, 70% (442) of the nucleotide positions were constant, 9.82% (62) were uninformtive (i.e. any variants were found in a single sequence and 20.12 % (127) were parsimony informative (Table 2). Informative sites in the 3rd codon position were the highest (16.9%), whereas those in 2nd codon position was the lowest (0.3%).

Sequence divergence

The mean pairwise sequence divergences of CO I gene based on K2P distance model within genera *Asota* and *Neochera* were 5.62% and 7.6%, respectively. While that of between genera was slightly higher than that of within genus *Neochera* (9.21%). The closest of it within genera was a pairwise between species *A. heliconia* with *A. heliconia zebrina* (1.58%). The most distant within genus *Asota* was between *A. javana celebensis* with *A. albiformis* and *A. plaginota* with *A. egens* (8.55%) whiles within *Neochera* was between *N.privata* and *N. dominia* (8.71%).

Figure 2 shows the relationship between pairwise distance for Transition (Ts) and Transversion (Tv) based on K2P distance model. Ts almost linearly increased with respect Tv and exceed Tv in all pairwise species comparison and its linear regression was $Y=0.2095+0.0248$, $R^2= 0.0157$.

Figure 3 shows the scatter plot of K2P distances between Transition/Transversion (Ts/Tv) and all substitutions in CO I gene. The means of Ts/Tv ratio in CO I gene was high (2.62) for insect mitochondrial gene, even within genera that value is higher (4.673). The highest ratio was found on the pairwaise *A. austalia* and *A. plaginota* (13.33), while the lowest on those *Peridrome* and *A. egens egens* (0.47).

Maximum Parsimony

Heuristic search option was applied in our Maximum Parsimony analysis to search the most parsimonious tree among million trees that are reconstructed from 21 taxa (18 ingroup taxa and 3 outgroup taxa). I evaluated several options such as weighting transition: transversion= 1:2, including transversion only, etc in order to obtain the most reliable parsimonious tree. The results of this evaluation are presented in Table 3.

The results of MP analysis showed that there was no single tree topology that was able to resolve the relationships either among species within genera or among the five genera with a confident strong bootstrap support. The strict consensus tree resulted from all substitutions (Fig. 4) and the weighting of transversion twice that of transitions provided strong support for the monophyly of the subfamily Aganainae (93-95% of bootstrap support) (Fig.5). Both these topologies consistently showed the same relationship among the five genera: *Neochera* was branched off first, then was followed by *Peridrome*, *Euplocia*, *Agape* and *Asota.*

Genus *Asota is* a monophyletic group even though it was supported by only a low bootstrap value (67-70%). Our two tree topologies clearly showed that *A. javana celebensis* branched off first. The relationship among other species within this genus were not consistent between the two topologies, except for the sister-group relationships between: *A. heliconia* and *A. heliconia zebrina*, *A. albiformis* and *A. heliconia lanceolata*, as well as between *A. plana* and *A. caricae*. The first sister group relationships was consistently supported by a high bootstrap value (100%).

Neighbor Joining (NJ)

To reconstruct the Neighbor Joining tree, I applied distance-based on Kimura-two parameter (K2P) and HKY 85 models (Kimura 1980; Hasegawa *et al*. 1985). There were no differences in topology, therefore only NJ tree based on K2P model is presented in Figure 6. Based on the tree, it seems that genus *Neochera* which branched off first was divided into two groups: (*N. mormorea* + *N. dominia*) and (*N. privata* +*Peridrome*). This grouping is also somewhat similar to MP trees in

which a sister group (*N. mormorea* + *N. dominia*) was branched off first and then was followed by *N. private* and other four genera.

Discussion

The results of this study showed that CO I genes from 18 species/ subspecies of five genera Aganaines moths were high A+T biased. It is consistent with mitochondrial genomes of other Lepidoptera previously reported by many authors (Reviewed in Simon *et al*. 1994) In other genera within families of Lepidoptera, high A+T contents have been found in *CO I* of *Choristoneura* (Arctiidae) (Sperling & Hickey 1994), *Hemileuca* (Arctiidae) (Rubinoff & Sperling 2002), *Glyphodes* (Crambidae) (Sutrisno 2003, Sutrisno *et al*. 2006) which ranged from 62 % to74 %. The average of A+T proportion in the present study (68.7%) was comparable with those found in other families of Lepidoptera. In addition, the bias in base compositions was found to be the greatest at third-base position.

The sequence divergence of CO I gene within subfamily Aganainae was relatively high (9.21%). It highlights the fact that genera within subfamily Aganainae are species-rich and very diverse, especially within genus *Asota* and *Neochera*. Within these two genera, the mean of sequence divergence is comparable to those found within (the species-rich) group of *Glyphodes* (5.92-7.55%) (Sutrisno 2006).

The present study revealed that the transition/transversion ratio of *CO I* within subfamily Aganainae was 2.62. It indicates that Transitions (Ts) occur more frequently than Transversions (Tv), and Ts values are usually expected to exceed Tv values (DeSalle *et al*. 1987); however, it has been reported for CO II that Tv values exceed Ts values (Simon *et al*. 1994, Goto & Kimura 2001). By contrast, the *COI* gene in this study indicated that this gene was not yet saturated with transitions (Fig 2). This finding also supports the general view that observed transitions exceed transversions only when recently diverged species or slowly evolving genes are compared (Irwin *et al*. 1991, Simon *et al*. 1994).

This MP and NJ analyses based on *CO I* supported the monophyly of Aganinae. The monophyly of this subfamily was consistently found in all tree-building methods and was supported by at least 95% bootstrap support values. The relationships among genera within Aganainae were consistent within MP and NJ analyses but they were supported by low bootstrap values for internal nodes (< 50%) except on the node of the monophyly of genus *Asota*. This finding contradicts a previous hypothesis proposed by Holloway (1988). His cladogram failed to resolve the relationship of the genus *Agape* with the rest of the genera within

Aganainae. No single synapomorphy proposed for each clade in Holloway's cladogram. The similarity between his cladogram and my hypothesis is only on the relationship between *Euplocia* and *Peridrome* and the division of *Neochera* into two clades. The synapomorphy of *Euplocia* + *Peridrome* is a large androconial patch on the forewing upperside at the costal base.

There are many explanations as to why the COI gene resulted in inconsistent tree topologies in different tree building methods (especially for the relationship within the genus *Asota*) and gave low support bootstrap for their relationships. It is often that MP and NJ tree building methods will produce different topologies since they use different algorithms. MP tree analysis usually produces more than one MP trees while NJ always produce in a single tree. Only very clean data will resulted in a similar topology tree with consistent strong bootstrap supports in different tree building methods (Sutrisno 2006). The number of informative sites were high (16%), there may be a lot of conflicting data which resulted in inconsistent trees in the MP analysis. Previous study showed that the mitochondrial gene *CO I* was very useful when combined with *CO II* to resolve the relationships in *Argyrotaenia franciscana* species group (Tortricidae) (Landry *et al*. 1999), *Choristoneura fumiferana* species group (Sperling & Hickey 1994) and genus *Papilio* (Caterino & Sperling 1999). In addition, the combination of the *CO I* and *EF-1α* increased resolution and supported most of the deeper phylogenetic relationships suggested by separate analyses of each gene in the genus *Hemileuca* and *Glyphodes* in various tree building methods (Rubinoff & Sperling 2002, Sutrisno *et al*. 2006).

The low bootstrap support for each node in various building methods was also possibly caused by many conflicts among the sequence in CO I due to the lack of species sampling in the analysis. The number of species of Aganainae in Indonesia is unclear but we believe that the Indonesian Aganainae is only a small part of the whole Aganainae in the world. This is a common problem in MP tree building method, that the lack of species sampling will result in inconsistent tree topologies and other problems such as a long branch length attraction (Nei & Kumar 2000). These problems can be resolved only by increasing the number of sample species in the analysis to potentially reduce the distance between sequences (Nei & Kumar 2000, Yang 2008). Small distances among sequences will produce strong phylogenetic relationship as indicated in the genus *Neochera*: the *N. mormorea* +*N. dominia* node always has a consistent strong bootstrap support in all tree building methods.

In general, all findings in the present study suggest that phylogeny of Indonesian *Aganainae* based on mitochondrial *CO I* gene

recovered five genera previously proposed by Holloway (1988). *Neochera* was divided into two clades and it was branched off first and then followed by *Peridrome*, *Euplocia*, *Agape* dan *Asota*. However, all internal nodes were only weakly supported. It indicates that the relationships among internal nodes proposed here were least valid, possibly due to the number of species included in the analysis which may not be sufficient to represent the real number of species in nature, but also due to the limited selection of analyzed genes and short sequence length. Further studies are needed to be done by including more species, other nuclear genes and genitalia characters in order to test the validity of the relationships proposed here.

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Table 1. Specimens used for molecular study and their genbank accession numbers:

No	Species	Date	Collector	Voucher specimen No	Genbank
1	*Neochera privata*	16.iv.2010	HS	MZB:Lepi slide No. 018	AB684334
2	*Neochera dominia*	6.vi.2010	AW	MZB:Lepi slide No. 019	AB684335
3	*Agape clhoropyga*	27.xi. 2010	UB	MZB:Lepi slide No. 020	AB684336
4	*Asota heliconia*	22.i.2010	AW	MZB:Lepi slide No. 021	AB684337
5	*A. javana celebensis*	29.vi.2010	UB	MZB:Lepi slide No. 022	AB684338
6	*A. egens*	27.xi.2010	UB	MZB:Lepi slide No. 023	AB684339
7	*A. albiformis*	28.iv.2007	D	MZB:Lepi slide No. 024	AB684340
8	*A. plana plana*	25.xi. 2008	D	MZB:Lepi slide No. 025	AB684341
9	*A. egens egens*	2.vii.2007	D	MZB:Lepi slide No. 026	AB684342
10	*A. caricae*	15.vii.2010	UB	MZB:Lepi slide No. 027	AB684343
11	*A. australis sinuosa*	26.vi.2010	AW	MZB:Lepi slide No. 028	AB684344
12	*A. orbana significan*	25.vi.2010	AW	MZB:Lepi slide No. 029	AB684345
13	*A.h.zebrina*	02.vii.2007	D	MZB:Lepi slide No. 030	AB684346
14	*Euplocia membliaria*	16.xii.2009	UB	MZB:Lepi slide No. 031	AB684347
15	*A.heliconia lanceolata*	21.xii.2009	UB	MZB:Lepi slide No. 032	AB684348
16	*A. plaginota plaginota*	2.vii.2007	D	MZB:Lepi slide No. 033	The sequence available on request

Note: AW= Awit Suwito, D= Darmawan, HS= Hari Sutrisno, UB= Ubaidillah

Table 2. Variable site percentages by codon position of CO I genes

	Total	1st codon	2nd codon	3rd-codon
Constant (%)	70 (442)	28.2 (178)	32.3 (204)	9.5 (60)
Uninformative (%)	9.82 (62)	2.2 (14)	0.7 (5)	6.8 (43)
Informatifve (%)	20.12 (127)	2.8 (18)	0.3 (2)	16.9 (107)

Table 3. Results of evaluation of several options on characters in parsimonious tree building method

No	Character included	No of MP trees	Tree length	CI	RI	HI	Note
1	All substitutions	2	485	0.4458	0.5542	0.5000	Reasonable
2	Transversion	18	184	0.4331	0.5669	0.6411	Worst
4	1^{st} +2^{nd} codon	7	424	0.4511	0.5489	0.4899	Worst
5	2^{nd} + 3^{rd} codon	2	472	0.4485	0.5515	0.5055	Reasonable
6	3^{rd} + 1^{st} codon	42	65	0.4667	0.533	0.6522	Worst
7	Tv:Ts=2:1	1	671	0.4578	0.5422	0.5504	Reasonable

Note: Worst= consensus tree resulted in a star tree, Reasonable= consensus tree resulted in good resolution tree

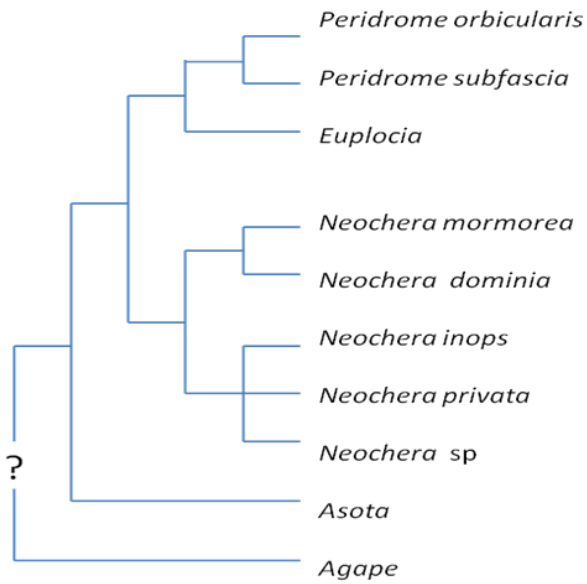

Fig. 1. Cladogram Aganainae (Holloway 1988)

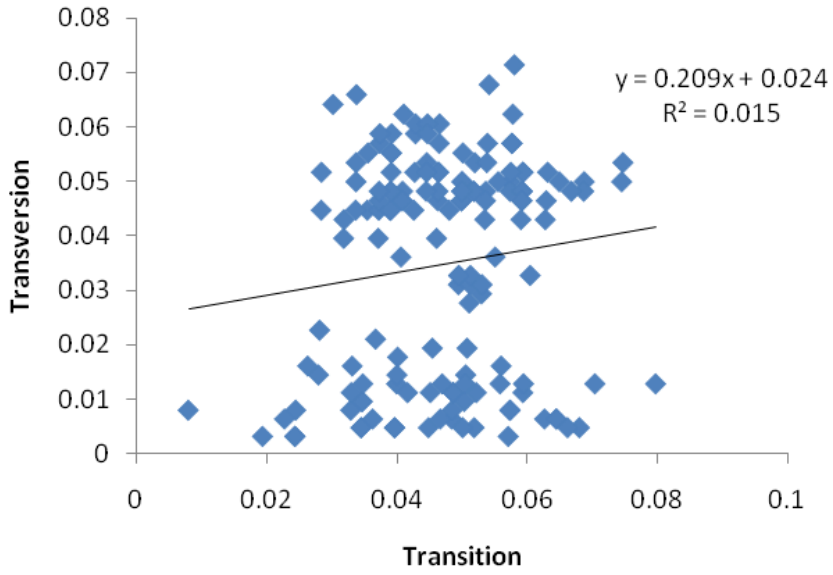

Fig.2. Scatter plots of K2P model distance for Transition (Ts) versus Transversion (Tv)

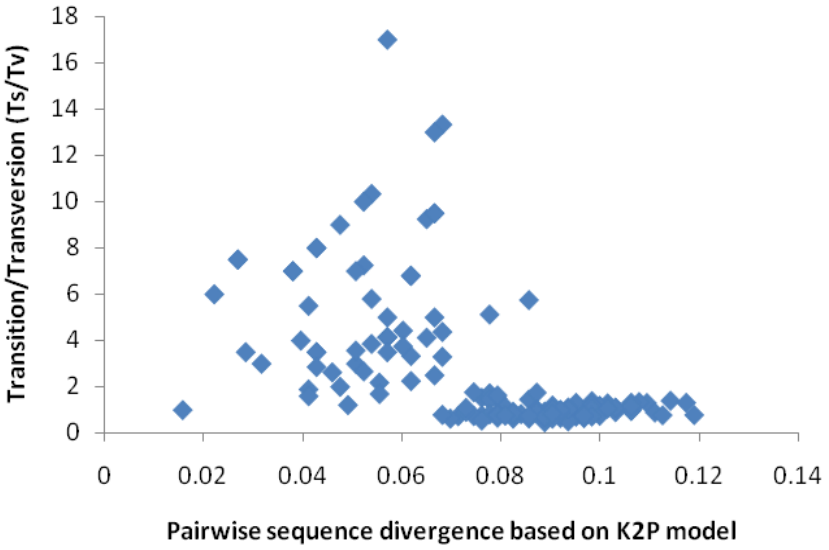

Fig. 3. Scatter plots of pairwise sequence devergence based on K2P model versus Transition/ Transversion (Ts/Tv)

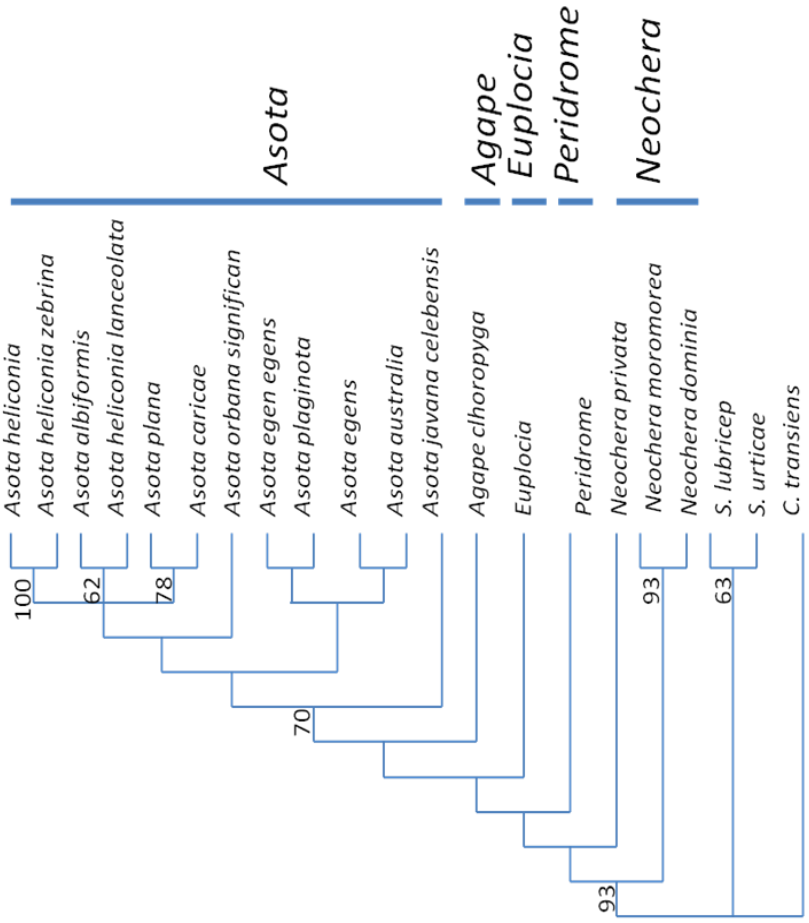

Fig. 4. Strict consensus of the two MP trees based on all substitutions of CO I gene (Bootstrap supports are shown only for the nodes which value > 50%)

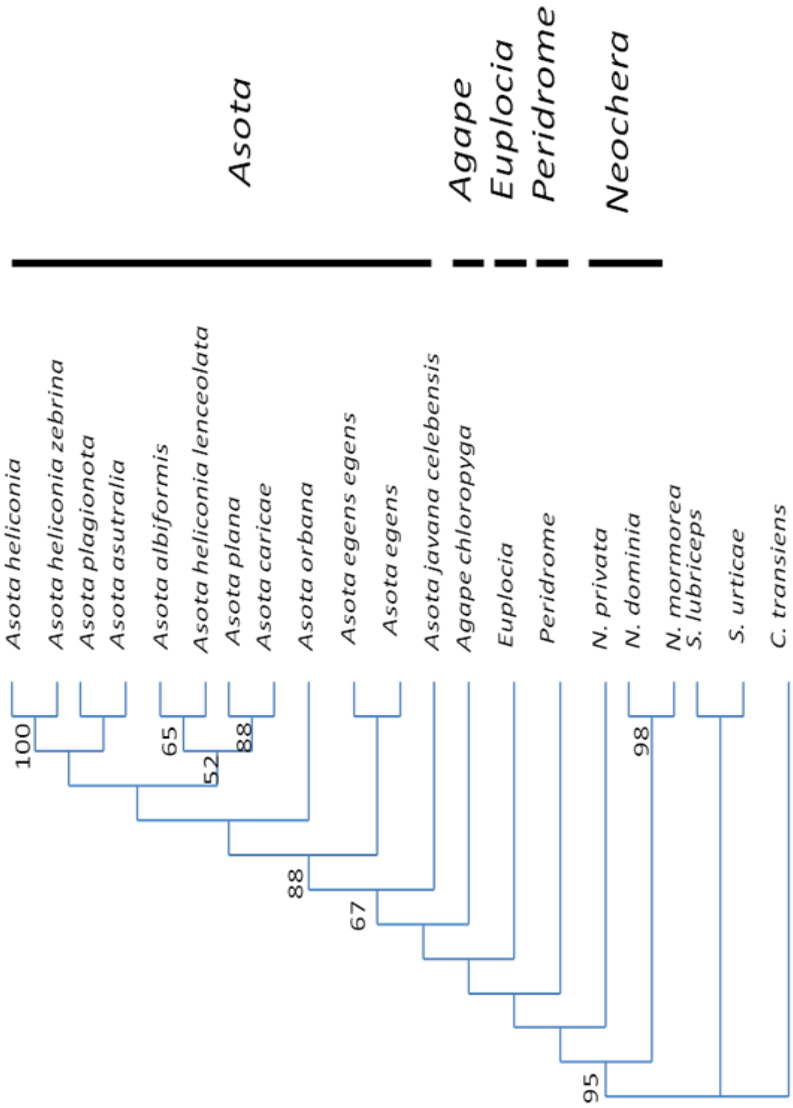

Fig. 5. A MP tree resulted from weighting Transversion: Transition (2:1) (Bootstrap supports are shown only for the nodes which have value >50%)

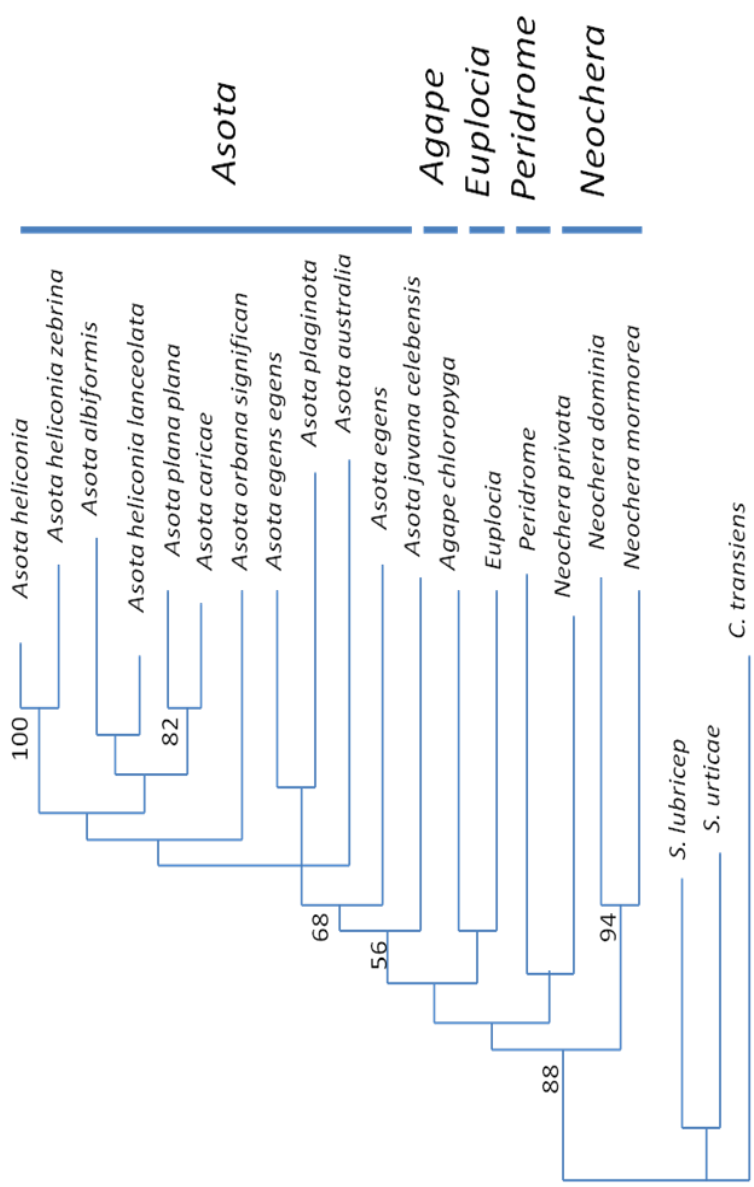

Fig. 6. NJ tree based on K2P distance model of all substitution of CO I gene (Bootstrap supports are shown only for the nodes which have value >50%)

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