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Studies on the Molecular Phylogeny of Coleoptera: Curculionidae, Staphylinidae and Carabidae Based on the Mitochondrial *Cytochrome oxidase* I Gene

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Abstract: Phylogenetic relationships among 146 species of Coleoptera (Families: Curculionidae, Staphylinidae and Carabidae) were estimated based upon mitochondrial *Cytochrome Oxidase* I gene sequences. The monophyletic of the polyphaga and Adephaga was not supported in our study using CO1gene sequences, as family Carabidae (Adephaga) was grouped with family Staphylidae (Polyphaga) with Staphylinidae paraphyletic. The subfamily Scolytinae is the most common ancestor for the subfamilies: Ceutorhynchinae, Curculioninae and Dryophthorinae and hence the oldest. The subfamily Cryptorhynchinae is the oldest among the five tested Curculionidae families. At the family level, the genetic distances and phylogenetic analysis obtained in this study showed that the family Carabidae was more related to family Staphylinidae than to family Curculionidae with the topology Staphylinida-Carabidae-Curculionidae. The topology was the same when *Micromus igorotus* from order Neuroptera was used as an outgroup taxon as it was Staphylinida, Carabidae, Curculionidae/Neuroptera. An alternative topology was obtained when *Acytolepis puspa* from order Lepidoptera was used as an outgroup that was Carabidae, Staphylinida, Curculionidae-Neuroptera/Lepidoptera, where the species of order Neuroptera placed within family Curculionida. According to the estimated genetic distances and to the standard mitochondrial DNA clock estimated at 2.3% MYA, family Curculionidae separated from family Staphylinidae and Carabidae approximately 112 and 115 MYA , respectively.

Key words: Coleoptera, beetles, Curculionidae, Cucujiformia.

1. Introduction

Beetles or Coleoptera species constitute 25% of all life forms e.g. 400,000 species (40% of described insect species) [1]. This large number of species allegedly led evolutionary biologist J.B.S. Haldane to quip, when some theologians asked him what could be inferred about the mind of the Creator from the works of His Creation, that God displayed “an inordinate fondness for beetles” [2].

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According to the phylogenetic analysis of living beetles, the oldest fossils of Coleoptera date back to the lower Permian (up to 299 million years old) [3-5]. The composition of the clad Coleoptera is not in dispute, with the exception of the twisted-wing parasites, Strepsiptera. These odd insects have been regarded as related to the beetle families Rhipiphoridae and Meloidae, with which they share first instar larvae that are active, host-seeking triungulins and later instar larvae that are endoparasites of other insects [6], or as the sister group of beetles [7], or more distantly related to insects.

Order Coleoptera is divided into four suborders of beetles diverged in the Permian and Triassic such as: (1) Polyphage, it is the largest suborder, containing more than 300,000 described species in more than 170 families, (2) Adephaga contains about 10 families of largely predatory beetles, (3) Archostemata contains four families of mainly wood-eating beetles and (4) Myxophaga contains about 100 described species in four families [8]. The phylogenetic relationship of the four suborders is uncertain and poorly known. There are several hypotheses regarding subordinal relationships. There are two hypotheses concerning the placement of the suborder Polyphaga. The first hypothesis suggests a close relationship of Polyphaga to Myxophaga as the two shared a reduction in the number of larval leg articles [6, 8]. Second, Polyphaga is a sister group of all remaining beetles, this based primarily on characters of wing structure, and on the loss of the cervical sclerites in the three suborders other than Polyphaga [7, 9]. The Adephaga was considered as sister to Myxophaga + Polyphaga, based on completely sclerotized elytra, reduced number of crossveins in the hind wings, and folded (as opposed to rolled) hind wings of those three suborders [7, 9].

Molecular studies on beetle phylogeny started in the 1990's but have not lead to great addition to beetles systematic yet especially for the suborder Polyphaga [10]. The basal relationships of Coleoptera suborders were inferred using 18 rDNA [11]. Based on his results he suggested this relationship: [(Archostemata (Myxophaga (Adephaga, Polyphaga)] with the root placed between Archostemata and the remaining suborders. Also the same authors observed that the Adephaga and Polyphaga grouped together with Polyphaga paraphyletic and suggested that Coleoptera suborders do not constitute a monophyletic group.

Results of the combined analysis of 18S rDNA and morphological data indicated that monophyly of and relationships among each of the weevil families are well supported with the topology (Nemonychidae, Anthribidae, Belidae, Attelabidae, Caridae, Brentidae, Curculionidae) [12]. At the subfamily level, Ithycerinae, Microcerinae, and Brachycrinae placed sequentially at the base of the cladogram, the remaining weevil subfamilies form two major natural groups: one constituted by the sister taxa Rhynchophorinae-Platypodinae; the other with Erirhininae at the base, as sister taxon of the "Curculionidae which show an unresolved trichotomy involving Curculioninae, Cossoninae-Scolytinae, and the clad including the Entiminae and allied subfamilies. This latter clad has Thecesterninae at the base; the next branch is Amycterinae, the sister taxon of the clade comprising two groups: one constituted by Aterpinae, Rhytirrhininae, and Gonipterinae; the other is Entiminae whose units form two main clades: one constituted by the sister tribes Pachyrhynchini-Ectemnorhinini, and the other by Alophini, Sitonini, and Entimini. When the analysis was done using only immature characters, results congruent with those based on the complete data set were obtained, except for the placement of Erirhininae. According to the results, the hypothesis of monophyly of broad-nosed weevils is not accepted, the Entiminae are justified as monophyletic and their natural classification into tribes is proposed and the phylogenetic position and relationships of higher taxa of Curculionidae are discussed [13].

In this study we used partial CO1gene sequences: (1) To investigate the phylogenetic relationships among two coleopteran suborders such as polyphage and adephaga, (2) To investigate the phylogenetic relationships among the curculionides and (3) To determine the systematic position of subfamily dryophthorinae.

2. Materials and Methods

2.1 Taxa

Few Sequences of the present study were obtained from scratch while others were obtained from GenBank (Table 1).

Table 1 Species and sequences used in this study.

Series	order	Suborder	Infraorder	Family	Subfamily	Species	GenBank no.
1	Coleoptera	Polyphaga	Cucujiformia	Curculionidae	Ceutorhynchinae	*Ceutorhynchus floralis*	AY837617.1
2					Cryptorhynchinae	*Cryptorhynchinae* spp.	FN429126.1
3						*Semiathe* spp.	FN429128.1
4						*Telaugia* spp.	FN429127.1
5						*Trigonopterus* spp.	FN429129.1
6					Curculioninae	*Miarus graminis*	AY837640.1
7					Dryophthorinae	*Rhynchophorus ferrugineus* (H1*)	HM043659
8						*R. ferrugineus* (H2*)	HM043660
9						*R. ferrugineus* (H3*)	HM043661
10						*R. ferrugineus* (H4*)	HM043662
11						*R. ferrugineus* (H6*)	HM043663
12						*R. ferrugineus* (H8*)	HM043658
13						*R. bilineatus*	HM043664
14						*R. cruentatus*	AY131113
15						*R. palmarum*	HM043666
16						*R. phoenicis*	HM043667
17						*Sphenophorus piceus*	HM043668
18					Scolytinae	*Ambrosiodmus aegir*	AF438519.1
19						*Coccotrypes advena*	AF444068.1
20						*Coleobothrus germeauxi*	AF375311.1
21						*Cyclorhipidion pruinosum*	AF375317.1
22						*Dendroctonus adjunctus*	AY040286.1
23						*Dryocoetes autographus*	AF438517.1
24						*Dryocoetoides cristatus*	AF375319.1
25			Cucujiformia	Curculionidae	Scolytinae	*Dryocoetiops cf. eugeniae*	AF438507.1
26						*Euwallacea validus*	AF375320.1
27						*Hylurdrectonus pinarius*	AY040290.1
28						*Hylurgops* spp.	AF375323.1
29						*Lymantor decipiens*	AF438512.1
30						*L. coryli*	AF438516.1
31						*Ozopemon uniseriatus*	AF438506.1
32						*Phloeotribus liminaris*	AF375325.1
33						*Pityophthorus* spp.	AF375326.1
34						*Sampsonius dampfi*	AF438520.1
35						*Sinophloeus porteri*	AF375314.1
36	Coleoptera	Polyphaga				*Taphrorychus bicolor*	AF375330.1
37						*Tomicus piniperda*	AY040296.1
38						*T. minor*	AY040297.1
39						*Webbia quattuordecimspinatus*	AF375331.1
40			Scarabaeiformia	Staphylinidae	Aleocharinae	*Acrotona* spp.	GQ980886.1
41						*Amidobia talpa*	GQ980898.1
42						*Amischa nigrofusca*	GQ980896.1
43						*A. analis*	GQ980895.1
44						*Atheta myrmecobia*	GQ980931.1
45						*Atheta vaga*	GQ980930.1
46						*Atheta vestita*	GQ980929.1
47						*Atheta graminicola*	GQ980927.1
48						*Atheta contristata*	GQ980926.1

(Table 1 continued)

Series	order	Suborder	Infraorder	Family	Subfamily	Species	GenBank no.
49						*Atheta ravilla*	GQ980924.1
50						*Atheta kenyamontis*	GQ980923.1
51						*Atheta bosnica*	GQ980922.1
52						*Atheta laticollis*	GQ980920.1
53						*Atheta subtilis*	GQ980919.1
54						*Atheta lippa*	GQ980918.1
55						*Atheta setigera*	GQ980917.1
56						*Atheta hampshirensis*	GQ980916.1
57						*Atheta cinnamoptera*	GQ980914.1
58						*Atheta aeneipennis*	GQ980913.1
59						*Atheta dadopora*	GQ980912.1
60	Coleoptera	Polyphaga	Cucujiformia	Curculionidae	Aleocarinae	*Atheta celata*	GQ980911.1
61						*Atheta modesta*	GQ980909.1
62						*Atheta crassicornis*	GQ980907.1
63						*Atheta pervagata*	GQ980906.1
64						*Atheta pallidicornis*	GQ980905.1
65						*Atheta membranata*	GQ980903.1
66						*Atheta klagesi*	GQ980900.1
67						*Bolitochara pulchra*	GQ980866.1
68						*Cordalia obscura*	GQ980864.1
69						*Dadobia immersa*	GQ980953.1
70						*Drusilla canaliculata*	GQ980874.1
71						*Geostiba circellaris*	GQ980954.1
72						*Gymnusa variegata*	GQ980860.1
73					Aleocarinae	*Halobrecta* sp. *cf. halensis*.	GQ980966.1
74						*Hydrosmecta* spp.	GQ980955.1
75						*Liogluta nigropolit*	GQ980938.1
76						*Lyprocorrhe anceps*	GQ980939.1
77						*Mocyta scopula*	GQ980891.1
78						*M. fungi*	GQ980888.1
79						*Myllaena audax*	GQ980881.1
80						*Nehemitropia lividipennis*	GQ980892.1
81		Polyphaga	Cucujiformia	Curculionidae		*Oxypoda praecox*	GQ980882.1
82						*Philhygra iterans*	GQ980946.1
83						*Ph. fallaciosa*	GQ980943.1
84	Coleoptera					*Ph. debilis*	GQ980941.1
85						*Placusa* spp.	GQ980883.1
86						*Thamiaraea cinnamomea*	GQ980963.1
87						*Th. brittoni*	GQ980962.1
88						*Th. Americana*	GQ980960.1
89						*Trichiusa ursina*	GQ980964.1
90					Tachyporinae	*Tachinus proximus*	GQ980859.1
91					Carabinae	*Carabus nitens*	GU347160.1
92						*C. nemoralis*	GU347156.1
93		Adephaga		Carabidae		*C. monilis*	GU347150.1
94						*C. auronitens*	GU347146.1
95						*Elaphrus riparius*	GU347213.1
96						*E. cupreus*	GU347209.1

(Table 1 continued)

Series	order	Suborder	Infraorder	Family	Subfamily	Species	GenBank no.
97					Carabinae	*E. aureus*	GU347205.1
98						*Nebria hellwigii*	GU347263.1
99						*N. jockischii*	GU347255.1
100						*Oreonebria castanea*	GU347252.1
101					Harpalinae	*Agonum viduum*	GU347037.1
102						*A. melleri*	GU347036.1
103						*A. micans*	GU347033.1
104						*A. marginatum*	GU347029.1
105						*Amara erratica*	GU347064.1
106						*A. similata*	GU347055.1
107						*A. quenseli*	GU347053.1
108	Coleoptera	Adephaga		Carabidae		*Anchomenus dorsalis*	GU347062.1
109						*Anisodactylus binotatus*	GU347064.1
110						*Dicheirotrichus obsoletus*	GU347177.1
111						*Dromius quadrimaculatus*	GU347183.1
112						*Harpalus rufipes*	GU347228.1
113						*H. affinis*	GU347222.1
114						*Licinus hoffmannseggii*	GU347232.1
115						*Molops piceus*	GU347251.1
116						*Philorhyzus sigma*	GU347273.1
117						*Ph. Melanocephalus*	GU347271.1
118						*Poecilus cupreus*	GU347274.1
119						*Pterostichus unctulatus*	GU347340.1
120						*P. rhaeticus*	GU347338.1
121					Harpalinae	*P. panzeri*	GU347332.1
122						*P. oblongopunctatus*	GU347327.1
123						*P. melanarius*	GU347302.1
124						*P. jurinei*	GU347294.1
125						*P. aterrimus*	GU347280.1
126						*P. anthracinus*	GU347279.1
127						*Stenolophus teutonus*	GU347352.1
128					Loricerinae	*loricera pilicornis*	GU347246.1
129					Omophroninae	*Omophron limbatum*	GU347268.1
130					Platyninae	*Limodromus assimilis*	GU347243.1
131					Scaritinae	*Clivina fossor*	GU347168.1
132						*C. collaris*	GU347164.1
133	Coleoptera	Adephaga		Carabidae		*Dyschirius thoracicus*	GU347192.1
134						*D. aeneus*	GU347187.1
135					Trechinae	*Bembidion punctulatum*	GU347136.1
136						*B. tetracolum*	GU347131.1
137						*B. semipunctatum*	GU347125.1
138						*B. ruficorne*	GU347121.1
139						*B. properans*	GU347117.1
140						*B. pallidipenne*	GU347110.1
141						*B. littorale*	GU347106.1
142						*B. elongatum*	GU347092.1
143						*B. decorum*	GU347088.1
144						*B. decoratum*	GU347078.1
145						*B. aspericolle*	GU347074.1
146						*B. articulatum*	GU347069.1
147	Neuroptera					*Micromus igorotus*	EF494180.1

2.2 DNA Extraction and PCR Amplification

Total genomic DNA was extracted from RPW samples using DNeasy Tissue Kit (Qiagen GmbH) according to the manufacturer's protocol.

PCR was performed in a total volume of 25 μL containing 1 × reaction buffer, 3 mM $MgCl_2$, 0.24 mM dNTPs, 1.4 μM of each primer (C1-J-1751(5'-GGATCACCTGATATAGCATTCCC-3') and C2-N-3014(5'-TCCAATGCACTAATCTG CCATATTA-3')) [14], 1U Go Taq Flexi DNA polymerase (Promega Corp.) and 2.5 μL of DNA (a 100 time dilution of the original DNA). The PCR program was as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corp). Purified PCR products were analyzed by Electrophoresis in 1% agarose gel. The molecular size of the amplified products was estimated using 100 bp DNA ladder (Invitrogen).

2.3 DNA Sequencing

Purified PCR products were sequenced using AB1 PRISM Dye Terminator Cycle Sequencing Ready Reaction System, version 1.1. (Applied Biosystems) Sequencing reactions were carried out in 20 μL volumes containing 1 μL of 3.2 μM primer, 2 μL of BigDye Terminator mix, and 3 μL of sequencing buffer and a template/sterile water mixture, containing 30 to 90 ng DNA. The program used was as follows: 96 °C for 1 min followed by 24 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min and a final extension at 60 °C for 5 min.

2.4 Data Analyses

Obtained sequences were aligned using MEGA4 software [15]. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) in order to perform sequence similarity searches, according to Zhang et al. [14]. Nucleotide frequencies were calculated using MEGA4 software [15], where missing data were eliminated from the dataset (Complete-deletion option). InDels (Insertion-Deletion polymorphism) was estimated using DnaSP software [16]. The genetic distances (number of nucleotide substitutions per site) among sequences were calculated using the Maximum Composite Likelihood model [17] in MEGA4 software [15]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding. All positions containing gaps and missing data were deleted from the dataset (Complete deletion option). The divergence time between the tested species was estimated according to the genetic distance and to the standard mitochondrial DNA clock estimated at 2.3% MYA [18].

2.5 Phylogenetic Analyses

Phylogenetic trees were reconstructed using maximum parsimony (MP) method. The MP tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [19] at a search level of 3. Initial trees for MP were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 software [15]. Bootstrap support values were obtained by 1,000 replications [20] using both methods. *Micromus igorotus* (Neuroptera) was used as an outgroup taxon.

3. Results and Discussion

Molecular phylogeny was investigated among 146 species belonging to order Coleoptera using partial sequences of *Cytochrome c oxidase* subunit I (COI). The tested species belonging to three families such as Curculionidae (39 species), Staphylinidae (51 species) and Carabidae (65 species). *Micromus igorotus* from order Neuroptera was used as an outgroup (Table 1).

3.1 Nucleotide Frequencies

The nucleotide composition was biased towards the adenine and thymine (AT) in all the tested CO1 sequences. AT frequencies ranged from 337 to 341 with frequencies of 60% to 70.3%. The guanine and cytosine (GC) frequencies ranged from 40% to 29.7%. This result for mitochondrial DNA was not surprising as it was found previously in sequences of other insect species [21-23].

3.2 Variable and Conserved Sites

After alignment, invalid end sequences were trimmed, this resulted in a final alignment of 458 bp in length. No insertion or deletion polymorphism was found. The variation among the obtained CO1 gene sequences due to nucleotide substitutions. From the total 458 bp, 11 were constant sites and 447 (98%) were variable. From the total of the 447 variable sites there were 436 parsimony informative sites (substitution shared by at least two sequences).

3.3 Genetic Pairwise Distances (GD)

The highest and lowest values recorded between the species of each family and between the species of the different families were as follows:

3.3.1 Curculionidae

The highest value of GD 4.946 was observed between *Ambrosiodmus aegir* (Subfamily: Scolytinae) and *Cryptorhynchinae* spp. (Subfamily: Cryptorhynchinae), while the lowest value 0.119 was observed between *R. bilineatus* and *R. ferrugineus* (H8*) (Subfamily: Dryophthorinae (Rhynchophorinae)).

3.3.2 Curculionidae and Staphylinidae

The highest value of GD 5.539, was observed between *Coleobothrus germeauxi* (Subfamily: Scolytinae) and *Atheta myrmecobia* (Subfamily: Aleocarinae), while the lowest value 2.647 was observed between *Sinophloeus porteri* and *Lyprocorrhe anceps*.

3.3.3 Curculionidae and Carabidae

The highest value of GD 4.665, was observed between *Coleobothrus germeauxi* (Subfamily: Scolytinae) and *Dicheirotrichus obsoletus* (Subfamily: Harpalinae), while the lowest value 2.587 was observed between *Sinophloeus porteri* (Subfamily: Scolytinae) and *Pterostichus anthracinus* (Subfamily: Harpalinae).

3.3.4 Staphylinidae

The highest value of GD 0.260 was observed between *Mocyta fungi* (Subfamily: Aleocarinae) and *Acrotona* spp. (Subfamily: Aleocarinae), while the lowest value 0.007 was observed between *Amischa analis* and *Amidobia talpa*.

3.3.5 Carabidae

The highest value of pairwise distances 0.220 was observed between *Loricera pilicornis* (Subfamily: Loricerinae) and *Amara erratica* (Subfamily: Harpalinae), while the lowest value 0.070 was observed between *Pterostichus melanarius* and *Philorhizus melanocephalus* (Subfamily: Harpalinae).

3.3.6 Staphylinidae and Carabidae

The highest value of GD 0.294 was observed between *Liogluta nigropolita* (Subfamily: Aleocarinae) and *Bembidion properans* (Subfamily: Trechinae), while the lowest value 0.144 was observed between *Cordalia obscura* (Subfamily: Aleocarinae) and *Agonum viduum* (Subfamily: Harpalinae).

3.3.7 Neuroptera

The lowest values of GD were 0.219, 3.139 and 3.070 with (*Tomicus piniperda*, Scolytinae, Curculionidae), (*Pterostichus jurinei*, Harpalinae, Carabidae) and (*Atheta subtilis*, Aleocharinae, Staphylinidae) respectively.

3.3.8 Lepidoptera

The lowest values of GD were 3.085, 0.204 and 0.206 with (*Cryptorhynchinae* spp., Cryptorhynchinae, Curculionidae), (*Atheta kenyamontis*, Aleocarinae, Staphylinidae) and (*Philorhizus melanocephalus*, Harpalinae, Caraidae) respectively.

3.4 Divergence Time

According to the estimated genetic distance and to the standard mitochondrial DNA clock estimated at 2.3% MYA [18]:

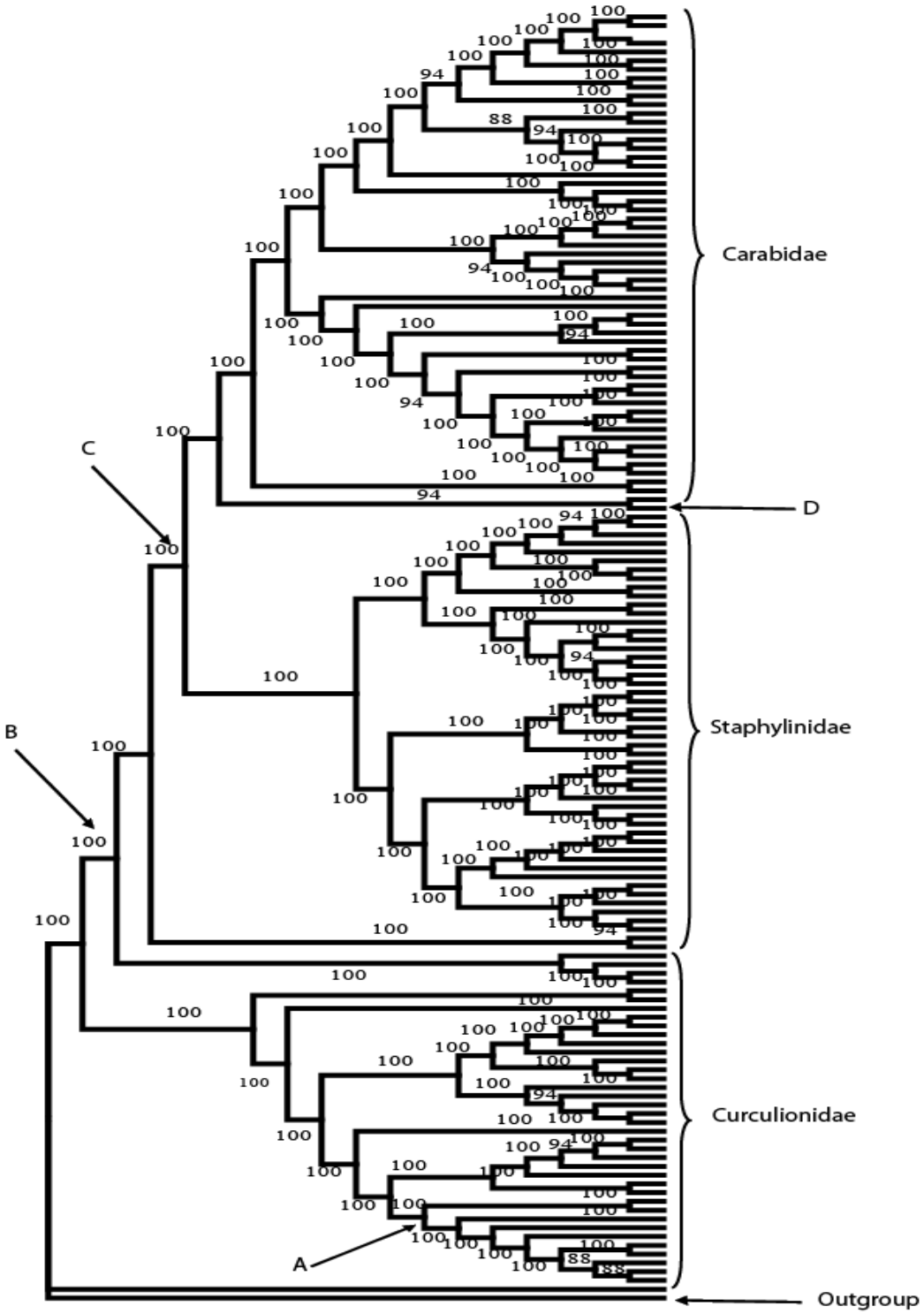

Fig. 1 Phylogenetic tree of COI gene sequences by MP methods. Bootstrap values were shown either above or down the branches. (A) The most common ancestor to the species of Dryophthorinae; (B) The most common ancestor for the three families: Curculionidae, Staphylinidae and Carabidae; (C) The most common ancestor for the two families: Staphylinidae and Carabidae; (D) A clade contained one species of Staphylinidae and another from Carabidae.

The order of the MP tree branches was as follow (from down to up):

(1) The outgroup-*Micromus igorotus* (Neuroptera);

(2) Curculionidae.

Sphenophorus piceus, *Rhynchophorus ferrugineus*(H6), *R. ferrugineus*(H2), *R. ferrugineus*(H1), *R. ferrugineus*(H4), *R. ferrugineus*(H3), *R. ferrugineus*(H8), *R. bilineatus*, *R. phoenicis*, *R. palmarum*, *R. cruentatus, Pityophthorus* sp., *Coleobothrus germeauxi, Dryocoetiops eugeniae*, *Euwallaceav alidus*, *Cyclorhipidion pruinosum*, *Webbia quattuordecimspinatus*, *Coccotrypes advena*, *Phloeotribus liminaris, Tomicus piniperda*, *T. minor*, *Dendroctonus adjunctus*, *Hylurgops* sp., *Ceutorhynchusf loralis*, *Dryocoetoides cristatus, Ambrosiodmus aegir*, *Sampsonius dampfi*, *Dryocoetes autographus, Ozopemon uniseriatus, Taphrorychus bicolor, Lymantor decipiens*, *L. coryli, Miarus graminis, Sinophloeus porteri*, *Hylurdrectonus pinarius*, *Telaugia* sp. *Semiathe* sp., *Cryptorhynchinae* sp. and *Trigonopterus* sp.

(3) Staphylinidae

Nehemitropial ividipennis, *Geostiba circellaris, Liogluta nigropolita, Atheta bosnica*, *Ath. Vestita, Ath. Laticollis*, Gymnusa variegata, *Amidobia talpa, Ath. Pervagata*, *Tachinus proximus, Acrotona* sp., *Ath. Kenyamontis*, *Mycota fungi*, M. scopula, *Ath. Membranata*, *Ath. Subtilis*, *Ath. Setigera*, *Trichius aursina, Ath. Crassicornis, Ath. Aeneipennis, Placusa* sp. *Ath. Ravilla, Philhygra Fallaciosa, Myllaena audax, Ath. Cinnamoptera, Ath. Myrmecobia, Ath. Lippa, Ath. Contristata, Ath. Graminicola, Ath. Vaga, Oxypoda praecox, Bolitochara pulchra, Halobrecta halensis, Ath. Modesta, Hydrosmecta sp., Ath. Klagesi, Ath. Pallidicornis, Tha. Americana*, *Phi. Debilis, Phi. iterans Dadobia immersa Ath. dadopora Tha. cinnamomea Cordalia obscura Drusilla canaliculata Ath. hampshirensis Atheta celata Thamiaraea brittoni, Amischa analis Ami. Nigrofusca, Oreonebria castanea Lyprocorrhea nceps.*

(4) Carabidae

Bembidion decoratum, B. elongatum, *Philorhizus melanocephalus*, *Phi. sigma*, *Dromius quadrimaculatus*, *Amara Quenseli*, *Poecilus cupreus, Pterostichus unctulatus*, *Pt. Melanarius*, *Pt. oblongopunctatus*, *Pt. aterrimus*, *Pt. anthracinus*, *Pt. rhaeticus*, *Am. Similata*, *Am. Erratica*, *Pt. Jurinei*, *Pt. Panzeri*, *Anchomenus dorsalis*, *Agonum muelleri*, *Ag. Marginatum*, *Ag. Micans*, *Ag. Viduum*, *Dicheirotrichus obsoletus*, *Elaphrus aureus*, *E. riparius*, *E. cupreus*, *Limodromus assimilis*, *Omophron limbatum*, *Carabus nitens*, *C. monilis*, *C. auronitens*, *C. nemoralis*, *Harpalus affinis*, *H. rufipes*, *Anisodactylus binotatus*, *Stenolophus teutonus*, *Licinus hoffmannseggii*, *Dyschirius* aeneus, *D. thoracicus*, *Clivina collaris*, *C. fossor*, *Molops piceus*, *B. ruficorne*, *Loricera pilicornis*, *Nebria jockischii*, *N. hellwigii*, *B. properans*, *B. punctulatum*, *B. decorum*, *B. tetracolum*, *B. semipunctatum*, *B. articulatum*, *B. aspericolle*, *B. litorale* and *B. pallidipenne*.

(1) Family Curculionidae separated from family Staphylinidae and Carabidae approximately 112 and 115 MYA respectively;

(2) Family Staphylinidae separated from family Carabidae approximately 6 MYA;

(3) Subfamily Dryophthorinae separated from the other subfamilies of family Curculionidae approximately 7 MYA where the lowest genetic distance between the species of subfamily Dryophthorinae and the species of other subfamilies was 0.172 (between *R. cruentatus* and *Phloeotribus liminaris* (Scolytinae).

3.5 Phylogenetic Inference

Phylogenetical analysis was investigated using maximum parsimony (MP) method. The complete nucleotide sequences were used in the analysis, a tree out of 18 most parsimonious trees was shown in Fig. 1. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index was (0.166549), the retention index was (0.723336), and the composite index was 0.122165 (0.120471) for all sites and parsimony-informative sites (in parentheses).

4. Conclusion

The monophyletic of the polyphaga and Adephaga was not supported in our study using CO1 sequences, as family Carabidae (Adephaga) was grouped with family Staphylidae (Polyphaga) with Staphylinidae paraphyletic. This result confirmed the previous results using 18 rDNA [11]. The subfamily Scolytinae is the most common ancestor for the subfamilies: Ceutorhynchinae, Curculioninae and Dryophthorinae and hence the oldest. The subfamily Cryptorhynchinae is the oldest among the five tested Curculionidae families.

At the family level, the genetic distances and phylogenetic analysis obtained in this study showed that the family Carabidae was more related to family Staphylinidae than to family Curculionidae with the topology Staphylinida-Carabidae-Curculionidae. The topology was the same when *Micromus igorotus* from order Neuroptera was used as an outgroup taxon as it

was Staphylinida, Carabidae, Curculionidae/Neuroptera. An alternative topology was obtained when *Acytolepis puspa* from order Lepidoptera was used as an outgroup that was Carabidae, Staphylinida, Curculionidae-Neuroptera/ Lepidoptera, where the species of order Neuroptera placed within family Curculionida.

According to the estimated genetic distance and to the standard mitochondrial DNA clock estimated at 2.3% My^{-1}, family Curculionidae separated from family Staphylinidae and Carabidae approximately 115 and 112 My^{-1}, respectively.

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