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Mitochondrial markers for identification and phylogenetic studies in insects – A Review

Abstract: Similar morphology and high genetic diversity poses problems in phylogenetic studies of insects. To solve these problems, mitochondrial based markers have been adopted and are increasingly used as molecular markers for phylogenetic studies. Varied markers have been used for different species of insects, viz., markers for 16S r RNA, 12S r RNA, ND (1-6 genes), ATPase and control regions. Among which protein coding gene, CO-1 is found to be best because of its advantage over others whereas, AT rich region of mitochondrial DNA is the least used marker. A recent advanced technology in phylogenetic analysis; namely mitogenomics have greatly improved this research field. This short review attempted to summarize recent studies on the application of various mitochondrial molecular markers for phylogenetic study of insects.

Keywords: insects, mitochondrial marker, molecular phylogeny, CO-1, mitogenomics

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Introduction

Mitochondria - the powerhouse of a cell plays a crucial role in respiration, genetic illness, aging and self-destruction of a cell [1-4].The genetic material in mitochondria, the mitochondrial DNA (mtDNA) contains genes involved in production of enzymes for oxidative phosphorylation and protein synthesis. Mitochondrial (mt) genome sequence and structure provides evolutionary and comparative genomics informations as well as, informations on molecular evolution and patterns of gene flow, phylogenetics and population genetics resources [5,6]. Like other animals, insect mitochondrial genome is a double stranded molecule with a range of 14,503 bp (*Rhopalomyia pomum*) to 19,517 bp (*Drosophila melanogaster*) in size [7]. It consists of 37 genes encoding the large and small subunit ribosomal RNAs, 22 transfer RNAs (*trnI, trnQ, trnM, trnW, trnC, trnY, trnL1, trnK, trnD, trnG, trnA, trnR, trnN, trnS1, trnE, trnF, trnH, trnT, trnP, trnS2, trnL2, trnV*) necessary to translate the protein-coding genes and 13 protein coding genes that are all components of the oxidative phosphorylation process (Figure 1). The insect mt genome also consists of a regulatory element known as AT rich region which plays important role in initiation of transcription and replication [8,9].

The arrangement of major genes in the mitochondrial genome is highly conserved across animal phyla. However insects are exceptionally different, having highly variable gene orders - Lepidoptera (butterflies and moths), Diptera (flies), Phthiraptera (lice), Thysanoptera (thrips), Psocoptera (bark lice) and Hymenoptera (wasps) (http://whitinglab.byu.edu/Research/MitochondrialGenomics.aspx). Eighty three insect species belonging to 11 orders

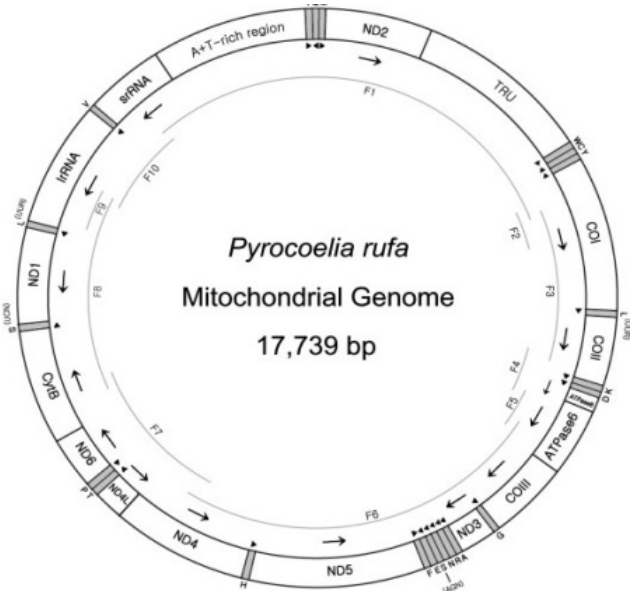

Figure 1: Map of the mitochondrial genome of *Pyrocoelia rufa* [82].

show mitochondrial gene rearrangement scenario, mostly in tRNA genes but rarely in protein-coding and rRNA genes [10-13] with an exception in insect orders Thysanoptera and Phthiraptera that showed both type of rearrangements [14,15]. In a separate study, Arif and Khan [16] have reported widely used mt DNA markers with decreasing order of conserved sequences: 12S rDNA> 16S rDNA> cytochrome b > control region (CR); showing that 12S rDNA is highly conserved, while the CR is highly variable.

Insects are ancient (>450 million years ago) and taxonomically diverse group having a worldwide distribution and a complex evolutionary history [17-19]. Many of them are considered as agricultural pests, major disease vectors, pollinator of crops, parasites of other insects and bio-indicator of environmental changes [20,21]. Identification of insects is crucial to manage endangered species, protected species, as well as invasive species in the ecosystem. This management is important for environmental quality indicators, basic research on evolutionary biology and ecology, agricultural pests/beneficial species and disease vectors / pathogens and for biodiversity study and conservation research. Until now, insect identification has been based on classical morphological and taxonomic studies. Eventually, difficulty in morphological identification has led to the use of molecular datasets instead of morphological analysis for identifying and characterization of different taxa (CBOL, Consortium for the Barcode of Life). This is again hindered by complexities in insect genomes. Due to a high genetic diversity it is difficult to understand the interrelationship between the insect orders by classical taxonomical methods [22]. Molecular methods therefore provide a better in-depth understanding to the variations and similarities among insects and even provide evolutionary explanations among different species. Molecular analyses and phylogenetic analysis using molecular markers can explain the relationships between different developmental stages, castes polymorphism in social insects, sexually dimorphic, polyphenic and polymorphic individuals [23-25].

Molecular phylogenetics uses the structure and function of molecules and how they change over time to infer these evolutionary relationships. In recent years, huge amount of sequence information is available in publically accessible online databases that enabled molecular phylogenetics to grow and find new applications [26-28]. Different types of mitochondrial and nuclear DNA markers are available for phylogenetic analysis of insects. However, the choice of a molecular marker in a particular analysis is crucial since sequence fragment, whose rate of substitution is inappropriate for the level of divergence under study, can be a source of misleading data [29]. Carefully planning is therefore necessary before any DNA barcoding experiments of insects.

In phylogenetic study, mitochondrial DNA has many advantages. They possess strict maternal transmission [30] with high mutation rate due to limited repair system (5-10 times that of nuclear DNA) [31] and conserved simple structure. These unique properties allow the development of universal primers and easy recovery from small or degraded biological sample due to its high copy number in most cells with a different evolution rate in different regions of mitochondrial DNA. Lack of recombination in mt DNA and its relatively infrequent gene rearrangements makes it a choice for the population genetic studies. In some cases, mitochondrial DNA sequence enters into nucleus and creates nuclear mitochondrial DNA used for interspecific diversity in insects [32]. Mitochondrial gene sequence based phylogenetic investigation is therefore restricted to closely related species, because of its high nucleotide substitution rates. Mitochondrial gene content and order variations have, however, been used to resolve phylogeny of distantly-related species, based on shared derived property [33-37]. Moreover, it is easy to amplify and sequence the different genes [38].But, appropriate bioinformatics tools for the analysis of these sequences is also a major factor for reliable phylogenetic inference using mitochondrial marker [39].Table 1 summarizes the properties of various mtDNA markers that have been applied to the study of insects [16].

Mitochondrial ribosomal RNA as markers

Mitochondrial ribosomal RNA sequence has been widely used for phylogenetic studies. Mitochondrial rRNA gene sequences in vertebrates appear to be as less conserved in evolution than their nuclear counterparts. DNA heteroduplex thermostability analysis and restriction-endonuclease cleavage-map comparisons have shown that they do change rapidly but not as rapid as the mitochondrial genes that code for proteins [40]. Insect mitochondria contain two ribosomal RNA (rRNA) genes, 12S rDNA and 16S rDNA. 12s rDNA is highly conserved in insects and used for the study of genetic diversity in phyla, but the large subunit of ribosomal RNA (16S rDNA) is often used for studies at the low and intermediate levels such as in families or genera [41,42].

Table 1: Characteristics of various mtDNA markers [16].

mtDNA	Inherited from the mother (maternal lineage); rare exceptions do exist
	Degrades slower than nuclear DNA. It can be used in degraded or old samples
	Evolves about 10–fold faster than nuclear DNA; no proof reading activity
12S rDNA	Highly conserved; used for high-category levels: phyla and subphyla
16S rDNA	Usually used in mid–category differentiation such as families
Protein-coding genes	Used in low–categories such as families, genera and species
Control region	Used for identification of species and sub-species

12S rRNA

Merzlyak *et al.* [43] showed that *Blastocrithidia triatoma* and *Leptomonas collosoma* were the earliest branching lineages among the insect trypanosomatids. An investigation on phylogenetic relationships, using 16s rRNA and 12s rRNA markers, within *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) supported the hypothesis of concerning evolution of different color forms of *H. axyridis* based on morphological data [44].In a separate study, phylogenetic status of the genus *Dactylopius* of Mexico was studied using 12S rRNA gene marker. This showed that the cochineal insects, *D. ceylonicus, D. confusus*, and *D. opuntiae* are closely related [45].

16S rRNA

Mitochondrial 16S rRNA has been used to reveal the evolutionary relationship of ten termite genera from five families [46]. Further, a molecular phylogeny of cockroaches and related insects based on 16S rRNA is presented by Kambhampati [47]. A study on 16S rRNA gene sequences from 65 taxa of Hymenoptera showed that the 16S rRNA gene is most informative for phylogenetic analysis among closely related species or populations, and among tribes, subfamilies, and families [48]. From the analysis of a phylogenetic tree based on 16S rRNA of mosquito, it was observed that genera and species in the tree are similar to the previously reported tree based on nuclear rRNA RFLPs [49,50].16S rRNA has also been used to resolve monophyly issues of the genus *Poecilimon* [51]. Moreover, 16S rDNA is useful for phylogeny study of ticks not only at the family level but also below this level [52]. Analysis of mt 16S rDNA gene fragments partially resolved relationships within the genus *Calopteryx* and between Calopterygid forms and seems to be promising for more exhaustive future analyses of this damselfly family [53].

Mitochondrial protein-coding gene markers

Due to their faster evolutionary rates compared to ribosomal RNA genes, protein-coding genes of mitochondria are used in evolutionary study in families, genera and species. Insect mitochondria contain 13 protein-coding genes[16]. According to the tests conducted by Zardoya and Meyer [54], mitochondrial protein-coding genes can be classified into three groups of good (ND4, ND5, ND2, cytb, and COI), medium (COB, COIII, NDl, and ND6) and poor (ATPase 6, ND3, ATPase 8, and ND4L) phylogenetic performers in recovering these expected trees among phylogenetically distant relatives. Among the protein coding genes, cytochrome oxidase subunit I (COI) is found to be the best molecular marker for evolutionary studies.

Mitochondrial CO1 gene together with ND5 has been used for phylogenetic studies of flies and only few genetic differences have been found among them. Although, having high substitution rate, CO1 gene can be used to resolve relationships between genera in two sub-families of Tetranichydae [55]. Intraspecific variation in *Ixodes pacificus* was studied using cytochrome oxidase III [56]. Mitochondrial cytochrome b sequences have been used in molecular phylogeny of Gryllidae [57]. Studies on ND4 gene of wild caught blackflies carrying *Onchocerca volvolus* infective larvae have led to the discovery of new alleles that allowed grouping of the individual flies carrying these alleles to the *Simulium damnosums* sibling species [58].Some application of CO1 gene in insect phylogenetic studies has been summarized in Table 2.

Mitochondrial protein coding genes in DNA bar-coding

Mitochondrial protein coding genes have often been used in DNA bar-coding. DNA bar-coding, proposed by

Table 2: Applications of CO1 gene in insect phylogenetic studies.

Name	Significance of the work	References
Beetle	Sequences from the CO1, 16S rRNA, tRNA and ND1 genes suggest that the new *Copelatus* species belongs to the subgenus *Papuadytes*, the morphological apomorphies of which are reduced in the new groundwater species.	Balke *et al*. 2004 [72]
Butterfly	The population genetic structure and phylogeography of *H. merope* using COI and ND5 shows that the subspecies are reciprocally monophyletic.	Norgate *et al.* 2009 [73]
Spider	Sequence diversity of CO1 gene is highly effective in discriminating spider species.	Barrett and Hebert, 2005 [74]
Moth	The groundnut leaf miner of South Africa is identified by mtDNA CO1 gene analysis as the Australian soybean moth (*Aproaerema simplixella*).	Buthelezi *et al.* 2012 [75]
Beetle	The mitochondrial genetic variation of Red Palm Weevil was investigated by using the CO1 gene from 310 individuals of 14 different countries showed eight different haplotypes.	El-Mergawy *et al.* 2011 [76]
Blowflies	Mitochondrial gene CO1, CO2, ND4 and ND4L have used for identification and evolutionary relationship of Australian carrion-breeding blowflies.	Wallman *et al*. 2006 [77]
Cotton bollworm	By compairing CO1 sequence between *H. armigera* and *H. assulta*, a PCR-RFLP based method that can distinguish the two pest at the egg stage.	Kranti *et al.* 2005 [78]
Honey bee	Phylogenetic position of *Apis nuluensis* is shown by using mitochondrial gene 16SrRNA, CO1and CO2 gene.	Tanaka *et al.*2001 [79]
Moth	Molecular Phylogenetic relationships between non-mulberry and mulberry silkworm species using the sequences from the cytochrome oxidase 1.	Mahendran *et al.* 2006 [80]
Stem Sawfly	Molecular phylogeny of Cephidae is constructed by mt CO1 gene sequences	Budak *et al.* 2011 [81]

Hebert *et al.* [59], is a rapidly accurate process of species identification using a short standardized gene sequence. This region shows 608 bp region in the mt CO1 gene is used for animal bar-coding and it showed high efficiency for the identification of bird, fish, flies and other animals where as chloroplast matK and rbcL are used for plant identification (CBOL, Consortium for the Barcode of Life, available http://www.barcoding.si.edu/ DNABarCoding.htm). DNA barcoding does not require any taxonomist for identification process. CO1 gene is used for bar-coding since it is the largest gene among the three mitochondrial genes encoding cytochrome oxidase subunit and has high insertion deletion events. It has a high rate of nucleotide substitution that helps to discriminate cryptic species. Moreover, universal primers for CO1 is very robust. Methodology for DNA barcoding involves sequencing followed by identification by comparing sequences that have been previously deposited into the database [59]. DNA barcoding has revealed novel taxa in the Albitarsis Group (*Anopheles*: *Nyssorhynchus*) of Neotropical malaria vectors [60]. Using DNA bar-coding, Weiblen and an international team of researchers studied the population of moth and butterfly species across Papua New Guinea [61]. In another study, Jones *et al.* [62] sequenced CO1 gene from 22 common pests that contribute to the spread of food borne pathogens. Table 3 list the DNA bar-coding library of each insect order stored in the BOLD system and the Global Mirror System of DNA Barcode Data (GMS-DBD).

Mitochondrial Noncoding region

Noncoding region of insect mitochondrial DNA called the AT rich region is responsible for transcription and translation of insect mitochondrial DNA located between rRNA and tRNA cluster [63, 64]. This region is known to be the less used molecular marker in phylogenetic study [65]. One reason for this may be that, the primer working for one taxa may not work for another, since control region may be different in different insect due to tRNA transposition. This region from different insect shows high level of divergence, concerted evolution of tandem repeats, reduced substitution rate and directional mutational pressure [66]. But its high level of variability and faster evolution rate compared to other mitochondrial genes makes it a potential marker for phylogenetic analysis of intraspecific or closely related species [67]. Bravo *et al.* [66] have sequenced mitochondrial control region of *Diatraea saccharalis* and found high similarity with Lepidoptera *Cydiapomonella*. Zhang *et al.* [67] cloned and sequenced

Table 3: DNA bar-coding library of insects stored in the BOLD system and the Global Mirror System of DNA Barcode Data (GMS-DBD)†.

BOLD System						GMS-DBD				
	Growth & Development	Wing development	Order	Example	Families	Specimen Records	Specimens with Sequences	Specimens with Barcodes	Species With Barcodes	Species With Barcodes
Insecta						1,890,117	1,494,243	1,352,907	131,406	519,733
Apterygota (wingless insects)	Ametabolus	No	Grylloblattodea	Grylloblatta	2	2	1	2	1	1
			Thysanura	Silverfish and firebrats	3	42	31	23	3	12
			Diplura	Two-pronged bristletails	2	16	11	10	4	8
			Archaeognatha	Jumping Bristle tails	2	205	107	102	9	73
			Protura	coneheads	4	92	99	89	16	0
			Collembola	Springtails	4	41,041	23,276	19,776	452	6,818
Pterygota (winged insects)	Hemimetabola (gradual development)	Paleopterous orders (primitive winged) orders	Odonata	Dragonflies, damselflies	22	11,151	7,343	6,367	611	2,637
			Ephemeroptera	May flies	28	17,290	13,145	11,770	730	7,439
		Orthopteroid orders (Polyneoptera)	Plecoptera	Stoneflies	13	6,891	5,504	5,176	556	1,824
			Embioptera	Web spinners	5	61	47	45	11	21
			Phasmatodea	Stick and leaf insects	9	719	675	437	80	102
			Phasmida	Stick and leaf insects	1	5	12	3	1	0
			Orthoptera	grasshoppers, crickets, weta, and locusts	29	13,004	10,225	8,956	1,031	3,619
			Dermaptera	Earwigs	6	572	275	248	19	51
			Blattaria	Cockroaches	6	1,906	1,379	1,238	104	509
			Mantophasmatodea	Rock crawlers, heelwalkers, mantophasmids	1	2	2	1	1	2
			Isoptera	Termites	8	1,701	1,324	1,111	252	757
			Mantodea	Praying mantis	13	857	676	636	161	228
			Zoraptera	Zorapterans	0	0	0	0	0	0
		Hemipteroid (sucking) orders (Paraneoptera)	Hemiptera	True bugs	117	84,874	58,624	48,100	5,081	11,619
			Pscoptera	Book lice	6	3,592	2,203	2,163	3	319
			Phthiraptera	Lice	9	850	827	733	55	575
			Thysanoptera	Thrips	4	4,707	3,646	2,958	153	1,132
	Holometabola (complete development)	Primitive Holometabola	Coleoptera	Beetles	131	154,046	95,125	74,080	12,089	22,097
			Neuroptera	Lacewings, mantidflies, antlions	16	4,380	3,489	3,220	230	1,414
			Megaloptera	Alderflies, dobsonflies and fishflies	2	1,619	1,433	1,382	64	827
			Raphidioptera	Snakeflies	2	74	62	34	7	10
			Hymenoptera	Sawflies, wasps, bees and ants	86	330,223	237,235	197,898	19,992	84,818
		Orders related to Lepidoptera	Trichoptera	caddisflies	48	46,318	37,160	32,340	3,780	18,197
			Lepidoptera	Butterflies & moths	129	831,968	738,216	697,622	73,032	287,825
		Orders related to Flies	Mecoptera	Scorpion flies	6	148	150	86	11	65
			Diptera	True flies	125	337,465	272,580	254,521	13,317	73,490
			Siphonaptera	Fleas	4	332	203	173	12	45
			Strepsiptera	twisted-wing parasites	4	56	45	12	6	12

†The *Barcode of Life Data Systems* (BOLD) and the *Global Mirror System of DNA Barcode Data* is developed by the Network Information Center, Institute of Microbiology, Chinese Academy of Sciences, International DNA Barcode of Life Project - Data accessed on 26th March 2013.

control region from 56 specimens of *Gampsocleis* to describe their phylogenetic relationship and structure of control region and proposed the possibility of more than one species in that group with two conserved motif.

Mitogenomics

Mitogenomics or complete mitochondrial genome study is a modern research area for molecular evolution providing a robust phylogeny with highly resolved tree having strong branch support. However, very few complete genome sequences are available, especially for insects. This can be improved by the advancement of PCR and sequencing technology like Long-range PCR and Next Generation Sequencing (NGS) such as 454 pyrosequencing, Solexa and SOLiD provided by Roche, Illumina and Applied Biosystems. These have the ability to generate a large number of sequences within a very short time when compared to Sanger's method of sequencing [68]. Rare genomic changes (RGC) obtained from NGS data can be used as a marker for robust phylogeny. Among NGS, 454 pyrosequencing is the most frequently used method as it can generate longer sequence requires for species identification and phylogenetic studies [69,70]. Kuhn *et al.* [71] described molecular phylogeny based on single nucleotide polymorphisms between four closely related species in the genus *Spathius* by using Illumina technology. An ongoing NGS based project 1KITE (1K Insect Transcriptome Evolution) is focusing on a phylogeny with more than 1,000 insect species encompassing all recognized insect orders (http://www.1kite.org). A summary of insect mitogenomic data is shown in Table 4.

Conclusion

Mitochondrial molecular markers are mostly utilized for systematic and phylogenetic studies. But the region of mitochondrial gene used for study is purely based upon the objective. Mitochondrial 16S rRNA gene is useful for genetic diversity study of higher categorical level while 12S rRNA is useful at family and generic level. Mitochondrial protein coding genes are used for the study of family, genera and species. Among them, CO1 has been found to be an important gene for species identification and has been the most widely used for DNA bar-coding. Still, the contemporary method of phylogenetic study has its own limitations. These limitations are thought to be ignored using mitogenomics method, which is more advanced technology in recent days.

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Table 4: Summary of insect mitochondrial genomes

	Order	Family/ Subfamily	Species	Length (bp)	Size of genic region (kb)	Size of inter-genic region (kb)	GC ratio (%)	Major amino acids ratio (%)
Hemimetabola	Orthoptera	Acrididae : Acridinae	*Acrida willemsei*	15,601	11	5	23.78	L (13.48) ,I (10.44)
		Tettigoniidae : Tettigoniinae	*Anabrus simplex*	15,766	11	5	30.55	L (16.24), S (8.89)
		Pyrgomorphidae	*Atractomorpha sinensis*	15,558	11	4	25.710	L (14.04), I (10.37)
		Acrididae : Gomphocerinae	*Chorthippus chinensi*	15,599	11	4	24.886	L (13.95), I (10.37)
		Rhaphidophoridae :Rhaphidophorinae	*Troglophilus neglectus*	15,810	11	5	26.629	L(15.91), F(9.26), I(9.07)
		Tetrigoidea : Tetrigidae	*Tetrix japonica*	13,104	10	3	24.908	L(13.69),I(10.01)
		Gryllidae : Gryllinae	*Teleogryllus emma*	15,660	11	5	26.884	L(13.84),F(9.73),I(9.14)
	Plecoptera	Pteronarcyidae	*Pteronarcys princeps*	16,004	11	5	28.543	L(17.43) ,S(8.85)
	Ephemeroptera	Heptageniidae	*Parafronurus youi*	15,481	11	4	33.622	L(17.19) , S(8.93)
	Phthiraptera	Philopteridae	*Campanulotes bidentatus*	14,804	11	4	29.884	F (14.28) , L(12.23)
		Philopteridae	*Bothriometopus macrocnemis*	15,564	11	5	29.202	L(12.89), S (12.67)
		Boopidae	*Heterodoxus macropus*	14,670	11	4	20.723	L(15.92), F(11.31),S(10.46)
	Blattaria	Blattellidae :Blattellinae	*Blattella germanica*	15,025	11	4	25.444	L(15.42),I (10.26)
		Blattidae : Blattinae	*Periplaneta fuliginosa*	14,996	11	4	24.853	L(14.63),I (10.05)
	Hemiptera	Coreoidea : Rhopalidae	*Aeschyntelus notatus*	14,532	11	4	24.29	L (13.95), I (10.11)
		Plataspidae	*Coptosoma bifaria*	16,179 b	11	5	28.673	L (14.18), I (9.42)
		Phylloxeridae	*Phylloxeridae*	12,349	9	4	16.034	L (14.59), F (12.20)
		Berytidae	*Yemmalysus parallelus*	15,747	11	5	22.823	L(14.15), I(10.25)
		Reduviidae : Triatominae	*Triatoma dimidiata*	17,019	11	6	30.431	L(14.24), S(10.03)
		Aleyrodidae : Aleyrodinae	*Trialeurodes vaporariorum*	18,414	11	8	27.696	L(13.42),F(11.34), S(10.26)
		Corixidae : Corixinae	*Sigara septemlineata*	15,724	11	5	24.841	L(13.45), I(10.97)
		Aphididae : Aphidinae	*Schizaphis graminum*	15,721	11	5	16.061	L(14.93), I(14.08)
		Alydidae	*Riptortus pedestris*	17,191	11	6	23.408	L(14.17), I(10.94)
		Largidae	*Physopelta gutta*	14,935	11	4	25.490	L(14.25), I(9.44)
		Pleidae	*Paraplea frontalis*	15,130	11	4	23.529	L(14.70), I(10.46)
		Psyllidae	*Pachypsylla venusta*	14,711	11	4	25.002	L(15.26), I(11.19)
		Aradidae	*Neuroctenus parus*	15,354	11	4	31.138	L(13.19), S(9.54)
		Gelastocoridae	*Nerthra sp. NKMT022*	16,079	11	5	25.823	L(13.12), I(10.38)
		Aleyrodidae : Aleyrodinae	*Neomaskellia andropogonis*	14,496	11	4	18.729	L(14.17), I(11.32)
		Malcidae : Malcinae	*Malcus inconspicuus*	15,575	11	5	22.202	L(13.41), I(11.05)
		Cydnidae: Cydninae	*Macroscytus subaeneus*	14,620	11	4	26.211	L(14.38), S(9.44)
	Odonata	Gomphidae	*Davidius lunatus*	15,913	11	5	29.888	L(14.28), S(9.32)
		Libellulidae	*Orthetrum triangulare melania*	14,033	11	3	26.089	L(13.93), I (9.13)
		Lestoidea	*Pseudolestes mirabilis*	15,314	11	4	17.337	L(14.4), I (13.05)
	Psocoptera	Lepidopsocidae	*Lepidopsocid Sp. RS2001*	16,924	11	6	20.976	L (15.45), I (11.84)
	Mantodea	Mantidae : Paramantinae	*Tamolanica tamolana*	16,055	11	5	24.727	L(15.52 , I(9.55)
	Thysanoptera	Thripidae: Thripinae	*Thrips imaginis*	15,407	11	4	23.431	L(13.25), F(13.25)
	Mantophasmatodea	Mantophasmatidae	*Sclerophasma paresisense*	15,500	11	4	24.935	L(15.18), I(9.95)
	Isoptera	Rhinotermitidae : Heterotermitinae	*Reticulitermes hageni*	16,590	11	5	34.451	L(14.36) S(9.86)
		Rhinotermitidae : Heterotermitinae	*Reticulitermes santonensis*	16,567	11	3	33.907	L(14.42)S(9.65)
		Rhinotermitidae : Heterotermitinae	*Reticulitermes flavipes IS13*	16,565	11	5	33.818	L(14.31) S (9.67)
		Rhinotermitidae : Heterotermitinae	*Reticulitermes virginicus*	15,966	11	5	34.369	L(14.23) S(9.89)
	Phasmatodea	Phasmatidae : Phasmatinae	*Ramulus hainanense*	15,590	11	5	26.902	L(13.03) S (9.47)
		Timematoidea (superfamily)	*Timema californicum*	14,387	11	3	27.857	L(15.10) I(10.00)
Ametabolus	Archaeognatha	Machilidae	*Pedetontus silvestrii*	15,879	11	5	25.657	L (15.61), I (9.51)
		Machilidae	*Petrobius brevistylis*	15,698	11	4	32.119	L (15.83), S (9.55)
		Meinertellidae	*Nesomachilis australica*	15,474	11	4	31.175	L (15.51) ,S (9.07)
	Collembola	Onychiuridae , Tetrodontophorinae	*Tetrodontophora bielanensis*	15,455	11	4	27.324	L (14.69),I (9.89)
		Neanuridae , Frieseinae	*Friesea grisea*	15,425	11	4	27.728	L(15.74) , S (10.04)
		Hypogastruridae	*Gomphiocephalus hodgsoni*	15,075	11	4	25.917	L (15.05), S (10.13)
		Entomobryidae , Orchesellinae	*Orchesella villosa*	14,924	11	4	27.821	L(14.50)I(10.13)
		Onychiuridae ,Onychiurinae	*Onychiurus orientalis*	12,984	11	2	30.894	L(15.56), S(10.43)
	Grylloblattodea	Grylloblattidae	*Grylloblatta sculleni*	29.711	10	6	29.711	L (16.22), S (9.80)

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