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GENETIC DIVERSITY ANALYSIS OF THE FLEA BEETLE, *PODAGRICA FUSCICORNIS* (CHRYSOMELIDAE) USING MITOCHONDRIAL CYTOCHROME OXIDASE SUBUNIT I GENE MARKER

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ABSTRACT

Podagrica fuscicornis referred to as Flea beetles are members of Family Chrysomelidae (leaf beetles) and occur in all plant life habitats. *Podagrica* species arise most frequently inside the open, namely within the location of grasslands, forests and water bodies. Molecular characterization and DNA barcoding is a taxonomic method that makes use of a short genetic marker in an insect DNA to identify a species, which include an unknown species. DNA barcoding for species identification of the Flea beetles, *P. fuscicornis* isolated from Malappuram district (Kerala: India) by using the mitochondrial cytochrome oxidase subunit I (CO I) gene have been checked. DNA sequence similarity searches of COI gene of *P. fuscicornis* (NCBI GenBank Accession No. KX 778629) revealed that it is genetically 87% identical to *Podagrica fuscipes* (Accession No. KF 655901) cytochrome oxidase I gene collected from Spain. The results indicate slow evolution of the CO I sequences among the morphologically distinct and geographically isolated group of *P. fuscicornis.*

KEYWORDS: Cytochrome oxidase I Gene Sequence, Molecular phylogeny, *Podagrica fuscicornis*

INTRODUCTION

Insects can be found in every environment on our planet. Many insects are herbivores, or plant-eaters, which makes them primary consumers. In India 59,353 insect species are found which corresponds to 6.83 % of world fauna (Varshney, 1997). The described taxonomic richness of insects is distributed unevenly among the higher taxonomic groups. Five orders stand out for their high species richness: the beetles (Coleoptera); flies (Diptera); wasps, ants and bees (Hymenoptera); butterflies and moths (Lepidoptera); and the true bugs (Hemiptera). Coleoptera is via some distance the maximum diverse order of insect and the biggest no longer most effective in phylum Arthropoda however also inside the whole animal kingdom; and it includes more species known to science than any other order. The most authoritative calculations indicate that already now more than 350,000 species of beetles were defined in approximately 190 households across the world; in India, approximately 15,501 beetle species are discovered.

Podagrica fuscicornis known as Flea beetles are members of Family Chrysomelidae (leaf beetles) and subfamily Alticinae (Aslan et al., 1998) arise in all plants habitats. *Podagrica* species occur maximum regularly inside the open, namely inside the vicinity of grasslands, forests and water bodies (Gruev and Döberl, 1997; Cilbiroglu and Gok, 2004). We describe flea beetles as small bugs with a size from 1.5 to 4 mm. They can jump due to the enlarged backside organ (hind legs) – Maulik's organ. They also have an ability to fly. When plant is heavily attracted by flea beetles, small round holes (up to 1 mm) caused by an individual flea beetles feeding may coalesce into larger areas of damage (Maceljski, 1999). Adult beetles of *P. fuscicornis* are 3.0 to 6.0 mm in size. Head and neck shield are coloured red. Front wings

(sheath-wings) are dark blue to blue green and rarely are detected metal coloured. Sheath wings contain hollows which are deep, very abundant and spread scarce. Hollows on neck shield are even more compacted. Legs are characteristically yellowish to bright brownish. Only few of the flea beetles cause damage on cultivated or local flowers permanently or on occasions, but not yet presenting special danger in plant production (Tanja Bohinc, *et al.*, 2011).

Taxonomy is the identification of species, primarily based on the morphological characters and it requires substantial knowledge about several organisms and their characters. The routine identification of known species can be tough, frequently requiring highly specialized information and representing a limiting factor in ecological studies and biodiversity inventories. Morphological statistics are normally time consuming and need experts, DNA barcoding techniques are a uniform and practical approach of species identification of insects and may be used for the identification of all developmental stages of insects, their food webs and biotypes and this could no longer be possible with morphology- based taxonomy. Hebert *et al.* (2003) focused this discussion by proposing that a DNA barcoding system for animal life could be based upon sequence diversity in cytochrome *c* oxidase subunit I (COI). Molecular characterization and DNA barcoding is a taxonomic method that uses a short genetic marker in an insect DNA to identify a species, including an unknown species. Insect mitochondrial genome is double-stranded circular genomes which range from 14,503 to 19,571 bp in size. Mitochondrial DNA sequences have already revealed a few surprising matters approximately the evolutionary interrelationships of many groups of creatures, from human beings to whales to sharks. Molecular phylogenetic is one aspect of molecular systematics, a broader term that also includes using molecular information in taxonomy and biogeography.

Molecular evolutionary studies are based on the idea that the accumulation of differences in the DNA sequences among homologous genes in two species displays the time elapsed due to the fact those species diverged from a commonplace ancestor. The comparison of number of sequences from diverse species is used to derive phylogenetic trees showing species relatedness and the relative amount of elapsed time since the species diverged. These trees are based on parsimony, where by species are positioned relative to one another by minimizing the number of nucleotide changes required to move between two positions. Molecular phylogeny analysis using mitochondrial COI gene sequences were extensively conducted in various insect groups ranging Odonata (Jisha *et al*., 2015), Hymenoptera (Rukhsana *et al*., 2014) Lepidoptera (Akhilesh *et al*., 2014), Heteroptera (Sreejith *et al*., 2014) and Diptera (Bindu *et al*., 2014; Priya *et al*., 2014).

The objective of the present work is to isolate and sequence the mitochondrial CO I gene of the Flea beetles, *P. fuscicornis* isolated from Malappuram district that can be used as a DNA barcode for its taxonomic identification and to analyse the phylogenetic relationships. The advent of molecular techniques has expanded greatly the availability of molecular characters useful in insect identification.

MATERIALS AND METHODS

Sample Collection, Identification and Preservation

The Flea beetles, *P. fuscicornis* are collected from the two different localities of North Kerala for the study. Two different methods are used for the sample collection for the study. Hand picking and sweep net methods are mainly used. The collected specimens are morphologically identified by using identification guides, keys and by expert consultation. The identified specimens are stored in 70% ethanol as voucher specimen.

Genomic DNA Extraction and PCR Amplification

The genomic DNA was extracted from one of the thoracic legs of the morphologically identified experimental insect, *P. fuscicornis* by using NucleoSpin XS ® Tissue Kit (Macherey- Nagel, Germany) as per the manufacturer's instruction. DNA was extracted from the leg piece of the experimental sample (Shere-Kharwar *et al*., 2013). Agarose gel electrophoresis is used for the conformation of the presence of DNA.

The amplification reaction was performed by using a DNA thermal cycler (Takara). About 2 ng of genomic DNA was amplified for mitochondrial cytochrome oxidase subunit I (CO I) gene using forward and reverse primers. PCR was carried out in a total volume of 50 µl containing 2 ng of genomic DNA(1 µl), 1 µl each forward and reverse primers with at a concentration of 10 µM, 1 µl of $dNTP_S$ (2mM), 5 µl of 10X reaction buffer with $MgCl_2$, 0.5 µl Taq polymerase (5 U/µl) and 41.5 µl of Water. After an initial denaturation at 95°C for 5 minutes, amplification was made through 30 cycles, each consisting of a denaturation at 95°C for 10 seconds, annealing at 50°C for 1minutes, extension step at 72°C for 45 seconds and a final extension at 72°C for 3 minutes.

Agarose Gel Electrophoresis

The PCR products were resolved on 2 % TAE agarose gel (Mahesh *et al.*, 2012) stained with EtBr (Sambrook and Russell, 2001) and photographed using a gel documentation system. A Gene Ruler (Thermo Scientific; GeneRuler 100bp DNA Ladder. #SM0242) was used to determine the size of the product. EtBr act as an intercalating agent on the bases of DNA molecules and imparts an orange colour to DNA under ultraviolet light.

PCR Product Purification

After ascertaining the PCR amplification of the corresponding CO I fragment, the remaining portion of the PCR product was column purified using GenElute™ PCR Clean up Kit. The GenElute™ PCR Clean up Kit is designed for rapid purification of single stranded or double stranded PCR amplification products from other components in the reactions such as excess primers, nucleotides, DNA polymerase, oils and salts from the PCR products. The purified product was again resolved on 2% agarose gel to check the presence of DNA.

DNA Sequencing and phylogenetic Analysis

The purified PCR product was sequenced from both ends using the forward and reverse primers used for the PCR using the Sanger's sequencing method at Sci Genom Labs Private Ltd., Cochin with ABI 3730XL automated sequencer. The DNA sequence of leaf beetles of North Kerala is presented here along with phylogeny analysis. The trimmed COI sequences of forward and reverse obtained were multiply aligned using ClustralW (Thompson *et al*., 1994). Take the aligned region as the final product sequence. The consensus sequence was searched for its similarity using BLAST n and BLAST p programme of NCBI (Altschul *et al*., 1990). The partial COI gene sequence was deposited in GenBank (NCBI) for worldwide accession. It can be used as a molecular barcode for the collected Chrysomelidae species.

Final nucleotide sequences were analyzed using MEGA6 (Tamura *et al*., 2013). The inter and intra specific genetic diversity were generated using Kimura 2 parameter model, and a phylogenetic tree was generated using the neighbor-joining algorithm (Saitou and Nei, 1987). Bipartitions in the neighbor-joining tree were examined by bootstrap analyses over 500 replicates (Felsenstein, 1985). This bootstrap analysis is an important for calculating the confidence

interval of monophyletic groups within phylogenies. Percentage nucleotide distances calculation were performed using MEGA6.

RESULTS AND DISCUSSION

The partial DNA sequence of mitochondrial cytochrome oxidase subunit I gene of *P. fuscicornis* isolated from Kannur and malappuram district, Kerala was PCR amplified using forward primer with DNA sequence 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse primer 5'TAAACTTCAGGGTGACCAAAAAATCA-3'. The PCR amplified sequences of mitochondrial COI gene fragment of *P. fuscicornis* isolated from Malappuram yielded a single product of 618 bp. The sequence has been deposited in the NCBI GenBank with Accession No. KX 778629. The phylogenetic tree plotted using neighbor joining method in rectangle format presented in Figure 1.

The DNA sequences in organisms are maintained from generation to generation with very little change. Although such genetic stability is crucial for the survival of individuals, the survival of organisms may depend on genetic variation through which they can adapt to a changing environment. DNA sequence based identification technique has revealed the morphological and ecological traits of many species during larval stages (Rukhsana *et al*., 2014).

The nucleotide BLAST against the nucleotide redundant database showed that the COI sequence obtained is novel (GeneBank Accession No. KX 778630). The nearest match (87%) to mitochondrial cytochrome oxidase subunit I (COI) gene of *P. fuscicornis* with *P. fuscipes* cytochrome oxidase subunit I (COI) gene (GenBank No. KF655901). The evolutionary history inferred using the Neighbor-Joining method indicated that *P. fuscicornis* is phylogenetically more closely to different species of same genus. The species *P. fuscipes*, *Longitarsus pratensis* (KF655025) and *Nanostrangalia chujoi* (FJ559029) are the nearest relative species of *P. fuscicornis* from other leaf beetle genus. The percentage of COI evolutionary divergence of *P. fuscicornis* with other beetle species was presented in Table No.1. The *P. fuscicornis* COI sequence showed 14.97% evolutionary divergences between *P. fuscipes* (GenBank No. KF 655901), 16.23% between *L. pratensis* (KF 655025) and 16.91% between *Calomicrus suturalis* (KF 654427). The species *P. fuscicornis* showed lowest divergence (14.97%) and the species *Belvosia* sp., highest divergence (18.35%) with *P. fuscicornis* among other leaf beetle genus used in this study. The nucleotide composition study showed the COI sequence of genus *P. fuscicornis* has 29.4%Adenine, 37.7% Thymine, 17.2% Cytosine and 15.7% Guanine nucleotides.

Variations within the mtDNA sequence are among the most extensively used genetic markers in animals. Leaf beetles are a far diversified because of their morphology and behavior. The molecular studies also assist the morphological and behavioral variation in the Leaf beetles. The most of the leaf beetle species used inside the phylogeny study shows widespread version with other leaf beetles and similarity in sequence with associated species probabilities of identity between the sequences also are an essential element in molecular systematics. Distantly related species might have 50% identity in a particular DNA sequence, while more closely related species might have 90% identity in the same sequence (Gurney et. al., 2000). All specimens of *P. fuscicornis* used in this study have a minimum of 87% identity with *P. fuscipes* COI sequence. All other *Podagrica* species used in this study showed 85.03% to 84.82% identity with *P. fuscipes*. The members of all species of *Podagrica* genus might have arouse from a single ancestor, hence it is a monophyletic. The estimated Transition/Transversion bias (*R*) of *P. fuscicornis* is 0.84. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model. The probability of substitution (r) from one base (row) to another base also

calculated for 24 nucleotide sequences shown in Table 2. The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating ML values, a tree topology was automatically computed. The maximum composite likelihood for this computation was -5577.651. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 590 positions in the final dataset

CONCLUSIONS

This study represents important progress towards understanding the evolution and biodiversity of leaf beetles and provides a foundation for similar future work. The *P. fuscicornis* provides an excellent study system as the order is huge and includes a wide variety of groups, some of which have a close association with agricultural habitat, living in or on for most of their lifespan.

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REFERENCES

1. Akhilesh, V.P. and Sebastian, C. D. 2014. Molecular barcoding and phylogeny analysis of *Herpetogramma stultalis* (Lepidoptera: Crambidae) using cytochrome oxidase subunit I gene sequence. *International Journal of Advanced Life Sciences*, **7**: 463-466.
2. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. *J Mol Biol*., 215 (3):403-10.
3. Aslan, I., Gruev, B., Ozbek, H. 1996. A preliminary review of the subfamiliy Alticinae (Coleoptera, Chrysomelidae) in Turkey. *Turk. J. Zool*., 23: 373-414.
4. Bindu, P. U. and Sebastian, C. D. 2014. Genetic structure of mitochondrial cytochrome oxidase subunit I gene ofthe mosquito, *Armigeres subalbatus. International Journal of Research*, 1(10): 49-56.
5. Çilbiroglu, E. G., Gok, A. 2004. Flea beetles (Coleoptera: Chrysomelidae) of Isparta, Turkey, with habitat use and host plant associantions. *Proceedings of Entomological Society*. 106: 858-864.
6. Felsenstein, J. 1985. Phylogenies and the Comparative Method. *The American Naturalist*, 125(1): 1-15
7. Gruev, B. and Doberl, M. 1997. General distribution of the flea beetles in the Palearctic subregion (Coleoptera, Chrysomelidae: Alticinae). *Scopolia*, 27: 1-496
8. Gurney, T. Jr., Elbel, R., Ratnapradipa, D. and Brossard R., 2000. Introduction to the molecular phylogeny of insects. *Ed. Proceedings of the 21st Workshop/ Conference of the Association for Biology Laboratory Education*, 21: 63-79.
9. Hebert, P.D.N., Ratnasingham and Dewaard, J. R. 2003. Barcoding animal life cytochrome c oxidase subunit 1 genes among closely related species. *Proc. R. Soc. Lond. B.* 270: 396 - 399.

10. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution.* 16:111-120.

11. Maceljski, M. 1999. Poljoprivredna entomologija. Cakovec, Zrinski: 464 p.

12. Mahesh, N., Balakumar, S., Indumathi,P., Ayyadurai, A. and Vivek, R. 2012. Production and Optimization of Mevastatin using Penicillium citrinum NCIM 768. *J Microbial Biochem Technol*., 4: 1.

13. Priya Bhaskaran, K. P. and Sebastian, C. D. 2014. Molecular barcoding of green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae) using COI gene sequences. *Journal of Entomology and Zoology Studies*, 3(1): 10-12.

14. Rukhsana, K. and Akhilesh, V. P. and Sebastian, C. D. 2014. Deciphering the molecular phylogenetics of the Asian honey bee, *Apis cerana* (Hymenoptera: Apidae) and inferring the phylogeographical relationships using DNA barcoding, *Journal of Entomology and Zoology Studies*, 2(4): 218-220.

15. Rukhsana, K., Ashitha, K.S., Mashoor, K. and Sebastian, C. D. 2014. Molecular phylogeny analysis of Southern house mosquito, *Culex quinquefasciatus* (Diptera: Culicidae) derived from mitochondrial DNA sequences. *International Journal of Research*, 1(5): 844-853.

16. Saitou, N. and Nei, M.1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4:406-425.

17. Sambrook , J. and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual *CSHL Press - Science*, 1: 2344.

18. Shere-kharwar , A.S., Magdum, S., Khedkar , G.D., Gupta, S. and Zambare, V. 2013. Moth legs: Excellent source of tissue for DNA extraction (Lepidoptera: Noctuidae) *Indian J. L. Sci*., 2(2): 35-37.

19. Sreejith, K. and Sebastian, C. D. 2014. Phylogenetic analysis and sequencing of the mitochondrial cytochrome oxidase subunit I (COI) of white backed plant hopper, *Sogatella furcifera* (Horvath). *International Research Journal of Pharmacy*, 5 (12): 887-890.

20. Tamura, K., Stecher, G., Peterson, D., Filipski A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

21. Tamura, K., Stecher, G., Peterson, D., Filipski,A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, 30 (12): 2725-2729

22. Tanja, B., Matej,V. and Stanislav, T. 2011. Massive occurrence of *Podagrica fuscicornis* (L.) (Coleoptera, Chrysomelidae) on common marshmallow (*Althaea officinalis* L.)

23. Varshney, R. K. 1997. Genera of butterflies from India and neighbouring countries (Lepidoptera: Lycaenidae). *Index Rhopalocera Indica. Part III*, 31: 83- 138.

APPENDIES

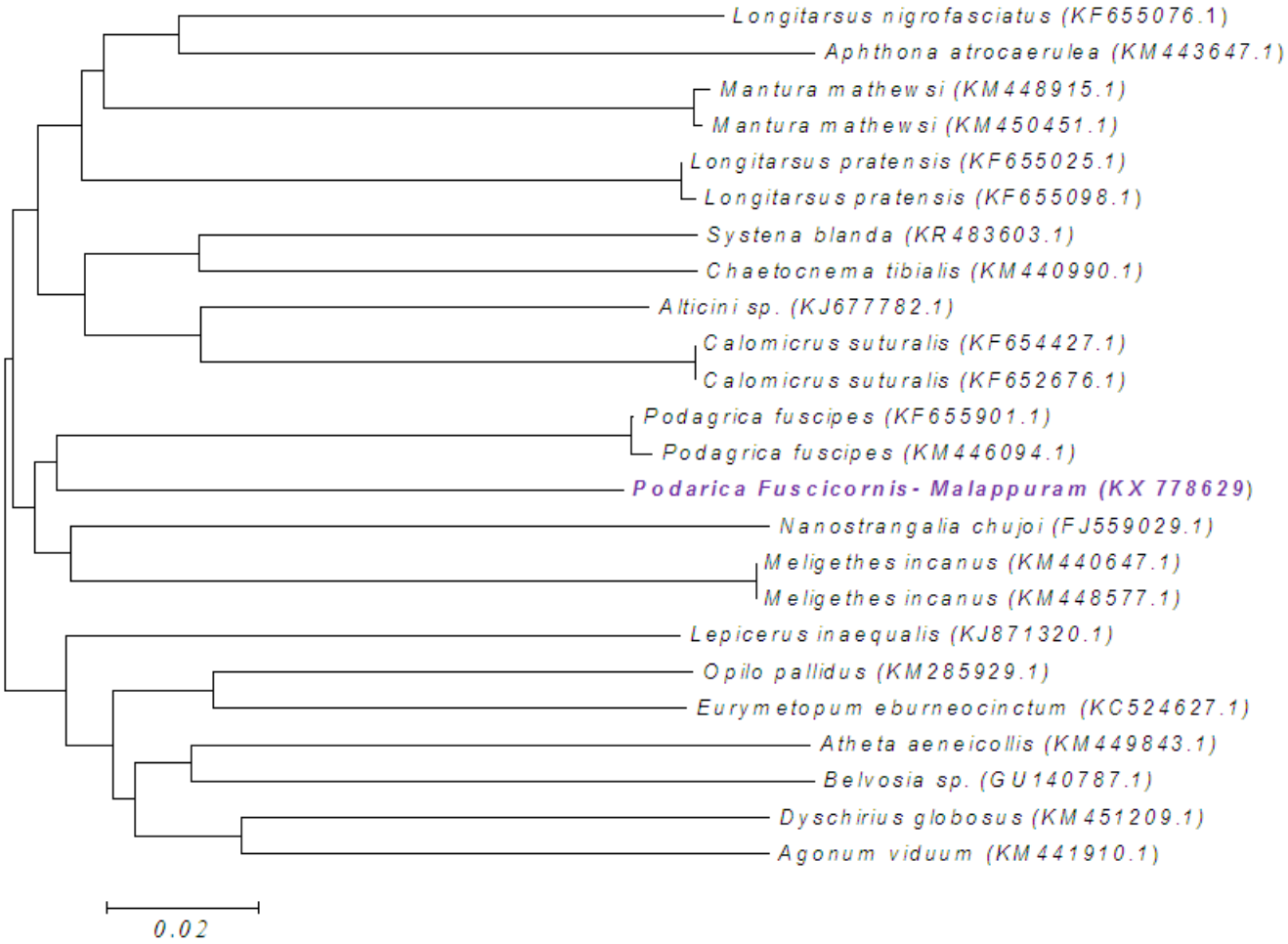

Figure 1: The phylogenetic Tree Plotted for *P. fuscicornis* was Inferred using COI Gene Partial Sequence by Neighbor Joining Method

Table 1: Evolutionary Divergence between Related Species on COI Gene Sequences of Podarica Fuscicornis

Species Name with GenBank Accession Number	% of Divergence
Podarica_Fuscicornis (KX 778629)	0
Podagrica_fuscipes_(KF655901.1)	14.97%
Podagrica_fuscipes_(KM446094.1)	15.18%
Longitarsus_pratensis_(KF655025.1)	16.23%
Longitarsus_pratensis_(KF655098.1)	16.43%
Calomicrus_suturalis_(KF654427.1)	16.91%
Calomicrus_suturalis_(KF652676.1)	16.91%
Meligethes_incanus_(KM440647.1)	16.88%
Atheta_aeneicollis_(KM449843.1)	16.66%
Nanostrangalia_chujoi_(FJ559029.1)	16.86%
Longitarsus_nigrofasciatus_(KF655076.1)	17.10%
Opilo_pallidus_(KM285929.1)	17.50%
Dyschirius_globosus_(KM451209.1)	17.70%
Alticini_sp._(KJ677782.1)	17.31%
Systena_blanda_(KR483603.1)	16.85%

Meligethes_incanus_(KM448577.1)	16.88%
Mantura_mathewsi_(KM448915.1)	17.34%
Aphthona_atrocaerulea_(KM443647.1)	18.04%
Chaetocnema_tibialis_(KM440990.1)	17.92%
Lepicerus_inaequalis_(KJ871320.1)	17.71%
Mantura_mathewsi_(KM450451.1)	17.12%
Agonum_viduum_(KM441910.1)	17.71%
Eurymetopum_eburneocinctum_(KC524627.1)	17.74%
Belvosia_sp._(GU140787.1)	18.35%

Table 2: Maximum Composite Like hood Estimate of the Pattern of Nucleotide Substitution. Each Entry Shows the Probability of Substitution (r) from one Base (Row) to another Base (Column)

	A	**T/U**	**C**	**G**
A	-	6.81	6.81	11.38
T/U	6.81	-	11.38	6.81
C	6.81	11.38	-	6.81
G	11.38	6.81	6.81	-