Molecular phylogeny of skipper butterflies (Lepidoptera: Hesperiidae) from the Western Ghats, India

A thesis submitted towards partial fulfillment of

BS-MS dual degree programme

by

Prabhdeep Singh

Under the guidance of

Dr. Krushnamegh Kunte, PhD
Post-doctoral Research Fellow,
FAS Center for Systems Biology,
Harvard University, Cambridge, MA, USA.

And

Prof. Milind Watve

Professor, Department of Biology,

IISER, Pune.

Department of Biology

Indian Institute of Science Education and Research, Pune

Certificate

This is to certify that this dissertation entitled "Molecular phylogeny of skipper butterflies (Lepidoptera: Hesperiidae) from the Western Ghats, India" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune, represents original research carried out by Prabhdeep Singh at Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Krushnamegh Kunte, Post-doctoral Research Fellow, FAS Center for Systems Biology, Harvard University, Cambridge, MA, USA, and Prof. Milind Watve, Professor, Department of Biology, IISER, Pune, during the academic year 2010-2011.

Supervisor: Krushnamegh Kunte, PhD

Date: 12 April 2011

Place: Cambridge, MA, USA

Head Biological Sciences

Date:

Place:

Internal Supervisor: Prof. Milind Watve

Date:

Place:

Molecular phylogeny of skipper butterflies (Lepidoptera: Hesperiidae) from the Western Ghats, India

Prabhdeep Singh [1*], Krushnamegh Kunte [2] and Milind Watve [1]

1. *Indian Institute of Science Education and Research, Sai Trinity, Garware Circle, Pune 411021, India.*

2. *FAS Center for Systems Biology, Harvard University, 52 Oxford St Northwest Lab, Room 458.40-3, Cambridge, MA 02138, USA.*

*for correspondence

p.singh@iiserpune.ac.in

Journal: Molecular Phylogenetics and Evolution.

Abstract

Butterflies and moths together comprise the second-most diverse order of insects on earth. Skipper butterflies (family Hesperiidae) form a natural biological link between butterflies and moths; they make up one of the largest families of butterflies, and are prominently represented in India's Western Ghats biodiversity hotspot. However, their phylogenetic relationships with other Oriental Hesperiidae are poorly known, which prevents us from developing a biogeographic understanding of diversification of butterflies in the Western Ghats. Here we propose higher (genus-level) phylogenetic relationships of many Western Ghats butterflies of family Hesperiidae based on molecular sequence data from two genes: the mitochondrial *cytochrome oxidase I* (*COI*) and nuclear *elongation factor-1α* (*EF-1α*). Phylogenetic analyses with the Maximum Likelihood, Maximum Parsimony and Neighbor-Joining methods resulted in similar relationships at subfamily and genus levels. Monophyly of family Hesperiidae was strongly supported and relationship among the traditionally recognized subfamilies was as follows: (Coeliadinae + (Pyrginae + Hesperiinae)). Coeliadinae was found monophyletic and similarly, Hesperiinae was also found forming monophyletic group. Pyrginae was found to be paraphyletic with several clades. We resolved phylogenetic positions of many genera of the diverse tribe *Baorini* (Hesperiinae), which has many Western Ghats endemics. This work aims to build towards a fuller taxonomic and phylogenetic framework that will contribute to understanding when and how the Western Ghats butterfly fauna formed and diversified. It will also help identify taxonomically unique species and contribute to their conservation.

Keywords: Molecular phylogeny; Hesperiidae; Skipper; Western Ghats

1. Introduction

The Western Ghats or Sahyadri mountains run along the western coast of peninsular India. These mountains have unique habitats comprising of tropical rain- and deciduous forests, grasslands and scrub forests, and montane evergreen forests that are known for their rich and unique flora and fauna with many endemic species. Western Ghats is amongst the 34 biodiversity hotspots of the world (Mittermeier et al., 2005) and one of only two biodiversity hotspots of South Asia (Myers et al., 2000). Due to rapid deforestation, the biodiversity of Western Ghats is declining and thus posing a serious threat to environment (Jha, et al., 2000). Western Ghats need ~~an~~ immediate attention for conserving its biodiversity and avoiding any further loss of biological resources. However, for conservation of any taxa it is necessary to understand its diversity, distribution and population dynamics. This can only be done if it is taxonomically well studied group. Among insects, butterflies are taxonomically and ecologically a well studied group (Thomas, 2005). This is partly because butterflies are most attractive and best loved group of organisms among invertebrates.

The Butterflies of Western Gats have been documented since the turn of 19th century and majority of species and sub species have probably already been described (Kunte, unpublished data). Due to its vast biological knowledge, butterflies are used as a model group of organism for various studies like ecology, evolution and developmental biology (Ellers and Boggs, 2003; Pollard, 1991). The ecology and diversity of Western Ghats butterflies has been studied to some extent (Kunte, 1999, 2005, 2008). They are dependent on their host plants and respond quickly to any type of habitat change (Blair, 1999; Mennechez et al., 2003). Further, because of their dependence on ~~the~~ plants, butterfly diversity indirectly reflects the overall plant diversity in a

given area and thus becoming a good indicator of health of environment (Kocher and Williams, 2000; Bobo et al., 2006; Akite, 2008).

Butterflies in the Western Ghats belong to five families, 164 genera and 334 species, with 33 endemic species (Kunte, 2008). However, their phylogenetic positions, generic, specific and subspecific assignments are still in flux (Kunte, unpublished data). Among butterflies, the family Hesperiidae (Lepidoptera: Hesperioidea) commonly known as "skipper butterflies" has lots of conflicts in relationships at various taxonomic levels. This family includes around 4000 recognized species all around the world (Bridges, 1993), which are currently distributed among 7 subfamilies and 567 genera (Warren et al., 2008). In Western Ghats, Hesperiidae has total 82 species (with 10 species endemic to this region) distributed among 46 genera and 3 subfamilies, namely Coeliadinae, Pyrginae and Hesperiinae (Kunte, 2000, 2008; Gaonkar, 1996).

For the past ~250 years, the classification of butterflies has been done based on the morphological characters of adult specimens (Wahlberg et al., 2005). The morphology-based classification has been very useful especially at the subfamily and higher levels, but it has limited utility in resolving precise phylogenetic positions at the genus and species levels. It has also left some higher-level taxonomic groups unresolved. For example, the position of "giant skippers" or Megathyminae is not confirmed yet. Freeman (1969) recognized them as a family, Mielke (2005) considered them a subfamily while some others authors put them deep within subfamily Hesperiinae (Ackery et al., 1999; Warren et al., 2008). Again, subfamily 'Pyrginae' always fails to form a monophyletic group and similarly, species of genus *Celaenorrhinus* are considered paraphyletic in many phylogenetic studies (Wahlberg et al., 2005; Warren et al., 2008, 2009).

In the last decade, there has been an enormous increase in the use of molecular data (DNA sequences) to assess genetic variation among various taxa. DNA sequences have now

become a popular means for identification and authentication of butterfly species. DNA sequences have also been used to resolve the phylogenetic relationship between families and subfamilies (Wahlberg et al., 2005; Warren et al., 2008). Warren et al. (2008) used DNA sequence data of one mitochondrial (*COI*) and two nuclear genes (*EF-1α* and *wingless*) to resolve the phylogenetic relationship between tribes and subfamily of Hesperiidae. They confirm the status of traditionally recognized subfamilies of Hesperiidae with following relationship: (Coeliadinae + ("Pyrginae" + (Heteropterinae + (Trapezitinae + Hesperiinae)))).However, DNA sequences have never been used to determine phylogenetic positions of Western Ghats butterflies.

In the current study, we tried to resolve phylogenetic relationships of family Hesperiidae at subfamily and lower levels using DNA sequence of two genes namely the mitochondrial *cytochrome oxidase I (COI)* gene and nuclear *elongation factor-1α (EF-1α*) gene, which are standard molecular markers for lepidopteran phylogenies (Wahlberg et al., 2005, 2008; Warren et al., 2008, 2009). We added 6 Oriental genera (*Baoris, Caltoris, Parnara, Borbo, Udaspes* and *Gomalia*) to the comprehensive Hesperiidae molecular phylogeny of Warren et al. (2008). Our work was mainly focused on Hesperiidae of Western Ghats which is currently divided into three subfamilies namely: Coeliadinae, Pyrginae and Hesperiinae (Kunte, 2000).

2. Material and methods

2.1 Study site and sampling

Our collection sites were mainly in Southern-Western Ghats of India. Butterfly specimens were collected from six Wild Life sanctuaries and National parks across Kerala: Shendurney Wild life Sanctuary, Munnar Division, Chinnar Wildlife Sanctuary, Eravikulam National Park, Peppara Wildlife Sanctuary and Thattekkad Bird Sanctuary. All the specimens were collected between June and August 2010.

Prabhdeep Singh collected adult butterflies in the field using a butterfly net and with help from our colleague S. Kalesh. Leg samples of species that we could not collect in the field were provided by S. Kalesh. All the specimens were identified by S. Kalesh based on morphological characters. A total of 43 species from 36 genera of Hesperiidae were collected. Specimens were preserved in glassine envelopes and legs were preserved in 100% ethanol for extracting DNA, Both the leg samples and specimens were stored at -40°C.

2.2 DNA extraction, gene amplification and sequencing

DNA was extracted from all species using QIAGEN DNeasy Blood and Tissue Kit (Cat.No: 69506) according to manufacturer's protocol. DNA was extracted from legs preserved in ethanol. Resultant DNA was eluted in 50 µl of AE buffer and was stored at -40°C.

For each specimen, 1058bp of the mitochondrial *cytochrome oxidase subunit I* (*COI*) gene and 744bp of the nuclear *elongation factor-1α* (*EF-1α*) gene was amplified. The *COI* fragment was amplified using the primers Rudy and Phyllis, and in some cases with the

Hesperiidae-specific primers Gary and Susan (Warren et al., 2008). *EF-1α* fragment was amplified using primers Al and Tipper. Further primer details are given in Table 1.

To amplify *COI* gene fragment, we used 50µL PCR solutions that included 5µL of 10X buffer, 7µL of 25µM $MgCl_2$, 1µL of 10µM dNTPs, 0.3µL Taq polymerase, 2µL of each primer (10 µM), 5µl of DNA template and 28.7 µL of distilled water. *EF-1α* PCR mix was similar: 5 µL of 10X buffer, 2 µL of 25 µM $MgCl_2$, 2µL of 10 µM dNTPs, 0.8 µL Taq polymerase, 2µL of each primer (10 µM), 6µl of DNA template and 30.2µL of distilled water. The thermocycling profile consisted of an initial denaturation of 4 min at 92°C, ~~and~~ 40 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C.

Amplified DNA sequences were cleaned using Promega PCR cleanup system (Cat. # A9282). Cleaned PCR products were sequenced by First BASE laboratories in Malaysia. Each gene was sequenced in both forward and reverse direction.

2.3 Data analysis

Sequences of all the species were cleaned by pairwise alignment of complementary sequences using the ChromasPro1.34 software and multiple sequence alignment was performed in BioEdit 7.0.8.0 (Hall, 1999). All small or incomplete sequences were removed. Phylogenetic trees were generated using three clustering methods: Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor-Joining (NJ). All these trees were generated by using MEGA 5.01 (Tamura et al., 2011). Maximum likelihood tree for both the genes was generated using the General Time Reversible (GTR) model. MEGA 5.01 was used to find best nucleotide substitution model for maximum likelihood tree, and the GTR plus gamma distribution plus invariable site (GTR+ Γ +*I*) model was found to be the best fit in all cases. Branch support was based on 500 bootstrap replicates. Neighbor-joining tree was generated based on Kimura 2-

parameter (K2P) model. Branch support was assessed with 1000 bootstrap iterations. Maximum parsimony tree was generated using Close-Neighbor-Interchange (CNI) on Random Trees. Branch support was assessed with 1000 bootstrap replicates. Separate analysis was performed for each gene and for the combined data set (*COI* + *EF-1α*).

3. Results

Analysis of the individual *EF-1α* gene region and the combined dataset (*COI* + *EF-1α*) resulted in a similar tree topology and phylogenetic relationships, while the *COI* tree disagreed with these trees. Out of the16 resolved ingroup branches of combined data, *EF-1α* supported 14 and disagreed with 2. Thus, the *EF-1α* region appeared to provide most of the phylogenetically informative characters. The *EF-1α* data consisted of 24 Hesperiidae species. Due to unavailability of *COI* sequence of 8 other species, we were left with a total of 16 Hesperiidae species for our combined data set.

3.1 Subfamily level relationship

Monophyly of Hesperiidae was strongly supported by all the three clustering methods and resulted in giving the following relationship between the three subfamilies: (Coeliadinae + (Pyrginae + Hesperiinae)). Coeliadinae was basal and monophyletic with high bootstrap support (Fig.1). Pyrginae was paraphyletic and formed several clades in the family and was supported by low bootstraps support only (Fig.1 and 2). Hesperiinae was monophyletic with high bootstrap support (71) in the combined data set (Fig. 1) and moderate bootstrap support (55) in the *EF-1α* data set (Fig. 2).

3.2 Genus level and species level relationship

All the four species of subfamily Coeliadinae (two in case of combined data) resulted in a monophyletic clade with good bootstrap support (see Fig. 1 and 2). In subfamily Pyrginae, two species of genus *Celaenorrhinus* form the monophyletic clade with high bootstrap values (>98) in cladograms of all analysis (Fig 1 and 2, also see supplementary information). Genus *Celaenorrhinus* is known to form a sister relationship with *Sarangesa* with a sufficiently high

bootstrap value (87). Five genera of subfamily Hesperiinae (*Parnara, Caltoris, Baoris, Borbo* and *Pelopidas)* formed a well-supported, monophyletic clade (Fig. 1 and 2). Relationships between other species of subfamily Hesperiinae were weakly supported by bootstrap value (Fig. 1 and 2).

4. Discussion

4.1 Subfamily level relationship

The *EF-1α* gene appeared to provide most of the phylogenetic information characters, as the relatively slow rate of evolution at *EF-1α* allowed the use of its sequences to resolve the phylogenetic relationship at the subfamily and tribe level. So we referred the results of both combined data and individual *EF-1α* data for our study. Our results show~~ed~~ that the family Hesperiidae was monophyletic and in agreement with the results of Warren et al. (2008, 2009) and Wahlberg et al. (2005). Monophyly of Coeliadinae was observed with its basal position sister to rest of family (bootstrap values of 94 (Fig.1) and 78 (Fig. 2) respectively), supporting the results of other authors (de Jong et al., 1996; Wahlberg et al., 2005; Warren et al., 2008, 2009)

Pyrginae was found paraphyletic; this result agrees with the study of many other authors (de Jong et al., 1996; Wahlberg et al., 2005) who also failed to recover Pyrginae as a monophyletic group. In their molecular study and molecular + morphological study, Warren et al. (2008, 2009) found that Pyrginae formed several clades within the group and failed to recover it as monophyletic group. In our results, bootstrap support for Pyrginae was low in both, the combined data set and the individual *EF-1α* data set (Fig.1 and 2). With such a low bootstrap values, the relationship of group was subjected to change with the addition of more information or characters. Further studies are needed on Pyrginae to get the satisfactory relationship within the group. A Phylogenetic study should be performed by adding more species of Pyrginae and more characters (morphology) to confirm its status.

Hesperiinae was found to be monophyletic with a bootstrap support of 71 in combined data (Fig. 1). We added few more genera to the subfamily Hesperiinae which were not included in the Warren et al. (2008) molecular study, which further supported the Monophyly of Hesperiinae.

4.2 Tribe-level relationship

Baorini, tribe of Hesperiinae: Monophyly of Evans' (1937, 1949) Gegenes group was strongly supported by our result, with high bootstrap value (Fig. 1 and 2). Warren et al. (2008) also recognize this group to be monophyletic, but the molecular data of genera *Parnara, Caltoris, Baoris* and *Borbo* of tribe *Baorini* was not included in his analysis. *Parnara, Caltoris* and *Borbo* are placed in tribe *Baorini* based on Evans' (1949) Gegenes group. In the present study, we added all the above mentioned genera for molecular analysis and our results confirmed the monophyly of *Baorini* with good bootstrap support (Fig. 1: 62, 47 and 97 for *Parnara, Caltoris* and *Borbo* respectively). This result was further supported by *EF-1α* analysis where *Pelopidas* (which is included in Warren et al., 2008) forms a monophyletic group with *Baoris, Caltoris, Parnara* and *Borbo* with high bootstrap values of 78, 83, 59 and 95 respectively(Fig. 2).

4.3 Genus-level relationship

The Pyrginae genus, *Celaenorrhinus* is not considered monophyletic by Warren et al. (2008, 2009). de Jong (1982) discussed that *Celaenorrhinus* shows morphological diversity in distribution of secondary sexual characters and considers this genus as paraphyletic based on morphological characters. In our study, we included two species of genus *Celaenorrhinus* (*C.ruficornis* and *C. leucocera)* and found that these two species form a monophyletic clade with very strong bootstrap support (all >98) in all cladograms (Fig. 1, Fig. 2 and also see

supplementary information). Our result based on two species of *Celaenorrhinus* supports the monophyly of this genus but more species of this genus should be included for further supporting our hypothesis.

There were few more genera in our study whose phylogenetic relationships are not yet confirmed by molecular study. These genera were *Udaspes, Arnetta, Matapa* and *Baracus.* In our analysis, we found that the branch supports for these genera were very poor (Fig. 2) and it was difficult to confirm their relationships based on this study, because with the addition of more characters the relationship among these genera may change. Further work should be done on these genera with the addition of more characters, both molecular and morphological, to confirm their taxonomic status.

4.4 Conclusions

In this study, we attempted to resolve various taxonomic conflicts within family Hesperiidae. Our results were based on two genes, *COI* and *EF-1α*. Based on our results (Fig. 1 and 2) the subfamily Hesperiinae and Coeliadinae were monophyletic while the subfamily Pyrginae failed to form monophyletic clad. *Baorini,* a tribe of Hesperiinae, was confirmed as monophyletic groups and similarly *Celaenorrhinus,* genus of Pyrginae was also confirmed as monophyletic groups. Further studies need to be performed by including more taxa, characters (morphology) and more information of entire *COI- tRNA leucine-CO-II* gene (total 2,200bp), *EF-1α* gene (1,200bp) and *wingless* gene (450bp). This will further reveal the taxonomic uncertainties of family Hesperiidae of Western Ghats.

Acknowledgments

We thank S. Kalesh for providing some valuable specimens, E. Kunhikrishnan for logistical support, Kerala Forest Department for issuing research and collecting permits (No.WL12-4354/2010). We are grateful to T.M. Manoharan (IFS), P. Pugazhendi (IFS), V. Radhakrishnan Nair, Sunil Babu. A. P, K.I. Pradeep kumar, P.I. Jose Panicker, Y. Muhammad Anwar, Afsal Ahammad and S.G. Mahesh Kumar for permits and logistical support during Prabhdeep Singh's field work in various protected areas across Kerala. We thank to Neelesh Dahanukar, Swapnil Gaikward and Farhat Habib for valuable technical advice and discussions, to all my lab mates, especially Manawa Diwekar for technical help. This research was funded by the Indian Institute of Science Education and Research, Pune.

References

Ackery P.R, de Jong, R., Vane-Wright, R.I., 1999. The butterflies: Hedyloidea, Hesperoidea, and Papilionoidea. in: Kristensen N.P. (Editor), *The butterflies: Hedyloidea, Hesperoidea, and Papilionoidea.* de Gruyter., Berlin, pp. 263–300.

Akite, P., 2008. Effects of anthropogenic disturbances on the diversity and composition of the butterfly fauna of sites in the Sango Bay and Iriiri areas, Uganda: implications for conservation. African Journal of Ecology 46, 3–13.

Bascombe, M.J., Johnston, G., Bascombe, F.S., 1999. The butterflies of Hong Kong. Academic Press, London.

Blair, R.B., 1999. Birds and Butterflies along an Urban Gradient: Surrogate Taxa for Assessing Biodiversity? Ecological Applications 9(1), 164-170.

Bobo, K.S., Waltert, M., Fermon, H., Njokagbor, J., Mühlenberg, M., 2006. From forest to farmland: butterfly diversity and habitat associations along a gradient of forest conversion in Southwestern Cameroon. Journal of Insect Conservation 10, 29–42.

Bridges, C.A., 1993. Catalogue of the Family-Group, Genus-Group and Species-Group Names of the Hesperiidae (Lepidoptera) of the World. Published by Author, Urbana, IL.

Ellers, J., Boggs, C.L., 2003. The evolution of wing color: male mate choice opposes adaptive wing color divergence in *Colias* butterflies. Evolution 57(5), 1100–1106.

Evans, W.H., 1937. A Catalogue of the African Hesperiidae. British Museum (Natural History), London.

Evans, W.H., 1949. A Catalogue of the Hesperiidae From Europe, Asia, and Australia in the British Museum (Natural History). British Museum, London.

Freeman, H.A., 1969. Systematic review of the Megathymidae. J. Lepidopterists' Soc. 23 (Suppl. 1), 1–58.

Gaonkar, H., 1996. Butterflies of the Western Ghats, India (including Sri Lanka: A Biodiversity Assessment of a Threatened Mountain System). Technical report submitted to the Centre for Ecological Sciences, Indian Institute of Science, Bangalor.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95-98.

Jha, C.S., Dutt, C.B.S., Bawa, K.S., 2000. Deforestation and land use changes in Western Ghats, India. Current Science 79, 231-238.

de Jong, R., 1982. Secondary sexual characters in Celaenorrhinus and the delimitation of the genus (Lepidoptera, Hesperiidae). J. Nat. Hist. 16, 695–705.

de Jong, R., Vane-Wright, R.I., Ackery, P.R., 1996. The higher classification of butterflies (Lepidoptera), problems and prospects. Entomol. Scand. 27, 65–101.

Kocher, S.D., Williams, E.H., 2000. The diversity and abundance of North American butterflies vary with habitat disturbance and geography. Journal of Biogeography 27, 785-794.

Kunte, K., Joglekar, A. P., Utkarsh, G., Pramod, P., 1999. Patterns of butterfly, bird and tree diversity in the Western Ghats. Current Science 77, 577-586.

Kunte, K., 2000. Butterflies of Peninsular India. Universities Press, Hyderabad and Indian Academy of Sciences, Bangalore.

Kunte, K., 2005. Species Composition, sex ratios and movement patterns in danaine butterfly migration in southern India. Journal of the Bombay Natural History Society 102 (3), 280-286.

Kunte, K., 2008. The Wildlife (Protection) Act and conservation prioritization of butterflies of the Western Ghats, southwestern India. Current Science 94, 729 – 735.

Kunte, K., (In press). Biogeographic origins and habitat use of the butterflies of the Western Ghats, south-western India. *In* D. R. Priyadarshan, K. A. Subramanian, M. S. Devy & N. A. Aravind (eds.) Invertebrates in the Western Ghats - Diversity and Conservation. Ashoka Trust for Research in Ecology and the Environment, Bengaluru.

Mennechez, G., Schtickzelle, N., Baguette, M., 2003. Metapopulation dynamics of the bog fritillary butterfly: comparison of demographic parameters and dispersal between a continuous and a highly fragmented landscape. Landscape Ecology 18, 279-291.

Mielke, O.H.H., 2005. Catalogue of the American Hesperioidea: Hesperiidae (Lepidoptera). Heteropterinae & Megathyminae. Vol. 6, pp. 1387-1536

Mittermeier, R.A., Gil, P.R., Hoffman, M., Pilgrim, J., Brooks, T., Mittermeier, C.G., Lamoreux, J., da Fonseca, G.A.B., 2005. Hotspots Revisited: Earth's Biologically Richest and most Endangered Terrestrial Ecoregions. Cemex, Conservation International and Agrupacion Sierra Madre, Monterrey, Mexico.

Myers, N., Mittermeier, R. A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853-858.

Pollard, E., 1991. Synchrony of population fluctuations: the dominant influence of widespread factors on local butterfly populations. Oikos 60(1), 7-10.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution (in press).

Thomas, J.A., 2005. Monitoring change in the abundance and distribution of insects using butterflies and other indicator groups. Philosophical Transactions of the R. Soc. B, Biological Sciences 360, 339–357.

Wahlberg, N., Braby, M.F., Brower, A.V.Z., de Jong, R., Lee, M.M., Nylin, S., Pierce, N.E., Sperling, F.A.H., Vila, R., Warren, A.D., Zakharov, E., 2005. Synergistic effects of combining morphological and molecular data in resolving the phylogeny of butterflies and skippers. Proc. R. Soc. B. 272, 1577–1586.

Warren, A.D., Ogawa, J.R., Brower, A.V.Z., 2008. Phylogenetic relationships of subfamilies and circumscription of tribes in the family Hesperiidae (Lepidoptera: Hesperioidea). Cladistics 24, 642–676.

Warren, A.D., Ogawa, J.R., Andrew V.Z., Brower, A.V.Z., 2009. Revised classification of the family Hesperiidae (Lepidoptera: Hesperioidea) based on combined molecular and morphological data. Syst. Ent. 34, 467–523.

Table 1

Oligonucleotide primers used in study

Gene	Name	Primer sequence	References
COI	Rudy	5′-GAAGTTTATATTTTAATTTTACCGGG-3′	Warren et al., 2008
	Phyllis	5′-GTAATAGCIGGTAAA⁄GATAGTTCA-3′	
COI	Gary	5′-TAGGAATAATTTATGCMATAATAGC-3′	Warren et al., 2008
	Susan	5′-TTGTTGTTCTAATARAAATCG-3′	
EF-1α	Al	5′-GAGGAAATYAARAAGGAAG-3′	Warren et al., 2008
	Tipper	5′-ACAGCVACKGTYTGYCTCATRTC-3′	

Figure legends

Fig. 1. Maximum likelihood tree based on combined sequences data of two genes (*COI* + *EF-1α*). This tree was generated by using software MEGA 5.01. Bootstrap value based on 500 bootstrap replications are shown above the branches. Colour codes represent subfamily as following: green- Hesperiinae; red- Pyrginae; blue- Coeliadinae and outgroup is represented in black colour.

Fig. 2. Maximum likelihood tree individual gene sequence *EF-1α.* This tree was generated by using software MEGA 5.01. Bootstrap value based on 500 bootstrap replications are shown above the branches. Colour codes represent subfamily as following: green- Hesperiinae; red- Pyrginae; blue- Coeliadinae and outgroup is represented in black colour.

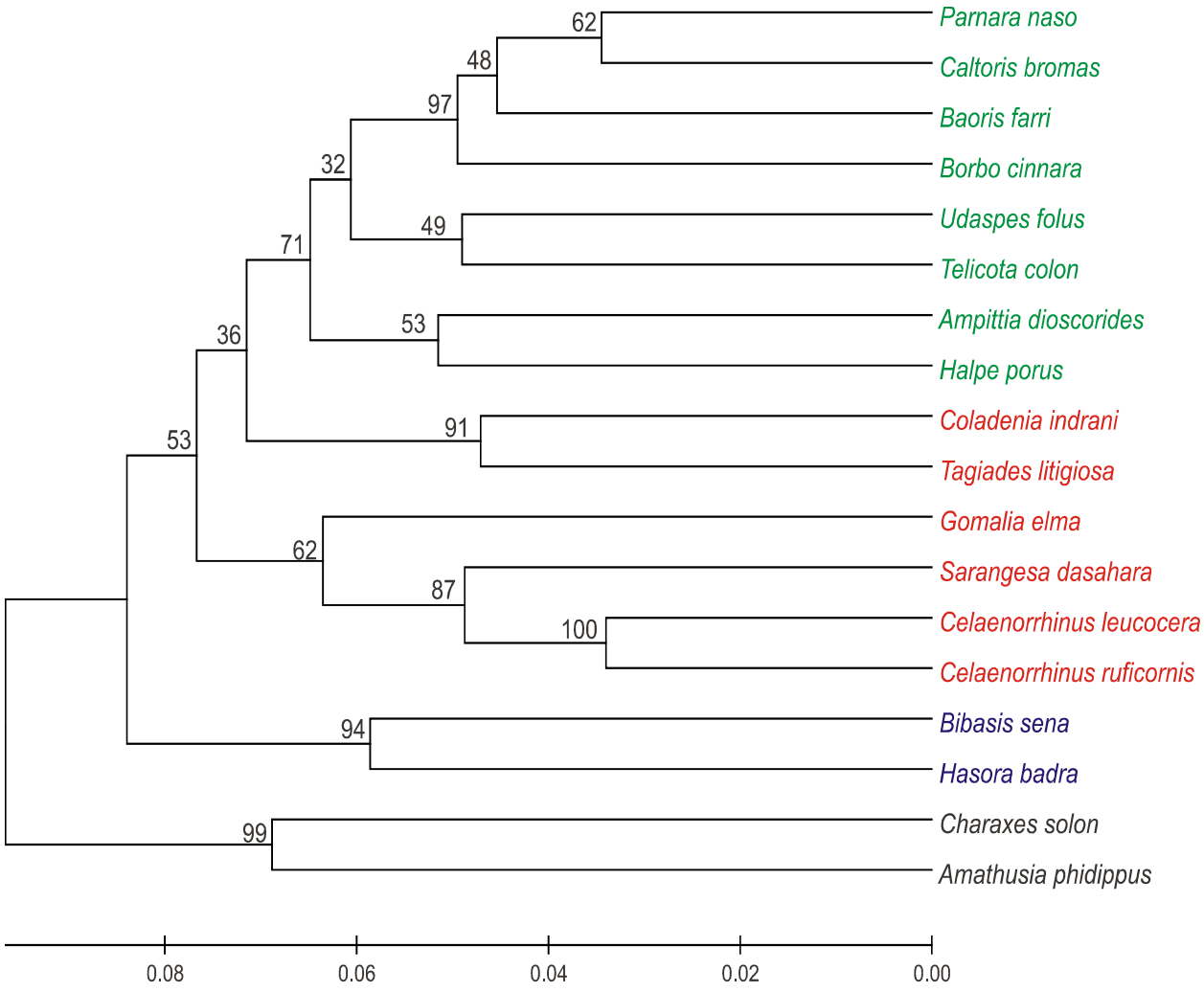

62
48
97
32
49
71
53
36
91
53
62
87
100
94
99
Parnara naso
Caltoris bromas
Baoris farri
Borbo cinnara
Udaspes folus
Telicota colon
Ampittia dioscorides
Halpe porus
Coladenia indrani
Tagiades litigiosa
Gomalia elma
Sarangesa dasahara
Celaenorrhinus leucocera
Celaenorrhinus ruficornis
Bibasis sena
Hasora badra
Charaxes solon
Amathusia phidippus
0.08
0.06
0.04
0.02
0.00

Fig. 1.

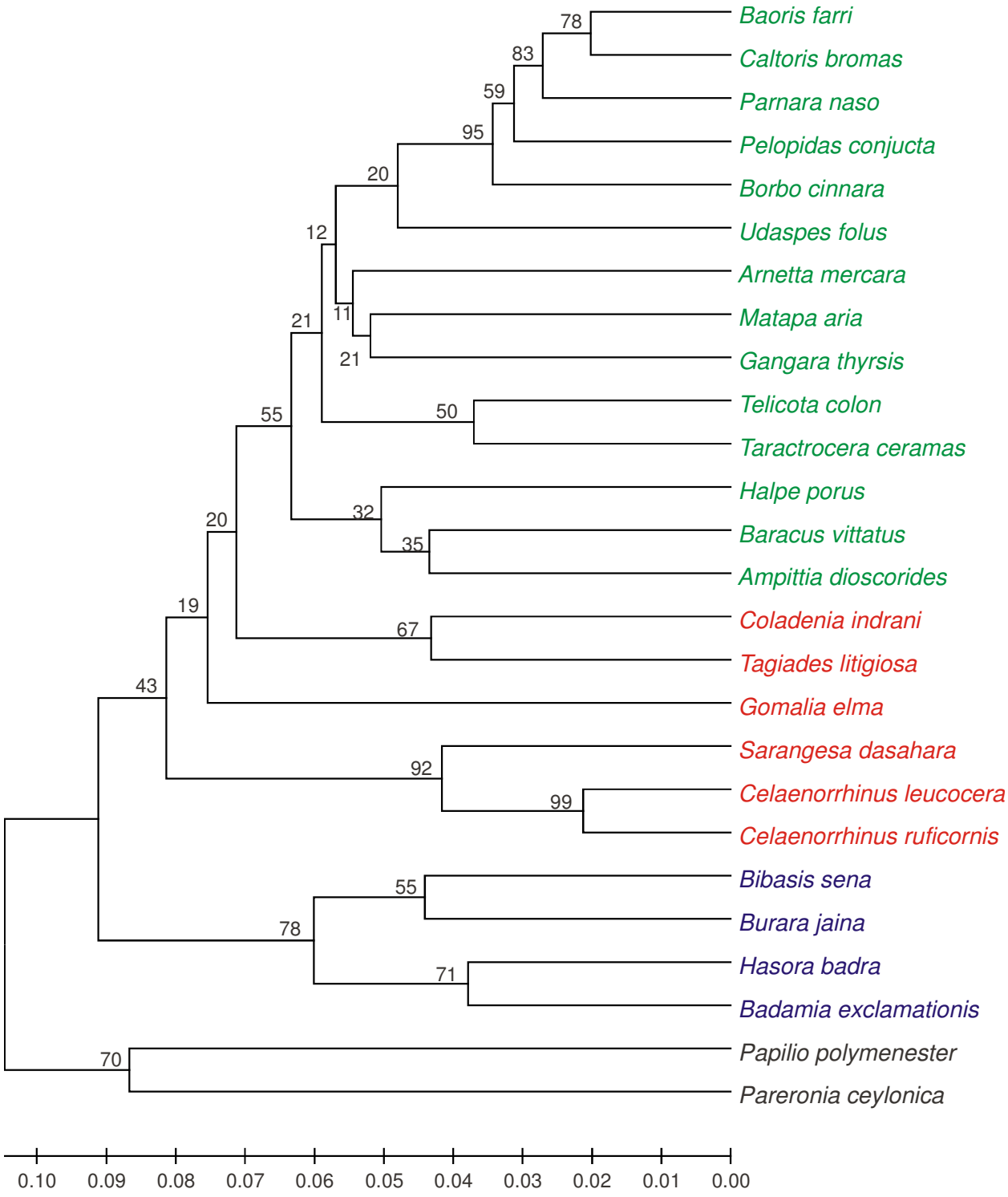

Baoris farri
Caltoris bromas
Parnara naso
Pelopidas conjucta
Borbo cinnara
Udaspes folus
Arnetta mercara
Matapa aria
Gangara thyrsis
Telicota colon
Taractrocera ceramas
Halpe porus
Baracus vittatus
Ampittia dioscorides
Coladenia indrani
Tagiades litigiosa
Gomalia elma
Sarangesa dasahara
Celaenorrhinus leucocera
Celaenorrhinus ruficornis
Bibasis sena
Burara jaina
Hasora badra
Badamia exclamationis
Papilio polymenester
Pareronia ceylonica
78
83
59
95
20
12
11
21
21
55
50
32
20
35
19
67
43
92
99
55
78
71
70
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00

Fig. 2.

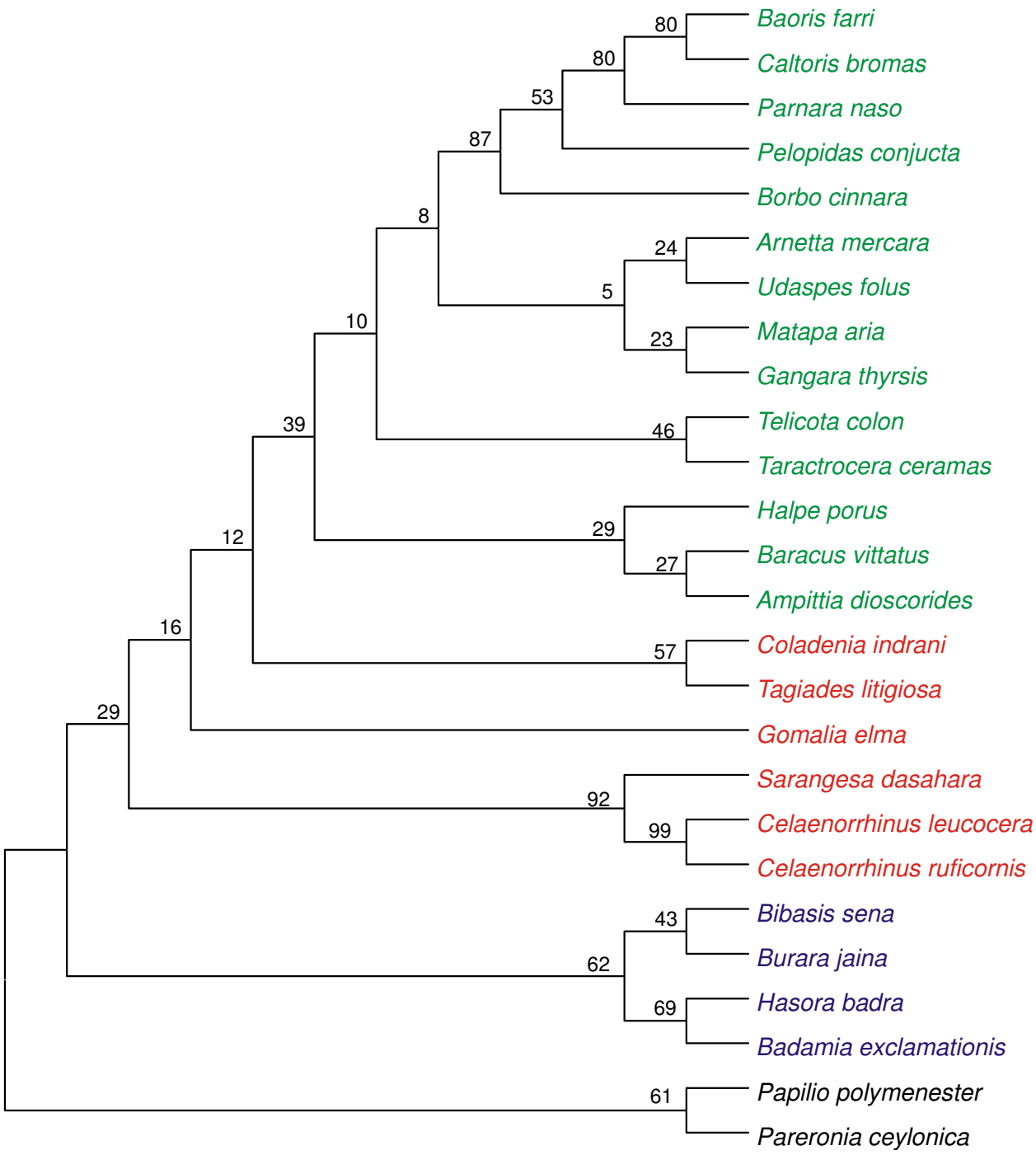
Supplementary Information

S.1. Maximum parsimony tree based on individual gene *EF-1α* (CI = 0.426451, RI = 0.447334). This tree was generated by using software MEGA 5.01. Bootstrap values based on 1000 bootstrap replications are shown above the branches. Colour codes represent subfamily as following: green- Hesperiinae; red- Pyrginae; blue- Coeliadinae and outgroup is represented in black colour.

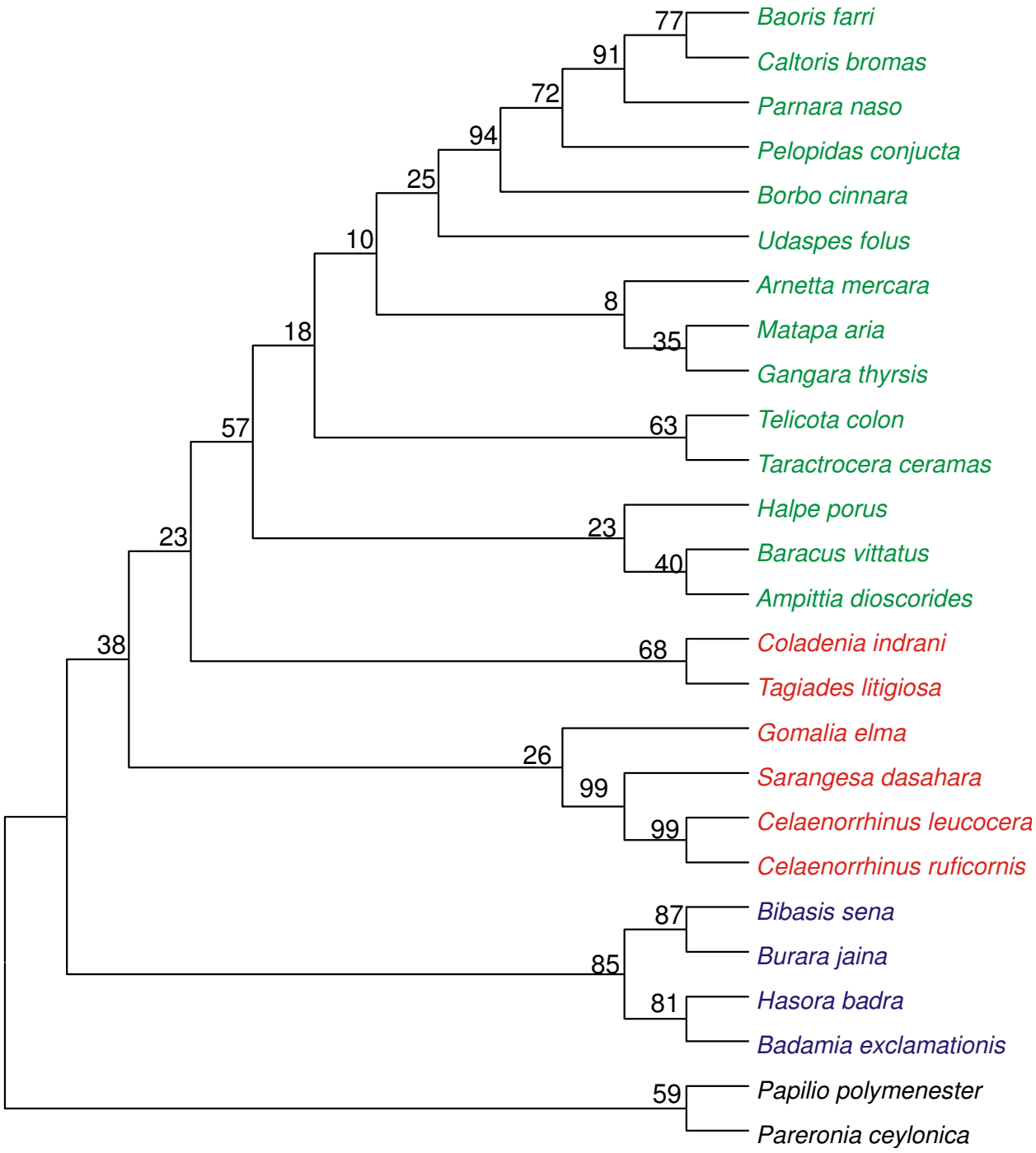
S.2. Neighbor joining tree based on individual gene sequence of *EF-1α*. This tree was generated by using software MEGA 5.01. Bootstrap values based on 1000 bootstrap replications are shown above the branches. Colour codes represent subfamily as following: green- Hesperiinae; red- Pyrginae; blue- Coeliadinae and outgroup is represented in black colour.

S.3. Maximum parsimony tree based on combine data (*COI* + *EF-1α*) (CI = 0.463256, RI = 0.356093). This tree was generated by using software MEGA 5.01. Bootstrap values based on 1000 bootstrap replications are shown above the branches. Colour codes represent subfamily as following: green- Hesperiinae; red- Pyrginae; blue- Coeliadinae and outgroup is represented in black colour.

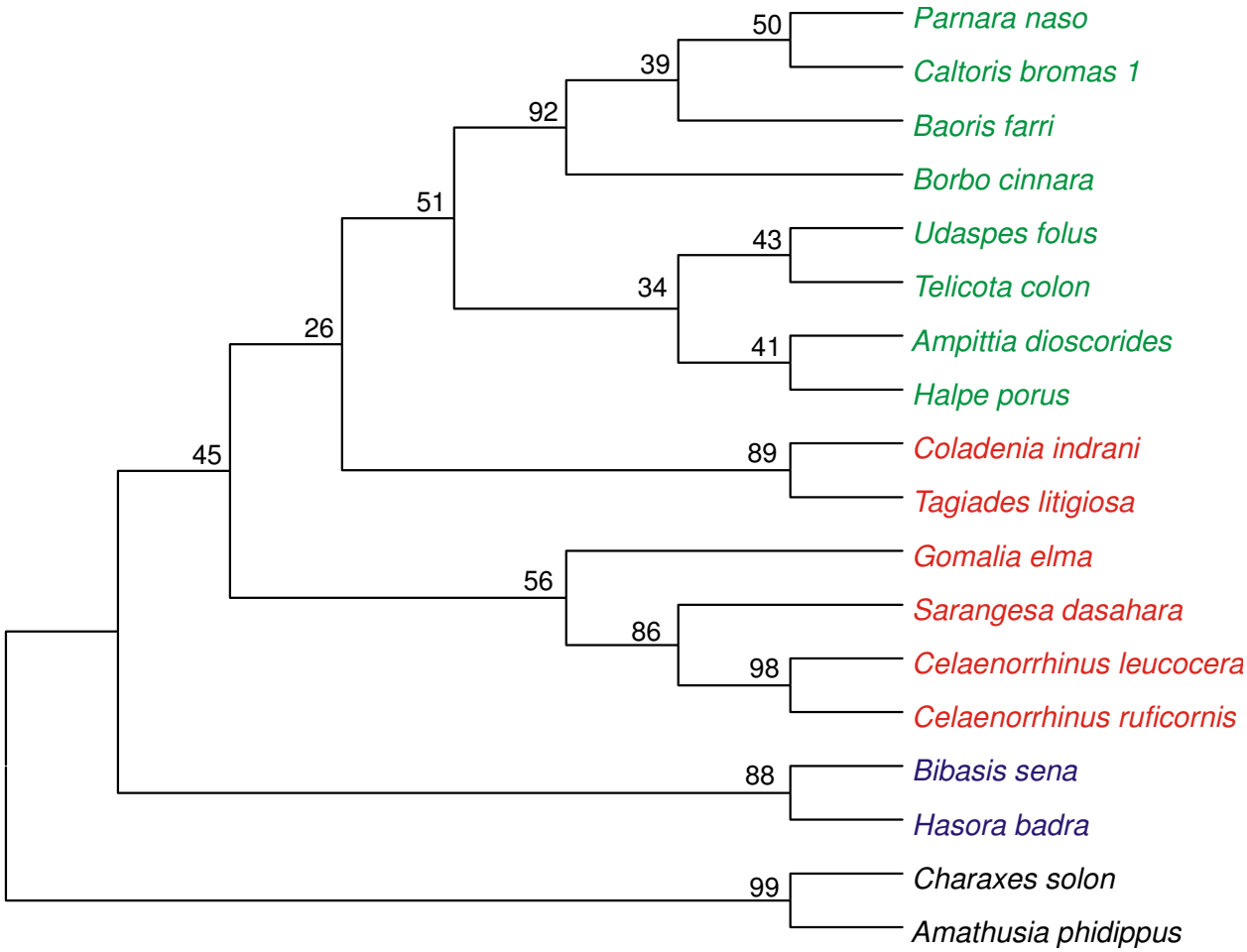
S.4. Neighbor-Joining tree based on combine data (*COI* + *EF-1α*). This tree was generated by using software MEGA 5.01. Bootstrap values based on 1000 bootstrap replications are shown above the branches. Colour codes represent subfamily as following: green- Hesperiinae; red- Pyrginae; blue- Coeliadinae and outgroup is represented in black colour.


Baoris farri
Caltoris bromas
Parnara naso
Pelopidas conjucta
Borbo cinnara
Arnetta mercara
Udaspes folus
Matapa aria
Gangara thyrsis
Telicota colon
Taractrocera ceramas
Halpe porus
Baracus vittatus
Ampittia dioscorides
Coladenia indrani
Tagiades litigiosa
Gomalia elma
Sarangesa dasahara
Celaenorrhinus leucocera
Celaenorrhinus ruficornis
Bibasis sena
Burara jaina
Hasora badra
Badamia exclamationis
Papilio polymenester
Pareronia ceylonica
80
80
53
87
8
24
5
10
23
39
46
29
12
27
16
57
29
92
99
43
62
69
61

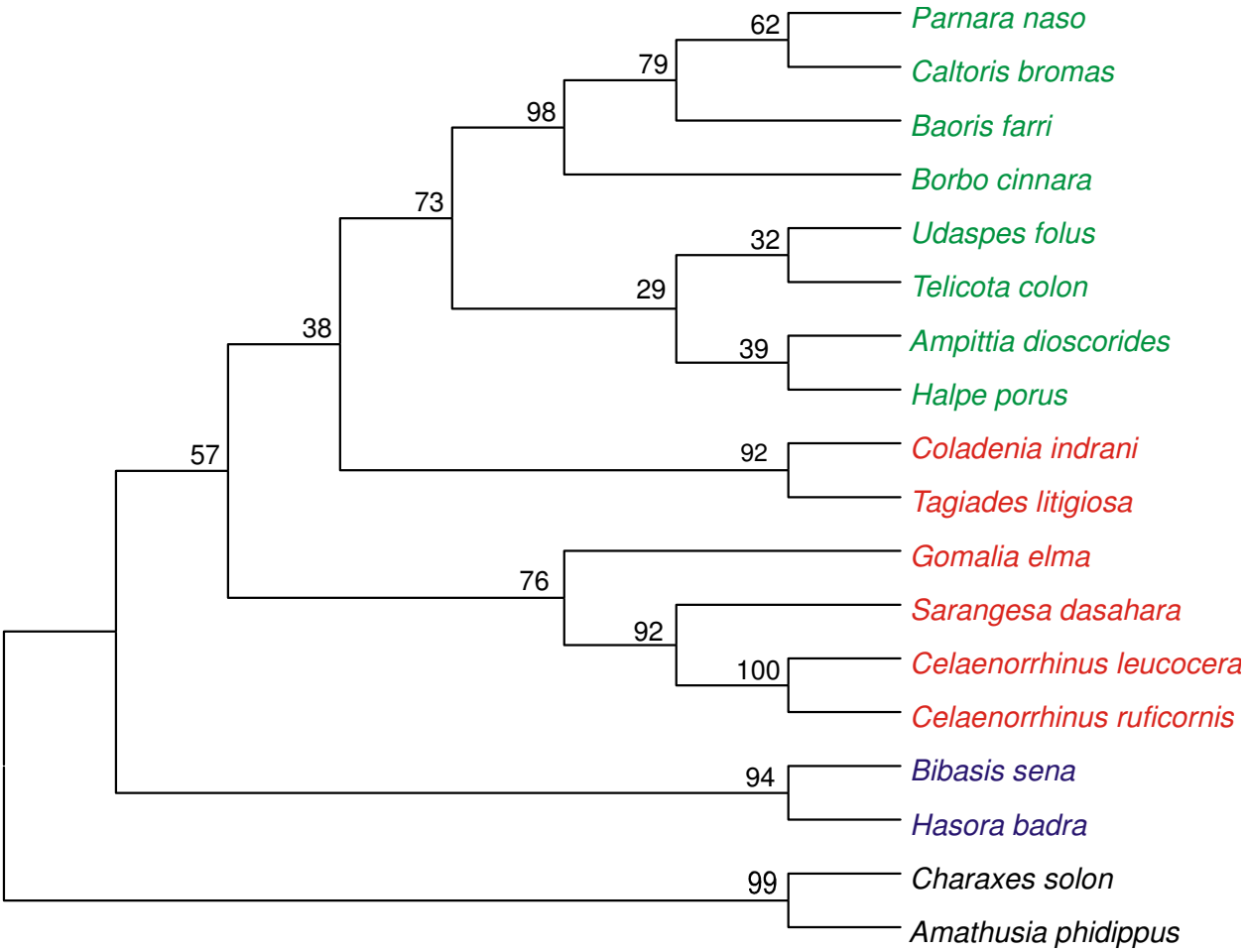
S. 1.


77
91
72
94
25
10
8
35
18
63
57
23
23
40
38
68
26
99
99
87
85
81
59
Baoris farri
Caltoris bromas
Parnara naso
Pelopidas conjucta
Borbo cinnara
Udaspes folus
Arnetta mercara
Matapa aria
Gangara thyrsis
Telicota colon
Taractrocera ceramas
Halpe porus
Baracus vittatus
Ampittia dioscorides
Coladenia indrani
Tagiades litigiosa
Gomalia elma
Sarangesa dasahara
Celaenorrhinus leucocera
Celaenorrhinus ruficornis
Bibasis sena
Burara jaina
Hasora badra
Badamia exclamationis
Papilio polymenester
Pareronia ceylonica

S.2.


50
39
92
51
43
34
26
41
45
89
56
86
98
88
99
Parnara naso
Caltoris bromas 1
Baoris farri
Borbo cinnara
Udaspes folus
Telicota colon
Ampittia dioscorides
Halpe porus
Coladenia indrani
Tagiades litigiosa
Gomalia elma
Sarangesa dasahara
Celaenorrhinus leucocera
Celaenorrhinus ruficornis
Bibasis sena
Hasora badra
Charaxes solon
Amathusia phidippus

S.3.


62
79
98
73
32
29
38
39
57
92
76
92
100
94
99
Parnara naso
Caltoris bromas
Baoris farri
Borbo cinnara
Udaspes folus
Telicota colon
Ampittia dioscorides
Halpe porus
Coladenia indrani
Tagiades litigiosa
Gomalia elma
Sarangesa dasahara
Celaenorrhinus leucocera
Celaenorrhinus ruficornis
Bibasis sena
Hasora badra
Charaxes solon
Amathusia phidippus

S.4.