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Mitogenomics of 'Old World *Acraea*' butterflies reveals a highly divergent '*Bematistes*' ☆

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ABSTRACT

Afrotropical Acraeini butterflies provide a fascinating potential model system to contrast with the Neotropical Heliconiini, yet their phylogeny remains largely unexplored by molecular methods and their generic level nomenclature is still contentious. To test the potential of mitogenomes in a simultaneous analysis of the radiation, we sequenced the full mitochondrial genomes of 19 African species. Analyses show the potential of mitogenomic phylogeny reconstruction in this group. Inferred relationships are largely congruent with a previous multilocus study. We confirm a monophyletic *Telchinia* to include the Asiatic *Pareba* with a complicated paraphylum, traditional (sub)genus *Acraea,* toward the base. The results suggest that several proposed subgenera and some species groups within *Telchinia* are not monophyletic, while two other (sub)genera could possibly be combined. *Telchinia* was recovered without strong support as sister to the potentially interesting system of distasteful model butterflies known as *Bematistes*, a name that is suppressed in some treatments. Surprisingly, we find that this taxon has remarkably divergent mitogenomes and unexpected synapomorphic tRNA rearrangements. These gene order changes, combined with evidence for deviating dN/dS ratios and evidence for episodal diversifying selection, suggest that the ancestral *Bematistes* mitogenome has had a turbulent past. Our study adds genetic support for treating this clade as a distinct genus, while the alternative option, adopted by some authors, of *Acraea* being equivalent to Acraeini merely promotes redundancy. We pave the way for more detailed mitogenomic and multi-locus molecular analyses which can determine how many genera are needed (possibly at least six) to divide Acraeini into monophyletic groups that also facilitate communication about their biology.

1. Introduction

The Heliconiinae (family Nymphalidae) contains some of the renowned aposematically colored Müllerian models that are textbook examples in evolutionary biology (Bates, 1863). The primarily Neotropical tribe Heliconiini that contains the highly diversified genus *Heliconius* (42 spp.) has by far received the most scientific attention (e.g. Merrill et al., 2015). A second species-rich tribe is the Acraeini, that is richest in the Afrotropics and also contains many primary models for mimicry, such as those found in the (sub)genus *Bematistes*. With its prominent rayed hindwing patterns, *Bematistes* species are distasteful models for mimics in other butterfly taxa such as *Hypolimnas* (Nymphalinae), *Pseudacraea eurytus* (Limenitidinae), *Elymniopsis bammakoo* (Satyrinae), *Papilio* (Papilionidae), *Mimacraea darwinia* and other Lycaenidae (Liptenidae). They thus provide a particularly interesting potential African model system that includes not only Batesian but, notably among *Bematistes* species and with *Telchinia jodutta*, Müllerian, dual sex and dual surface mimicry (Carpenter, 1948; Owen, 1971; Owen and Chanter, 1972; Pringle et al., 1994; Punnett, 1915; Vane-Wright et al., 1977). Yet, compared to the Heliconiini, which in all consists of around 10 clearly defined genera with 77 species, the systematics and genus-level nomenclature of Acraeini remains largely unresolved, hampering the usage of this tribe for comparative research.

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In the Afrotropics, several generic names still contend for usage at differing ranks (Fig. 1), but commonly, just two genera are used, with sometimes *Bematistes* even sunk. Van Son and Vári (1963, pg. 2) stated "A remarkable peculiarity of the Acraeinae is the exceedingly small number of genera, two of which contain most of the known species of the subfamily. The neotropical region has about 50 species, all of which are placed in a single genus, *Actinote* Hubner. The Ethiopian region is richest in species, over 170 being known at present; of these, over 150 are placed in the genus *Acraea* F., 22 in *Bematistes* Hemming". In this work, Van Son and Vári (1963) noted that overall structural similarity in Acraeini contrasts with great genitalic diversity, particularly among *Acraea*. This genitalic variation likely accounts for the large number of species groups with at least 13 major groups and many subgroups proposed among the ~228 Afrotropical species (Bernaud, 2014). A very simple formal generic taxonomy was also used by Ackery et al. (1995) who followed the earlier cladistic studies of Pierre (1987); see also Pierre (1992). These authors employed a single genus, *Acraea* with *Actinote* as subgenus (including Neotropical species), while *Bematistes* were totally subsumed within subgenus *Acraea*. In contrast, Henning and Williams (2010) advocated much greater formal subdivision of the tribe Acraeini, for the Afrotropics with four subgenera within *Acraea* Fabricius, 1807 (including *Bematistes*), and with three subgenera within *Telchinia* Hübner, [1819], detailed under Taxon sampling and in Fig. 1. Henning and Williams (2010) stressed their classification was provisional, acknowledging the possible problem of a paraphylum within *Acraea*.

DNA sequence data has been helpful to further resolve relationships within the clade pantropically. The most comprehensive molecular analysis of the tribe is still that of Silva-Brandão et al. (2008), which focused on Neotropical acraeine taxa, but included 30 African and three Asian taxa. This study used the nuclear genes EF-1α (elongation factor 1 alpha; 240 bp) and *wg* (wingless; 403 bp) as well as *cox*1 (mitochondrial cytochrome c oxidase 1; 1508 bp) to infer phylogenetic relationships among 68 species of Acraeini, and suggested that *Actinote* might be expanded to include all Neotropical species, while the Afrotropical *Actinote* might be replaced by *Telchinia*. The revival of the latter genus (type species, *Papilio serena* Fabricius, 1775) emerges from their finding that the neotropical (largely Compositae-feeding) *Actinote* (within which *Altinote* and *Abananote* should be subsumed) is sister to this Afrotropical (largely Urticaceae-feeding) group. However, the position of *Bematistes* was not robustly resolved in this study, and was either sister to other Acraeini or to a small clade including '*Acraea*' *egina*, in turn together sister to the rest. These authors concluded that additional sampling was needed, especially of Afrotropical members, to clarify the systematics of earlier diverging elements, which as their study highlighted, appeared to form a grade.

As a prelude to more detailed evolutionary studies of acraeine butterflies, we were motivated to obtain an initial set of Old World acraeine mitogenomes and test their effectiveness in resolving phylogenetic relationships. This approach would seem promising since mitogenomes are useful for reconstructing families within the Lepidoptera as a whole (Timmermans et al., 2014) as well as at lower taxonomic levels. High levels of nodal support have been found using mitogenomes for phylogenetic reconstructions of the butterfly family Nymphalidae and its subfamily Limenitidinae, a sister group to Heliconiinae (Wu et al., 2014). Currently, there is only one acraeine mitogenome available (the oriental '*Acraea*' *issoria* (Hu et al., 2010)), although there has been no attempt to place this taxon within an overall framework, and sampling is so far inadequate even for a representation of subgeneric-level taxa within the entire tribe.

Recent developments in high-throughput sequencing have made full mitogenome sequencing extremely straightforward,

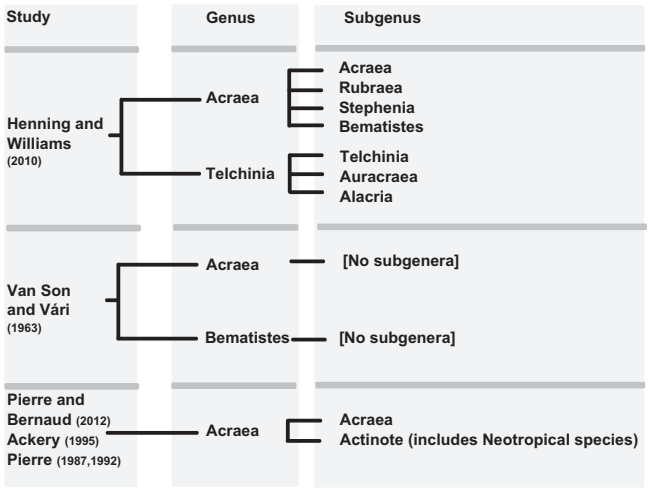

Fig. 1. (Sub)generic names proposed in recent treatments for Afrotropical Acraeini butterflies.

especially now that protocols have been developed that allow cost-efficient assembly of full mitochondrial genomes from pools of mixed genomic DNA (Gillett et al., 2014; Andujar et al., 2015; Crampton-Platt et al., 2015). Although this "mitochondrial metagenomics" approach (Tang et al., 2014) requires sufficient divergence of the included mitogenomes to allow for correct assembly (Dettai et al., 2012), the method seems to work even on mixtures of congeneric DNA (Gómez-Rodríguez et al., 2015). Yet, as chimeric assembly remains an obvious risk (e.g. see Timmermans et al., 2015), stringent checks need to be implemented to ensure the obtained mitogenomes are correct.

In this study, using the mitochondrial metagenomics approach, we obtained a set of 19 new Old World acraeine mitogenomes from pooled genomic DNA, which we use to resolve species relationships. Because its recognition as a genus has been the most historically contentious among authors, we were specifically interested in the position of *Bematistes*.

2. Methods

2.1. Taxon sampling, DNA extraction and sequencing

Butterflies of the tribe Acraeini were sampled from various locations in Africa (Table 1). A total of 23 species were available for genetic analysis. DNA was extracted from small pieces of butterfly tissue using the DNA Blood and Tissue kit (Qiagen). DNA concentrations were measured on a Qubit 2.0 fluorometer using the High-Resolution kit (Invitrogen). The samples were mixed by adding equal amounts of each to a single pool. This mixture was sent to the NHM London sequence facility for library preparation (Illumina TruSeq Nano) and sequencing (2 × 250PE) on an Illumina MiSeq. In addition, two mitochondrial regions were targeted using PCR. The 5′ region of *cox*1 was amplified using primers LepCox1 and JerryR and the 5′ region of *cob* was amplified using primers SytbF and SytbR. Primer sequences and PCR conditions are given in Timmermans et al. (2014). Amplicons were Sanger sequenced in both directions using an Applied Biosystems 3730xl sequencer.

Employing here (and in Fig. 1) the nomenclature of Henning and Williams (2010), our samples include four members of *Acraea* (*Bematistes*), one member of *Acraea* (*A.* (*A.*) *zetes* although not the type species, *Papilio horta* Linnaeus, 1764), plus members of *Stephenia* (*A.*(*S.*) *rogersi*) and the type species of *Rubraea* (*Papilio egina* Cramer, 1775; *A.*(*R.*) *egina*). The sampling of *Telchinia* is denser, including *Telchinia* (*Telchinia*) (*T. acerata*, *T. alciope*, *T. bonasia*,

Table 1
Species of the tribe Acraeini sampled. Specimens were sampled from various locations in Africa. Nomenclature uses Henning and Williams (2010) for genera/subgenera and Bernaud (2014) for species groups. Mean coverage (stdev): mean sequencing depth as estimated using Geneious.

Specimen ID	Genus	Subgenus	Species	Collection locality	GenBank acc. no.	Mean coverage (stdev)
ACR-010	*Acraea*	*Stephenia*	*A. (S.) rogersi*	East Nimba Nature Reserve, Nimba county, Liberia	KT371365	142 (28)
ACR-017	*Acraea*	*Acraea*	*A. (A.) zetes*	East Nimba Nature Reserve, Nimba county, Liberia	KT371361	323 (42)
ACR-030	*Acraea*	*Bematistes*	*A. (B.) alcinoe*	Mount Beeton Nimba Mountains, Western range, Liberia	KT371373	169 (40)
ACR-024	*Acraea*	*Bematistes*	*A. (B.) epaea*	East Nimba Nature Reserve, Nimba county, Liberia	KT371368	31 (12)
OSK-11	*Acraea*	*Bematistes*	*A. (B.) poggei*	Kibale, Uganda	KT371366	149 (31)
ACR-029	*Acraea*	*Bematistes*	*A. (B.) vestalis*	East Nimba Nature Reserve, Nimba county, Liberia	KT371375	110 (21)
ACR-016	*Acraea*	*Rubraea*	*A. (R.) egina*	East Nimba Nature Reserve, Nimba county, Liberia	KT371359	39 (8)
OSK-16	*Telchinia*	*Alacria*	*T. (Al.) penelope*	Kibale, Uganda	KT371367	403 (55)
ACR-012	*Telchinia*	*Alacria*	*T. (Al.) perenna*	East Nimba Nature Reserve, Nimba county, Liberia	KT371369	184 (33)
OSK-13	*Telchinia*	*Alacria*	*T. (Al.) parrhasia*	Kibale, Uganda	KT371374	498 (98)
OSK-10	*Telchinia*	*Telchinia*	*T. (T.) acerata*	Kibale, Uganda	KT371360	103 (19)
OSK-1	*Telchinia*	*Telchinia*	*T. (T.) alciope*	Kibale, Uganda	x	
OSK-8	*Telchinia*	*Telchinia*	*T. (T.) aurivillii*	Kibale, Uganda	x	
ACR-003	*Telchinia*	*Telchinia*	*T. (T.) bonasia*	East Nimba Nature Reserve, Nimba county, Liberia	KT371364	68 (14)
ACR-007	*Telchinia*	*Telchinia*	*T. (T.) circeis*	East Nimba Nature Reserve, Nimba county, Liberia	KT371362	254 (47)
ACR-027	*Telchinia*	*Telchinia*	*T. (T.) encedana*	Coldwater, ENNR, Liberia	x	
ACR-020	*Telchinia*	*Telchinia*	*T. (T.) encedon*	East Nimba Nature Reserve, Nimba county, Liberia	x	
ACR-004	*Telchinia*	*Telchinia*	*T. (T.) jodutta*	East Nimba Nature Reserve, Nimba county, Liberia	KT371358	185 (39)
ACR-005	*Telchinia*	*Telchinia*	*T. (T.) lycoa*	East Nimba Nature Reserve, Nimba county, Liberia	KT371370	216 (42)
ACR-011	*Telchinia*	*Telchinia*	*T. (T.) pharsalus*	East Nimba Nature Reserve, Nimba county, Liberia	KT371363	117 (19)
ACR-025	*Telchinia*	*Telchinia*	*T. (T.) polis*	East Nimba Nature Reserve, Nimba county, Liberia	KT371371	285 (52)
OSK-15	*Telchinia*	*Telchinia*	*T. (T.) kalinzu*	Kibale, Uganda	KT371376	341 (52)
ACR-001	*Telchinia*	*Telchinia*	*T. (T.) serena*	East Nimba Nature Reserve, Nimba county, Liberia	KT371372	151 (26)

T. circeis, *T. jodutta*, *T. kalinzu*, *T. pharsalus*, *T. polis*, *T. serena*) and *T.* (*Alacria*) (*T. parrhasia*, *T. penelope*, *T. perenna*). For a full testing of the Henning and Williams subgeneric-level treatment, we lacked only a representative of *T.* (*Auracraea*) (type species *Acraea anacreon* Trimen, 1868).

2.2. Sequence processing, mitogenome assembly and identification

Geneious 8.0.3 (Kearse et al., 2012) was used to manually edit the Sanger traces, to clip primer sequences and to generate a consensus sequence for each targeted fragment. *Cox*1 (COI-5P) DNA barcode sequences were used to confirm species identifications using BOLD (Ratnasingham and Hebert, 2007). For both gene fragments, *p*-distances (proportion of sites that are different between two sequences) were calculated for each pairwise species comparison using MEGA6 (Tamura et al., 2013).

The Illumina proprietary MiSeq Control Software (version 2.4.0.4) was used for initial read processing. The resulting Fastq files were parsed through TRIMMOMATIC (version 0.3) (Bolger et al., 2014) to clip remaining adapter sequences and bases with a quality value <20 from the sequence starts and ends.

Two different approaches were used to assemble individual mitogenomes from the mixed data. For the first approach, reads of putative mitochondrial origin were extracted by comparing the sequence data to a dataset of 107 publicly available lepidopteran mitogenomes using BLAST (blastn, e-value: 1e−5) (Altschul et al., 1990). Retrieved reads were subsequently assembled using IDBA-UD (Peng et al., 2012) using a "mink" value of 100 and a "maxk" value of 220. For the second approach, mitochondrial reads were extracted by mapping all reads onto a single mitogenome (Genbank Accession: NC_013604) using the Geneious 8.0.3 (Kearse et al., 2012) mapper using custom settings and iterating up to five times. Three different mapping stringencies were used: (1) Minimum overlap 100 bp, Maximum mismatches per read: 20%, maximum gap size: 15 bp, Minimum overlap identity: 80%, (2) Minimum overlap 100 bp, Maximum Mismatches per read: 35%, Maximum gap size: 15 bp, Minimum overlap identity: 65%, (3) Minimum overlap 100 bp, Maximum Mismatches per read: 30%, Maximum gap size: 50 bp, Minimum overlap identity: 65%. For each of the three mappings, the mapped reads were subsequently assembled in Geneious (setting: Medium Sensitivity/Fast). To assign species names to the assembled mitogenomes, contigs were aligned to the *cox*1 and *cob* Sanger sequences. Geneious supports circular assembly of circular molecules, whereas IDBA-UD does not. Linear contigs with overlapping ends were circularized manually in Geneious. For each species, one full mitogenome was selected for further analyses, prioritizing the IDBA-UD assembly over the Geneious ones. To obtain estimates of mean coverage, reads were mapped onto the circularized genomes using the Geneious read mapper (settings: minimum overlap of 100 bp; maximum sequence divergence of 1%).

2.3. Genome validation and annotation

The quality of the full mitogenomes was evaluated using REAPR (Hunt et al., 2013). Paired-reads were re-mapped onto the mitogenomes using SMALT using the default REAPR settings. Improperly mapped read-pairs were removed using SAMTOOLS (Li et al., 2009) (setting: view -b -f 2) and the resulting datasets (Bam file format) were analyzed using REAPR's 'pipeline' function.

The validated mitogenomes were subsequently annotated. Protein coding gene annotations were obtained by aligning the contigs to the mitochondrial genome of the type species of the Asiatic acraeine *Pareba*: '*Acraea*' ('*Pareba*') *issoria* (NC_013604), in Geneious 8.0.3. The tRNAs were annotated using COVE (Eddy and Durbin, 1994) as described in Timmermans and Vogler (2012) and Crampton-Platt et al. (2015). To obtain secondary structure predictions for *trnE* and *trnQ* the sequences were also analyzed using ARWEN (Laslett and Canback, 2008). These secondary structures were then drawn using the VARNA Java applet (Darty et al., 2009). Possible gene rearrangement scenarios were investigated using the heuristic algorithm implemented in the program CREx (Bernt et al., 2007).

We also checked for the presence of a second Isoleucine tRNA, which has been reported for '*A.*' ('*P.*') *issoria* (Hu et al., 2010). Because this putative tRNA is distinct from the standard *trnI* (and contains a 10 bp 'intron'), it is not recognized using the custom tRNA covariance models used here. To pinpoint the second Isoleucine tRNA all sequences were analyzed using the tRNAscan-SE (mitochondrial/chloroplast setting) webserver (Lowe and Eddy,

1997; Schattner et al., 2005). Annotated mitogenomes are deposited on GenBank (accession numbers: KT371358–KT371376).

2.4. Phylogenetic analyses and tests of molecular evolution

All annotated gene sequences were extracted using the FeatureExtract (Wernersson, 2005) webserver. The data was combined with data on the full mitogenome of '*A*' ('*P.*'). *issoria* (NC_013604) and five outgroups. A member of the Limenitidinae (*Athyma sulpitia*) was used to root the tree (Tian et al., 2012). Limenitidinae is the sister group of Heliconiinae (Wahlberg et al., 2009). Four Asian species from the Old World Heliconiinae, tribe Argynnini (*Issoria lathonia, Argynnis hyperbius*, *A. paphia* and *Fabriciana nerippe*) (Timmermans et al., 2015; Kim et al., 2011; Wang et al., 2011; Xu et al., unpublished) were also used as outgroups. See Fig. 4 for Accession numbers. Alignments were constructed for each of the 37 genes. The two tRNA and 22 rRNA genes were aligned using MAFFT (Katoh et al., 2002) using the "auto" and L-INS-i settings, respectively. The 13 protein coding genes were aligned using CLUSTALW (Thompson et al., 1994) and the TRANSALIGN script (Bininda-Emonds, 2005). To test whether saturation resulted in loss of phylogenetic signal in the dataset the test of Xia et al. (2003) was applied, as implemented in DAMBE (Xia and Lemey, 2009; Xia and Xie, 2001). Tests were conducted on the full dataset and on each of the three codon position of the concatenated protein coding genes. The full dataset was then partitioned in 42 initial partitions (one partition for each codon position in each protein coding gene, one for each of the rRNAs and one for the combined tRNAs) and analyzed using PartitionFinder (Lanfear et al., 2012) to obtain a suitable reduced partition scheme and fitting models. Tree searches were then performed in MrBayes (Huelsenbeck and Ronquist, 2001) (2 runs with 4 chains each; 10 million generations; a tree sampled every 1000 generations). Tracer (Rambaut et al., 2014) was used to check convergence. In addition, Maximum Likelihood based tree searches were performed in IQ-TREE (Nguyen et al., 2015). For these latter searches, the same partition scheme and models were used. Branch support was determined using 1000 ultrafast bootstraps.

To investigate changes in dN/dS ratio the SelectionLRT test (Frost et al., 2005) as implemented in HYPHY (Pond et al., 2005) was used with the GTR model (model character designation: 012345). We specifically tested whether the branch leading to the *Bematistes* clade and that clade itself showed evidence for divergent selective pressures. Finally, we tested for evidence of episodal diversifying selection using a Branch-site REL analysis (Pond et al., 2011) as available on the Datamonkey webserver (Pond and Frost, 2005). This analysis was performed on the ingroup taxa only and used a Neighbour-Joining tree.

3. Results

3.1. Mitogenome assembly

Genomic DNA of 23 species was mixed and a sequencing library was constructed. A total of 18,997,194 paired-end sequences were obtained. After quality control and BLAST-based enrichment of mitochondrial reads this set was reduced to 7,819,190 sequences with a mean length of 249.2 bp (sd: 7.6 bp). IDBA-UD and Geneious were used for sequence assembly. Neither IDBA-UD nor Geneious assembled all genomes in full. Yet, when combining the results of the different assemblies, 19 out of 23 mitogenomes were assembled seemingly correctly (Table 1). The remaining four genomes are those of two pairs of highly related *Telchinia* species from the *encedon* group, i.e. *T.* (*T.*) *encedana* and *T.* (*T.*) *encedon*, and *T.* (*T.*) *alciope* and *T.* (*T.*) *aurivillii*. The *cob* and *cox*1 sequences for these species pairs were (nearly) identical. The *p*-distance for the Sanger data on *cob* and *cox*1 was 0.000 and 0.005 for the first pair and 0.003 and 0.000 for the second pair. These values were lower than any of the other pairwise comparisons, which were 0.2460 (stdev: 0.1147) for *cob* and 0.165 (stdev 0.0442) for *cox*1.

3.2. Genome validation and annotation

The 19 selected genomes were verified using REAPR (Hunt et al., 2013). No breakpoints were detected by the software, suggesting the genomes were assembled correctly. For three mitogenomes (OSK-11, ACR-24, ACR-30) putative "insertion/deletion" assembly errors were flagged up in or near the AT-rich control region. We therefore inspected the reads that were re-mapped onto these contigs. As no obvious errors could be detected it was decided to include these sequences in all further analyses.

Annotation of tRNAs revealed gene order rearrangements specific to the *Bematistes* clade. In the four *Bematistes* species *trnM* and *trnI* have moved to the 3′-end of ND2 and *trnE* has moved from a position between *trnP* and *trnN* to a position at which *trnQ* is found in the 'standard' Ditrysian mitogenome and is now coded on the other strand (Fig. 2). *trnQ* on its turn is located in between *trnV* and *rrnS*. Secondary structures were obtained for *trnE* and *trnQ* which were visualized using the VARNA applet. These structure predictions suggest all translocated tRNAs can form functional secondary structures (Fig. 3). Sequence similarity among the different *trnE* molecules (and their stems) is high, indicating the translocation of this tRNA in *Bematistes* is not due to an anticodon mutation in *trnQ*, but involved an actual sequence translocation. CREx (Bernt et al., 2007) predicted two translocation events to underlie the changes observed, involving a reverse transposition event for *trnE* and involving a tandem-duplication–random loss event for *rrnS, trnM, trnI, trnQ* and ND2. Finally, we investigated the presence of a *trnI* duplication within the new mitogenomes. This duplication was first reported for '*A.*' ('*P.*') *issoria* (Hu et al., 2010), and here detected in five additional species using tRNAscan-SE (Lowe and Eddy, 1997), but not using ARWEN (Laslett and Canback, 2008). No obvious phylogenetic pattern was detected (Fig. 4) and sequence alignment revealed the alleged tRNA sequences not to be highly conserved (Supplementary Fig. 1).

3.3. Phylogenetic analyses and tests of molecular evolution

The concatenated dataset of protein coding genes, tRNAs and rRNAs had a total length of 15,075 base pairs. The test of Xia et al. (2003) and Xia and Lemey (2009) indicates saturation not to be a problem in the dataset (full dataset: Iss: 0.2428 < Iss.c: 0.8484, $P = 0.0000$; first codon position: Iss: 0.1814 < Iss.c, 0.8123, $P = 0.0000$; second codon position: Iss: 0.0911 < Iss.c: 0.8123, $P = 0.0000$; third codon position: Iss: 0.5173 < Iss.c: 0.8123, $P = 0.0000$). The dataset was partitioned in 10 partitions and Maximum Likelihood and Bayesian trees were generated. The inferred relationships were identical for the two analyses (Fig. 4).

With *Athyma* (Limenitidinae) as an outgroup, Heliconiinae (including Acraeini) are monophyletic, as are Argynnini (including *Issoria*). *Fabriciana nerippe* should be placed in the genus *Argynnis* according to some recent studies (e.g. Kim et al. (2011)) and our study supports that placement. Strongly supported (pp = 1; bootstrap = 100%) as sister to other Acraeini was a pair consisting of '*A.*' *rogersi* and '*A.*' *egina* (type species of *Rubraea*; *P. pseudoegina* and *P. abdera* also belong with the last species according to Silva-Brandão et al. (2008)). '*A.*' *rogersi* has not been placed in previous molecular studies (it was placed in its own group by Pierre and Bernaud (2012) and is placed in the subgenus *Stephenia* Henning 1992 by Henning and Williams (2010)). Sister to the rest (pp = 0.99; bootstrap = 92%) was *A. zetes* (usually placed in the

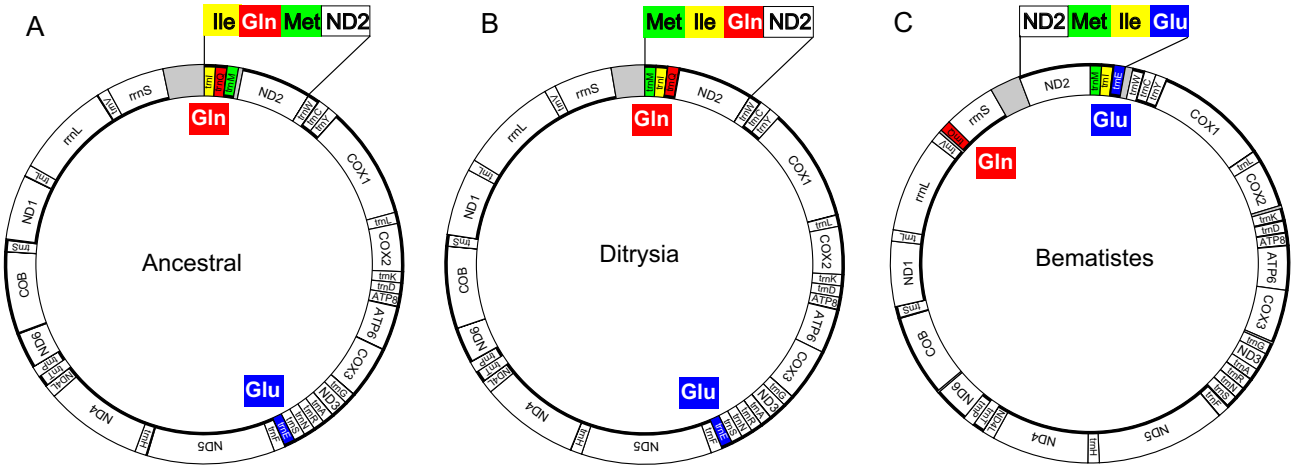

Fig. 2. Observed mitochondrial gene order for (A) Lepidoptera ("ancestral"), (B) Ditrysia, (C) '*Bematistes*'.

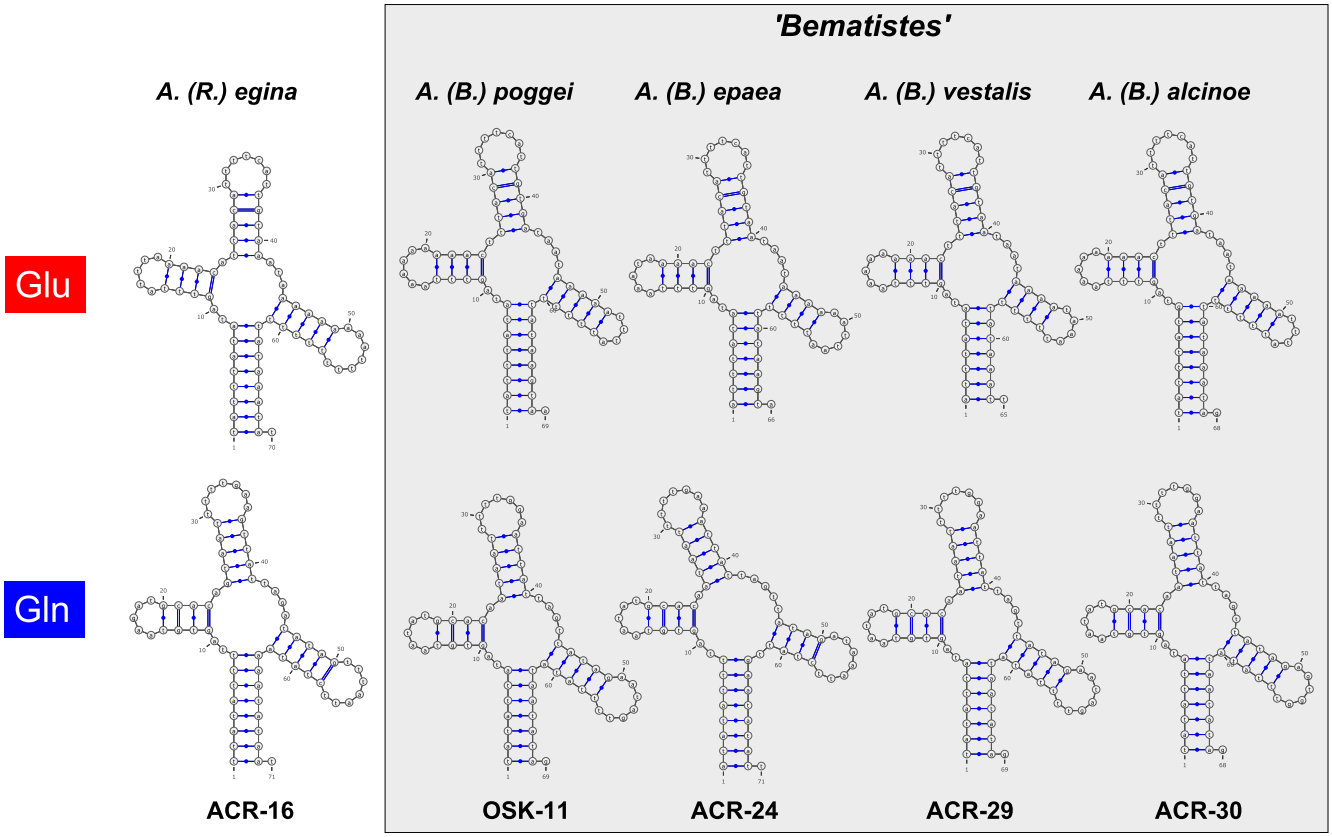

Fig. 3. Predicted secondary structures for tRNA-Gln and tRNA-Glu for the four '*Bematistes*' species and one other species (*A.* (*R.*) *egina*) for comparison. Glu = Glutamate. Gln = Glutamine. Compared to the ancestral state, tRNA-Gln (*trnQ*) and tRNA-Glu (*trnE*) are translocated in '*Bematistes*'. These secondary structure predictions, which were generated using ARWEN (Laslett and Canback, 2008), suggest that all translocated tRNAs are functional.

subgenus *Acraea*, but our sole representative of the distinctive *A. zetes* group). *A. zetes* was sister to a weakly supported relationship (pp = 0.6; bootstrap = 76%) of *Bematistes* (four species forming a clearly supported clade, pp = 1, bootstrap = 100%) + *Telchinia* (13 species also forming a clearly supported clade, pp = 1, bootstrap = 100%). Within *Telchinia*, members of the *serena* species group ('*A.*' *acerata*, '*A.*' *bonasia* and '*A.*' *serena*, placed by Henning and Williams (2010) in *Telchinia* (subgenus *Telchinia*)) formed a variably supported group (pp = 0.99, bootstrap = 43%) which was sister to other *Telchinia* species for which the Asian '*A.*' ('*P.*') *issoria* was poorly supported (pp = 0.91, bootstrap = 36%) as sister to a clade containing other *Telchinia* species groups. Within this latter clade, members of the *encedon* group (*T. pharsalus* + *T. lycoa* + *T. jodutta*) formed a well supported clade (pp = 1, bootstrap = 99%), separated from *T. polis* (*pentapolis* group) + (*T. perenna* (*perenna* group) + *T. kalinzu* (*iturina* group)); these last two species groups being sister to *T. penelope* + *T. parrhasia* + *T. circeis* (*parrhasia* group). The nomenclature of these species groups are as utilized by Bernaud (2014); see Fig. 1 for subgenera of Henning and Williams (2010). Note that a supported relationship for *Bematistes*

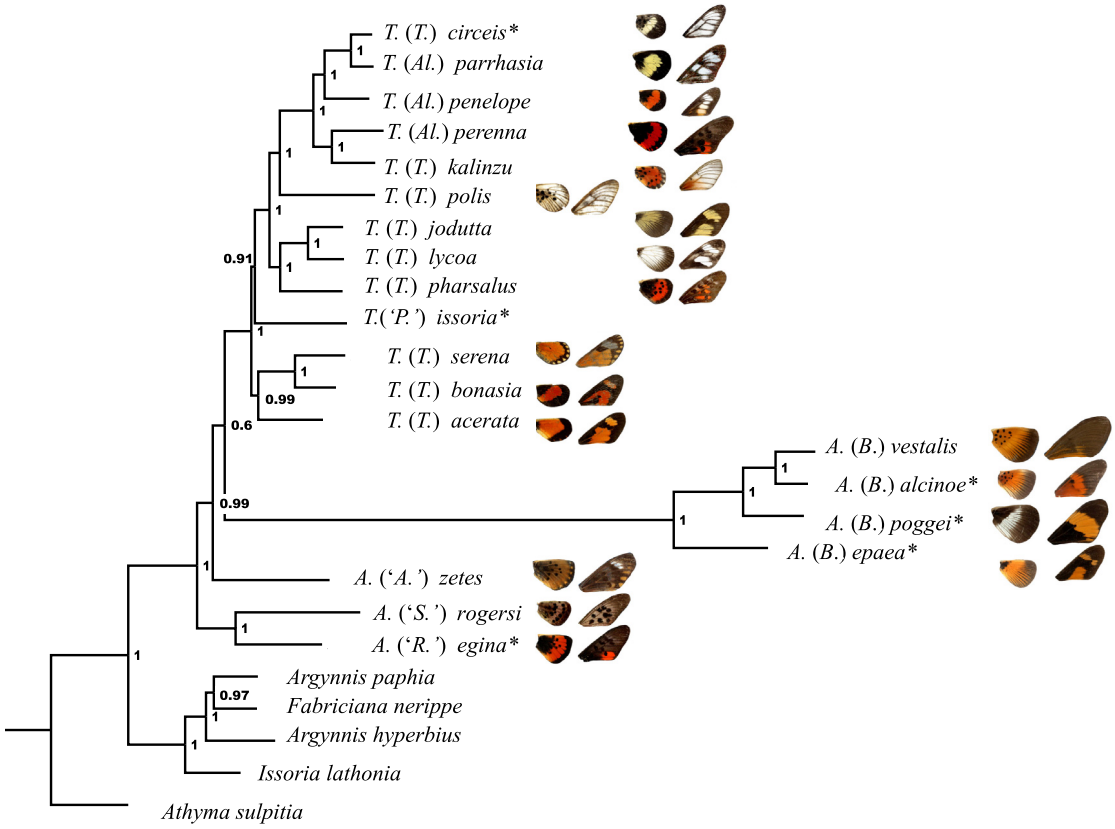

Fig. 4. Mitogenomic phylogeny of Acraeini rooted with Limenitidinae (*Athyma*) and with Heliconiinae (Argynnini: *Issoria*, *Argynnis* and '*Fabriciana*') outgroups. For convenience we use the existing nomenclature of Henning and Williams (2010) in this figure, as it is the most finely divided at a higher taxonomic level. Genera: *A.* = *Acraea*; *T.* = *Telchinia*. Subgenera: *R.* = *Rubraea*; *S.* = *Stephenia*; *A.* = *Acraea*; *B.* = *Bematistes*; *T.* = *Telchinia*; *P.* = *Pareba*; *Al.* = *Alacria*. GenBank accession numbers for the outgroup sequences are: KM592975, NC_016419, NC_015988, NC_018030, NC_017744. *Genomes for which tRNAscan-SE (Lowe and Eddy, 1997) detected additional tRNA-Ile copies. Numbers: Bayesian posterior probabilities.

was neither found by our study nor in that of Silva-Brandão et al. (2008) who had it next to the *egina* group suggesting the need for more markers.

Most interestingly however, the trees also reveal a long branch for the *Bematistes* clade, indicating their mitogenomes are highly divergent. Test for selection were performed to determine whether this divergence is driven by divergent selection pressures. A SelectionLRT test indicates that dN/dS ratios within *Bematistes* are somewhat higher than those found elsewhere in the three. It also reveals the branch leading to *Bematistes* to show a very different ω. The test therefore suggests that *Bematistes* mitogenomes evolve differently to those of other *Acraeini* and that selection on the ancestral *Bematistes* mitogenome deviated from that observed in the other acraeine butterflies (Table 2). This is supported by the Branch-site REL analysis that revealed evidence for episodic diversifying selection for the same long branch (Supplementary Fig. 2).

4. Discussion

Up to present, the extent of application of the generic name *Acraea* has remained controversial and in some recent faunal catalogues (Ackery et al., 1995) is used synoptically, i.e. *Acraea* is sometimes equivalent to Acraeini (minus *Pardopsis*: Silva-Brandão et al., 2008). The phylogenetic analysis of Silva-Brandão et al. (2008) focused on neotropical members and was not able to fully clarify acraeine genus-level nomenclature. However, it is clear from their study that the tribe Acraeini as redefined is monophyletic and also that larval Passifloraceae-feeding is the primitive state of the tribe. Although a possible resolution is to apply the generic names *Actinote* for neotropical members and *Telchinia* for the paleotropical members, the monophyly of other (earlier diverging) members of Acraeini and notably the position and even status of *Bematistes* has remained unresolved.

Clearly a much more detailed study than conducted here is needed to provide formal evidence for abandoning the broad concept of the genus *Acraea* advocated by Pierre and colleagues (a large series of papers summarized by the website of Bernaud (2014)). Our results, however, show that there is considerable phylogenetic information from the mitogenome that may help to resolve the conflict of using genus *Acraea* for all Acraeini or to refine a potentially more divided application of the genus in terms of natural groups. The grouping of *A. rogersi* with *A. egina* (the type species of *Rubraea* Henning 1992) is interesting and not suggested before (*A. rogersi* has been placed in its own isolated group by Bernaud (2014), or as part of the subgenus *Stephenia* by Henning and Williams (2010)). Generic subdivision within the early diverging lineages of Acraeini indeed provides a future avenue for avoiding any uncomfortable paraphylum. In such a multi-genus system, it would appear at present a minimum of five genera with available names (*Rubraea/Stephenia*, *Acraea*, *Bematistes*, *Telchinia*, *Actinote*) might be required, even if *Stephenia* were to be synonymised with *Rubraea* or vice-versa (apparently, neither name has priority; ideally, an exemplar of its type species *Papilio caecilia* Fabricius, 1781, that Bernaud (2014) placed in the *natalica* group along with

Table 2
Results of SelectionLRT test evaluating differences in dN/dS (ω) values between the four '*Bematistes*' species (Clade A), their Acraeini relatives (Clade B) and the branch separating them.

Model		dN/dS (error bounds)	*P*-value	AIC
One dN/dS model		0.0560< 0.0575 <0.0590		188834.09727
Separating branch vs Two Clades	Clades (A + B) Branch	0.0536< 0.0551 <0.0566 0.2171< 0.2328 <0.2493	0	188621.66301
Clade A + branch vs Clade B	Clade A + branch Clade B	0.0732< 0.0768 <0.0805 0.0525< 0.0541 < 0.0558	3.23E−14	188778.50838
Clade A versus Branch + Clade B	Clade A Clade B + branch	0.0579< 0.0618 <0.0660 0.0551< 0.0567 <0.0583	0.06	188832.69134
Clade A, Clade B, branch	Clade A Clade B Branch	0.0572< 0.0612 <0.0653 0.0524< 0.0540 <0.0556 0.2119< 0.2272 <0.2434	0	188616.74752

A. pseudoegina, should be sequenced). Also, *A. zetes* (and probably its entire species group) might require an additional genus according to its isolated position in both Silva-Brandão et al. (2008)'s results and ours. Our results support the view that the Asian (sub)genus *Pareba* Doubleday, 1848 (its original combination, *Telchinia issoria* Hübner, [1819] is the type species) really belongs in *Telchinia* (it was sister to the other *Telchinia* in Silva-Brandão et al. (2008)), but they also clearly suggest that further subdivision as suggested by Henning and Williams (2010) should probably be abandoned (neither the subgenera *Telchinia* nor *Alacria* as defined in the last work is monophyletic. Furthermore, in the Silva-Brandão et al. (2008) study, their one representative of *Auracraea*, *A. rahira*, is sister to a member of the *encedon* group also sequenced here, '*A.*' *acerata*). Key questions for future resolution regarding the "*Acraea* paraphylum" is where in the tree the type species of *Acraea*, *A. horta* and the Oriental *Miyana* Fruhstorfer, 1914 (type species *Papilio moluccana* Cramer) fall. Bernaud (2014) places both taxa in the *neobule* group of their subgenus *Acraea* along with the Asiatic *A. andromacha, A. terpsicore*, and *A. meyeri*. In the phylogeny of Silva-Brandão et al. (2008), all exemplars of the *neobule* group fall as sister to *A. zetes* + (*Telchinia* + *Actinote*). It would seem possible therefore that *Miyana* could be a synonym of *Acraea* (s.s.). A joint and well sampled analysis of the neotropical *Actinote* and paleotropical *Telchinia* (that Henning and Williams (2010) place as sisters in their subtribe Actinotina) is also needed to test if these are truly sister groups as suggested by Silva-Brandão et al. (2008).

The divergent nature of the mitogenomes of *Bematistes*, with its gene rearrangements, is striking, and provides genetic support for resurrecting the name (see Pierre and Bernaud, 2012) as a distinct genus of acraeines. Their diversity is significant: COI-5P DNA barcoding has facilitated the recognition of 30 *Bematistes* species (called there "ex-*Bematistes*" or the "*Acraea* (*Acraea*) *epaea*"-group; Pierre and Bernaud, 2012). Gene rearrangements and duplications appear to be rare in lepidopteran mitogenomes; for an example see Wang et al. (2014). At a deep level the only rearrangement reported to date is the synapomorphic arrangement order of the tRNAs *trnM, trnI, trnQ*, observed in Ditrysia, Palaephatoidea and Tischerioidea, which differs from the ancestral state *trnI, trnQ, trnM*. Very atypically, the four *Bematistes* genomes show three synapomorphic gene translocations: (1) *trnM* and *trnI* moved from the 5′-end to the 3′-end of ND2, (2) *trnE* moved from the cluster of tRNAs in-between ND3 and ND5 to the 3′ end of the *trnM–trnI* block and (3) *trnQ* moved to a position between *trnV* and *rrnS*. The sequence of these events cannot currently be inferred. We would like to point out that sequence conservation (Fig. 3) indicates that the rearrangement of *trnE* is not due to an anticodon mutation in *trnQ*, but involves an actual genetic transposition. The tandem duplication–random loss (TDRL) model, which assumes gene order changes follow from a tandem duplication event and the subsequent random loss of duplicated gene copies, is a popular model to reconstruct translocations and agrees with many mitogenomic rearrangements (Boore, 1999). However, considering that *trnE* has been inverted, the TDRL model is insufficient to explain all rearrangements observed in *Bematistes*. CREx predicted that in addition to a TDRL event, a reverse transposition of *trnE* would have been required to get the observed gene order.

A gene duplication has been reported for one species that is included in this study ('*A*'. ('*P.*') *issoria*; *trnI*) (Hu et al., 2010). We investigated the distribution of this putative duplication over the phylogeny. This alleged tRNA is quite distinct from the standard *trnI* (and contains a 10 bp 'intron') and was not recognized with our custom tRNA covariance models, or by the ARWEN software (Laslett and Canback, 2008). In contrast, tRNAscan-SE (Lowe and Eddy, 1997) detected similar *trnI*-like structures in five other species (all in the AT-rich control region). However, the presence/absence of this supposed duplication shows no phylogenetic pattern and sequence alignment revealed the pinpointed sequences not to be highly conserved (Supplemental Fig. 1), which raises the question whether this alleged tRNA is actually a genuine and functional gene.

The accelerated divergence of the *Bematistes* mitogenomes has most likely affected a robust placement of the clade in the tree, as indicated by the low bootstrap values. Interestingly, previous studies that included nuclear data did not reveal long branches for this taxon, which suggests that the divergence is restricted to the mitochondrial genome. It also indicates that nuclear data will be needed to reliably resolve relationships of the *Bematistes* clade to other Acraeini.

Our quite limited taxon sampling using mitogenomes, combined with the results of Silva-Brandão et al. (2008) shows that both *Telchinia* (within which we confirm *Pareba* should be included) and *Bematistes* are likely monophyletic, and those two names could be used at generic level. Apart from the precise position of '*A.*' ('*P.*') *issoria*, our topology of *Telchinia* is fully congruent with the multi-locus phylogeny of Silva-Brandão et al. (2008), and so is that of *Bematistes*. This lends confidence in our mitogenomic phylogeny. Our sampling is insufficient to resolve the grade that comprises other Afrotropical and a few Oriental acraeines, for which several generic names are available. Also, a few of the subgenera of Henning and Williams (2010) seem likely not to represent natural groups. Clearly a much more comprehensive sequencing study is needed to provide a clearer picture of the Old World Acraeini.

We also confirm that simultaneous mitogenomic sequencing is feasible in cases such as the pooling of a large number of species of the same tribe or genus, as long as relatively divergent genomes are being combined. Such work may be a more efficient route to assessing phylogeny in a large radiation than using multiple genes

with the classical Sanger method, but sequencing of a range of nuclear markers are certainly also needed to see if their histories are congruent or not with the unusual history reported here for the mitochondrial locus. Our study also looks ahead to the prospect of more detailed genomic and biological studies of Acraeini, including investigation of mimicry and the role of endosymbionts in mitochondrial evolution (not yet reported in *Bematistes*, but see Jiggins (2003) and Jiggins et al. (2000, 2001)).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.12.009.

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