Interrelationships and diversification of Argynniss Fabricius and Speyeria Scudder butterflies

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Abstract. Diverse radiations of insects are often associated with adaptations to host plants, and well-resolved phylogenetic relationships are required to fully understand them. Palearctic Argynnis and related subgenera, together with North American Speyeria butterflies make up a radiation whose species hypotheses are confounded by shared wing colour patterns between sympatric populations of closely related recognized species. Previous studies of this group indicate that Speyeria is a lineage within Argynnis, but sampling in these studies has either involved too few Speyeria species or incomplete sampling of Argynnis species. Thus, no comprehensive phylogenetic analysis exists for all members that answers the question of monophyly of Speyeria, or other subgeneric taxa, and their relationship to Argynnis species. We completed a phylogenetic analysis of all North American Speyeria species and all but one species within Argynnis, using one mitochondrial (cytochrome c oxidase I, COI) and four nuclear genes [elongation factor 1 alpha (EF1α), wingless (WG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ribosomal protein S5 (RPS5)]. The results indicate three major lineages within Argynnis s.l.: two Palearctic and one containing both Palearctic and Nearctic species. In summary, the phylogenetic analyses suggest the need for reorganization into three natural groups: Argynnis, Fabriciana and Speyeria. Within each of these genera the phylogenetic hypothesis indicates an evolutionary history marked by rapid diversification and potential extinction, followed by ongoing lineage sorting. The position of North American Speyeria is nested within the Palearctic lineages, which indicates that the radiation began in Asia and was fuelled by existing Viola diversity in North America. Dating analyses of Viola and Speyeria corroborate this hypothesis. The current North American Speyeria species are mixed on the tree, indicating a recent and ongoing radiation. These results provide needed clarity on the evolution of this group, which contains species of conservation concern.

Introduction

Adaptive radiations provide an excellent window through which to study biological diversification because they can link both the patterns and processes of evolution (Robichaux et al., 1990; Craddock & Kambysellis, 1997; Losos et al., 1998; Gillespie, 2004; Seehausen, 2006; Givnish et al., 2009; McGlaughlin & Friar, 2011; Ebel et al., 2015; Kozak et al., 2015). Within insects, diverse radiations often co-evolve with their food plants and involve associated benefits of that ecological interaction, such as unpalatability, aposematism and Müllnerian mimicry (Ehrlich & Raven, 1964; Trigo & Brown, 1990; Brown et al., 1991; Trigo et al., 1996; Beccaloni, 1997; Willmott & Mallet, 2004; Engler-Chaouat & Gilbert, 2007; Ebel et al., 2015). However, a full understanding of the process of adaptive radiation requires detailed systematic and biogeographic knowledge, and phylogenetic analyses that can be used to put host associations into an evolutionary context.

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Butterflies in the genera *Speyeria* Scudder and *Argynnis* Fabricius (Nymphalidae: Heliconiinae: Argynnini) (together = *Argynnis* s.l.) are an enigmatic and challenging radiation of temperate species that spans the Holarctic region. These genera are associated with *Viol* larval food plants and have been the subject of numerous systematic (Warren, 1944; dos Passos & Grey, 1947; Simonsen, 2006a; Dunford, 2009; McHugh et al., 2013), morphological (Urbahn, 1913; Barth, 1944; Williams, 2001; Simonsen, 2006b, 2007; James, 2008; Zaman et al., 2014) and ecological studies (Boggs, 1987; McCorkle & Hammond, 1988; Boggs & Ross, 1993; Kelly & Debinski, 1998; Bierzychudek et al., 2009; Zimmermann et al., 2009; James & Pelham, 2011; Zaman et al., 2015), as well as the focus of recent conservation studies (Shepherd & Debinski, 2005; Salz & Fartmann, 2009; Schultz et al., 2011; Zima et al., 2013; Wells & Tonkyn, 2014), of which the references here are only a few. Despite this long history of study, a detailed understanding of the patterns and processes of this radiation is hampered by classification and systematic challenges.

The diverse *Argynnis* s.l. radiation harbours extensive morphological variation within and among species that has created difficulties in identifying species boundaries. *Argynnis* s.l. includes 39 species in total, with 23 of them being Palearctic *Argynnis* and associated subgenera, and 16 being North American *Speyeria* (Tuzov, 2003; Pelham, 2008). *Argynnis* s.l. are medium- to large-sized butterflies and the group is generally easy to recognize, with black-spotted orange dorsal wing colouration (Fig. 1), although some sexual dimorphism exists. The ventral side is patterned with dark lines and net-like markings or conspicuous spots that are sometimes silvered, depending on the lineage. Species identification is typically based on colour pattern of both dorsal and ventral wings, with the ventral side being especially diagnostic in many cases. However, species delimitation in this group is clouded by extensive variation in wing pattern within populations that is comparable to the amount of variation between recognized species, making certain populations difficult to identify consistently (Dunford, 2009; McHugh et al., 2013). In addition, species often tend to co-vary in colour pattern such that several species will have similarly coloured hindwings in particular localities. There is also some taxonomic disagreement among workers about where to draw lines between species, with *S. atlantis* and *S. hesperis* being prime examples from North America. *Speyeria atlantis* and *S. hesperis* are considered different species by many (Scott et al., 1998; Opler & Wright, 1999; Guppy & Shepard, 2001; Brock & Kaufman, 2003; Warren, 2005; Pelham, 2008; James & Nunnallee, 2011), but some consider *S. hesperis* a junior synonym of *S. atlantis* (Glassberg, 2001; Pyle, 2002). Differences in the ventral patterns and larvae are used to justify the split between *S. atlantis* and *S. hesperis* with consistent differences in sympatry supporting two species. However, some authors find the ventral patterns too inconsistent where populations of *S. atlantis* and *S. hesperis* overlap, and maintain there is a single species (Dunford, 2009). As a result of all these issues, species boundaries can be unclear, some subspecific taxa have been difficult to place in the appropriate species, and establishing the exact number of species has been a challenge.

In addition to the challenges in alpha taxonomy and species delimitation, the morphological variation in *Argynnis* s.l. has resulted in variable classification schemes above the species level. Historically, many subgenera have been used in classifying the Palearctic species, with two subgenera used in North America. Current workers vary in their use of generic names applied to these species. Palearctic workers commonly use *Argynnis* to circumscribe all the Palearctic subgenera, or use *Argynnis* for subgenera such as *Childrena*, *Nephargynnis*, *Panordiana*, *Damaora*, *Argyronome*, or *Argyreus* (hereafter *Argynnis* s.s.) and recognize additional subgenera (i.e. *Mesoacidalia*, *Fabriciana*) as genera. North American workers use *Speyeria* for species there, or use *Argynnis* based on phylogenetic work showing one or two *Speyeria* species were nested within *Argynnis*, and the fact that they share aspects of morphology (Tuzov, 2003; Simonsen, 2006a; Simonsen et al., 2006). *Argynnis* s.s. has many members with vertical bands, net-like patterns or opaque solid green hindwing discs that lack the ventral spotted/silver spotted morphology, supporting their congeneric status (Fig. 1). In addition, many members of *Argynnis* s.s. and *Argynnis* subgenus *Fabriciana* have male genitalia that differ markedly from those of *Speyeria* (Tuzov, 2003; Simonsen, 2006a; Simonsen et al., 2006). dos Passos & Grey (1945) were responsible for the generic recognition of the North American genus *Speyeria*, on the basis of divergent male genitalia morphology and continental isolation. However, several *Argynnis* species (subgenera *Mesoacidalia* and *Fabriciana*) possess similar wing patterns with silvered ventral hindwing spots, and species of subgenus *Mesoacidalia* have male genitalia similar to North American *Speyeria* (Tuzov, 2003; Simonsen, 2006a), making this arrangement unsatisfying.

Since the recommendation by dos Passos & Grey (1945), the taxonomic recognition of *Speyeria* has largely been followed and, although morphological, ecological and phylogenetic evidence indicates that *Argynnis*, its related subgenera, and *Speyeria* are closely related, questions remain about their interrelationships. Simonsen and co-workers (Simonsen, 2006a; Simonsen et al., 2006) examined the phylogenetic relationships within the Argynnini, including *Speyeria*, based upon morphological and genetic evidence (COI, EF1α, and WG), and found *Speyeria* to be part of a lineage containing the old world *Argynnis* species. However, these two studies are limited in their ability to conclusively establish the interrelationships within *Argynnis* s.l. because they sampled only a small fraction of recognized North American *Speyeria* species. Furthermore, the sampled *Speyeria* (*S. cybele* and *S. idalia*) are rather divergent in wing morphology, leaving open the slight possibility that *S. cybele* and *S. idalia* are actually more closely related to Palearctic *Argynnis* than they are to other North American *Speyeria*. Another recent phylogenetic study is also limited in its ability to establish *Argynnis–Speyeria* interrelationships (McHugh et al., 2013). McHugh et al. (2013) focused on establishing the monophyly of the endangered *S. zerene hippolyta* in relation to other *Speyeria* species, using data for COII, WG, GAPDH, triosephosphate isomerase (TPI), RpSS, and eight of 16 *Speyeria* species, all of which are likely to form a clade. However, the dataset was incomplete for many exemplars and they included only a single
species of *Argynnis* and *Brenthis* as outgroups. *Speyeria zerene hippolyta* and species other than *S. cybele* for which multiple subspecies were included, were not found to be monophyletic, corroborating the difficulties in this genus discussed earlier. Given the taxonomic sampling in these studies, the position of *Speyeria* relative to *Argynnis* and the interrelationships within this lineage have not been adequately assessed.

The goal of this study was therefore to increase taxonomic sampling from past studies and analyze a complete multi-gene dataset to investigate the phylogenetic relationships within *Argynnis* s.l. based on all North American *Speyeria* species, all but one Palearctic *Argynnis* species, and 4307 bp of nuclear (EF1α, WG, GAPDH, and RPS5) and mitochondrial (COI) DNA sequence data. Given the Holarctic distribution of this lineage, we also conducted a dating analysis in order to estimate the age of different lineages and their concordance with the putative colonization route across the Bering land bridge. We sampled multiple subspecies for several taxa to examine presently
accepted species hypotheses. If the results indicate species are monophyletic, it would indicate that multiple gene analyses are a viable way to validate species hypotheses that are based on differences in wing colour patterns. This is of interest given the conservation concern for multiple species in this lineage (Hammond & McCorkle, 1983; Shepherd & Debinski, 2005; McHugh et al., 2013; Wells & Tonkyn, 2014). Alternatively, if recognized species are not monophyletic, it may indicate that further study is needed to recognize the biological and genetic diversity that exists in this radiation and that additional genetic tools will be necessary to help identify unique lineages.

**Material and methods**

**Sampling plan**

All recognized species of Speyeria were sampled for analysis, along with all but one species of Argynnis. We used the taxonomic arrangement of Pelham (2008) and Tuzov (2003), with the exception that Argynnis corenpippe was synonymized with Argynnis xipe following Lee (2005). Issoria Hübner (1819) and Brenthis Hübner (1819) were chosen to serve as outgroups, because previous work has found Brenthis to be the sister genus to Argynnis, with Issoria sister to Brenthis and Argynnis (Simonsen, 2006a; Simonsen et al., 2006). Table S1 lists the individuals sampled and their collection locality.

**DNA sequence acquisition**

Sequence data for several taxa were available through GenBank, using previously established primers and polymerase chain reaction (PCR) protocols (Wahlberg & Wheat, 2008), and accession numbers are indicated in the supplementary materials. For taxa without available sequences, extraction of genomic DNA was done with a DNeasy extraction kit (Qiagen Inc., Germantown, MD, U.S.A.) using thoracic or leg tissue and modified by doing two final elution steps of 100 μL AE elution buffer. PCR reactions were performed at a final volume of 20 μL that consists of 10 μL Multiplex PCR Master Mix 2x (Qiagen), 7 μL PCR water (Qiagen), 1 μL of each primer, and 1 μL of the DNA template. Secondary PCR reactions may also have been performed at a final volume of 20 μL of 10x Advantage II buffer (Qiagen), 14.4 μL PCR water (Qiagen), 1 μL of each primer, 0.4 μL dNTPs, 0.2 μL platinum Taq DNA polymerase, and 1 μL of DNA template. To obtain product from degraded material, DNA template volume may have been increased to 2 μL. Details on specific primer pairs, primer sequences and melting temperatures are listed in Table 1. A semi-nested reaction was employed to amplify the EF1α region. First the primers 40 and Tipper were used according to the protocol described and summarized in Table 1. The subsequent PCR product was then diluted with PCR water (Qiagen) at a 1:10 ratio. This dilution was employed as the template in a second reaction, utilizing the primers 40 and Gennifer to obtain the final product. This semi-nested protocol was also used to amplify degraded COI material, with the internal primer Rudy. PCR reactions were run on a BioRad C1000 (Hercules, CA, U.S.A.) thermocycler. All PCR products were checked with gel electrophoresis and run on a 1% agarose gel. Sequest Corporation (http://www.sequestech.com) performed sequencing reactions in both forward and reverse directions. A subset of the specimens were sequenced in the Wahlberg laboratory using previously published protocols (Wahlberg & Wheat, 2008).

Chromatograms were assembled into a contiguous sequence, manually inspected for quality, and edited, if necessary, using seqman pro 2.1.0 (DNastar, 2011), and finally checked for stop codons and trimmed in mesquite 3.04 (Maddison & Maddison, 2010). Sequence data were aligned with the online version of muscle 3.8.31 (Edgar, 2004). Terminal gaps or missing sequence data were treated as ambiguous unknown sequence data. For each individual, several variable sites across each gene were evaluated and compared with the respective chromatogram to double-check for sequencing errors.

**Phylogenetic analysis**

In total, 4307 bp of COI, WG, GAPDH, RPS5 and EF1α were analyzed. We obtained the best-fitting models of sequence evolution using MrModelTest2 (Nylander, 2004) for two partitioning strategies, by gene and by codon for each gene (15 partitions). For each partition, the best-fitting model selected had a ΔAIC value > 2 (Table 2). MrBayes 3.2.5 (Ronquist & Huelsenbeck, 2003) was used for Bayesian inference, with default parameters employing four coupled chains for 50 million generations with a sample frequency of every 1000th generation. Gamma shape, proportions of invariant sites, state frequencies and substitution rates were all unlinked across gene partitions. The first 25% of the samples were discarded as burn-in. Bayes factors [= 2 x difference in –harmonic mean likelihood (HML)] were used to assess which partition strategy was preferred (not over-parameterized) with values > 10 supporting the more parameter-rich strategy (Kass & Raftery, 1995).

Maximum likelihood and parsimony analyses were also performed on the complete concatenated dataset to examine the sensitivity of the topology to models of evolution and optimality criteria. A maximum likelihood analysis was run using the online version of raxml (Stamatakis et al., 2008). The data set was unpartitioned and a GTR+G+I model of evolution was implemented with 100 bootstrap replicates. Parsimony analysis was performed in TNT by running the ‘aquickie.run’ script (Goloboff et al., 2008).

**Dating analysis**

We explored temporal patterns of diversification in Argynnis and Speyeria by estimating lineage divergence times using the Bayesian Markov chain Monte Carlo relaxed clock method in BEAST 2.1.3 (Bouckaert et al., 2014). We used the same combined sequence dataset as in the MrBayes analysis, with the
Table 1. Primer information for the amplified genes.

| Gene    | Primers       | Primer sequence 5′−3′                | Anneal temp. | Citation            |
|---------|---------------|--------------------------------------|--------------|---------------------|
| COI (part 1) | LCO           | GGTCAAACACTATAAGATGATTTG            | 50°C         | Folmer et al. 1994 |
|         | Lep3.1r       | TCCTAAATCTAAAGATGATTTGGAAC          |              | Dasmahapatra et al. 2007 |
| COI (part 2) | Pat           | AAGTGCACTAATCGGATATTG              | 48–52°C      | Brower & Jeanson 2004 |
|         | Jerry         | CAAATTTTTTGGATTTTTTTTTTT             |              | Brower et al. 2006  |
|         | Rudy          | GAAGTTTTATTTTTATTTTTTACCGG          |              | Brower & Jeanson 2004 |
| WG      | WG1           | GARTGYAARTGYCAGYGTGACTCTTG          | 55°C         | Brower & de Salle 1998 |
|         | WG2           | ACTGCGCARCCACGCTGAAATGTERCA         |              | Brower & de Salle 1998 |
| EF1α    | 40            | GTCGTSATYGGWCACGTMGATT             | 55°C         | Cho et al. 1995    |
| GAPDH   | HybFrigga     | TAAATACCCTCACTATAGGGGACGCTG          | 50°C         | Wahlberg & Wheat 2008 |
|         | HybBurre      | ATTTAACCCTCACTAAAGGGGTTGAATGACTCT |              | Wahlberg & Wheat 2008 |
| RPS5    | HybR5degF     | TATACGACTCATATAGGGGACGCTG           | 55°C         | Wahlberg & Wheat 2008 |
|         | HybR5degR     | ATTAACCCTCACTAAAGGGGTTGAYTCAACACG   |              | Wahlberg & Wheat 2008 |

COI, cytochrome c oxidase I; WG, wingless; EF1α, elongation factor 1 alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPS5, ribosomal protein S5.

Table 2. Molecular models chosen for each gene partition using MrModelTest and >2ΔAIC for model selection.

| Partition       | Model          | AIC   |
|-----------------|----------------|-------|
| COI position 1  | GTR+G+I        | 3456.7|
| COI position 2  | GTR+G+I        | 1725.4|
| COI position 3  | GTR+G+I        | 13134.8|
| EF1α position 1 | GTR+G+I        | 1397.8|
| EF1α position 2 | HKY+I          | 1264.7|
| EF1α position 3 | HKY+G          | 4296.2|
| GAPDH position 1| HKY            | 699.1 |
| GAPDH position 2| HKY            | 661.3 |
| GAPDH position 3| GTR+G          | 2928.6|
| RPS5 position 1 | GTR+G          | 664.9 |
| RPS5 position 2 | F81            | 563.2 |
| RPS5 position 3 | GTR+G          | 2047.1|
| WG position 1   | K80            | 497.6 |
| WG position 2   | K80            | 405.6 |
| WG position 3   | SYM+I          | 1593.0|

AIC, Akaike information criterion; COI, cytochrome c oxidase I; EF1α, elongation factor 1 alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPS5, ribosomal protein S5; WG, wingless.

data partitioned by gene and codon, as this partitioning strategy was favoured in the MrBayes analysis. The same models of sequence evolution were used as employed in the mrbayes analysis with the following four exceptions, in which a simpler model (HKY) was employed to avoid over-parameterization and to reach adequate effective sample sizes (ESS) > 200: position 1 of RPS5 and EF1α, position 2 and 3 of COI. We used the uncorrelated relaxed lognormal clock model, with exponential priors for ucldMean and ucldSD, birth death model tree prior, and lognormal priors for birth rate and relative death rate. As no fossils are available to calibrate the nodes in this lineage of nymphaids, we used the estimate of Wahlberg et al. (2009) to enforce a normal prior with a mean of 11.0 million years ago (Ma), and a standard deviation of 1.3 Ma for the divergence of Brenthis + Argyrnis + Speyeria (constrained to be monophyletic) from Issoria (estimated at 11 Ma in Wahlberg et al., 2009). The analysis was run twice for 20 million generations each, sampling every 1000 generations, and 25% of samples were discarded as burn-in. Tree annotator was used to create the maximum clade credibility tree with mean height selected.

Results

Results of the MrBayes analysis indicated that partitioning by gene and codon was a better strategy than partitioning by gene alone (2 × −HML = 2340). Thus, the Bayesian phylogenetic and dating results discussed in the following are for the analysis by gene and codon.

Phylogenetic analyses produced highly resolved relationships with similar topologies, high posterior probabilities or bootstrap values, and recovered three major lineages within Argynnis s.l. (Fig. 2; Figures S1, S2). The first major lineage sister to all the rest comprised species exclusively found in Argynnis s.s. and included the subgenera Argyrnys, Argyreus Scopoli, Argyronome Hübner, Childrena Hemming, Damora Nordmann, Nephargynnis Shirozo & Saigusa and Pandoriana Warren. The second major lineage corresponded to Fabriciana Reuss, which was sister to a third major lineage that included members of the subgenus Mesoacidalia Reuss and Speyeria. There was high clade support in all analyses for the monophyly of Fabriciana and Speyeria within the Argynnis s.l. clade (Fig. 2, posterior probability = 1.0; Figures S1, S2, bootstrap values = 100%). The monophyly of subgenus Mesoacidalia was not supported by our analyses (Fig. 2; Figures S1, S2).

Within the Argynnis s.s. lineage there was a lack of resolution among the basal long branches, but many relationships between species were well supported (Fig. 2; Figures S1, S2). Argyrnys pandora (subgenus Pandoriana) was estimated to be sister to the remaining species in all analyses, but with support varying by analytical method. Among the remaining species,
several historically recognized subgenera were recovered in all analyses (McHenry, 1963). The subgenus Argyronome was a well-supported monophyletic lineage containing A. ruslana and A. iadice. There was high support for a lineage containing A. hyperbius (subgenus Argyreus) with two species in the strongly supported subgenus Childrena (A. zenobia and A. childrena). A well-supported sister relationship between A. sagana (subgenus Damora) and A. paphia (subgenus Argynnis) was recovered in all analyses. The phylogenetic position of A. anadyomene (subgenus Nephrargynnis) was ambiguous with no clear sister taxon.

The Fabriciana lineage was almost completely resolved, with the same relationships in all analyses (Fig. 2; Figures S1, S2). Argygnis kamala was always well supported as sister to the rest of the Fabriciana species. A second well-resolved lineage within Fabriciana included Argygnis vorax and Argygnis nerepis as sister taxa, together with Argygnis adippe. Among the remaining species, A. xipe and A. niobe were always sister taxa, but the position of Argygnis auresiana and sister relationship of Argygnis elisa and Argygnis jainadeva were dependent on the type of analysis. The two species of Fabriciana for which multiple exemplars were sampled were both monophyletic. The two A. adippe samples were from different geographic regions (Spain and Finland) and were more genetically distinct than the two A. auresiana, which were both from Morocco.

Within the third lineage containing Speyeria and Mesoacidalia, deep-level relationships depended on the method of phylogenetic analysis. Support for the monophyly of Mesoacidalia was inconsistent as the position of Argygnis clara was unclear. Argygnis clara was weakly supported as the sister to Speyeria in the Bayesian (posterior probability = 0.86; Fig. 2) and maximum likelihood analysis (bootstrap = 69%; Figure S1), but the position of A. clara was unresolved in the parsimony analysis (Figure S2). The other individuals from subgenus Mesoacidalia (Argynnis aglaja and Argygnis alexandra) were grouped together. However, the sister relationship between the A. aglaja

Fig. 2. Inferred phylogenetic relationships for Argygnis and Speyeria butterflies resulting from Bayesian analysis. Posterior probabilities are 1.0 unless indicated. ‘M’ denotes subgenus Mesoacidalia.
individuals, which were from different geographic areas (Primorsky Krai province Far East Russia, and Sweden), was poorly supported.

Species of North American Speyeria were always strongly supported as a monophyletic lineage arising from within Argytnis s.l. However, within Speyeria the higher relationships among species were not well resolved, given the weak support for several nodes and conflict among analytical methods. The basal lineages comprised the most morphologically discrete species, with S. idalia always sister to the rest, followed by Speyeria nokonis and Speyeria diana. The relationship between S. nokonis and S. diana differed based on the analysis, and it was not clear which branched first (Fig. 2; Figures S1, S2). The putative subgenus Semnopsyche Scudder (1875), proposed for S. idalia, S. diana, S. cybele and S. aphrodite on the basis of an accessory bursal sac in the female genitalia (dos Passos & Grey, 1947; Dunford, 2009), was never recovered as monophyletic (Fig. 2, Figures S1, S2). Relationships among the remaining species tended to form a grade, but the weak support for many of the higher nodes made relationships unclear.

Although the higher relationships within Speyeria were generally not well resolved, several species groups were common to all analyses and generally well supported. Speyeria adiaste was strongly supported as sister to Speyeria hydaspe. Speyeria carolae was always part of a lineage containing the sampled subspecies of S. coronis. Speyeria atlantis and S. hesperis were sister taxa in the Bayesian analysis, but this relationship was weakly supported in the maximum likelihood and parsimony sister taxa in the Bayesian analysis, but this relationship was weakly supported in the maximum likelihood and parsimony analyses. Additional tentative species groups which involved paraphyletic species and weaker support were: S. zerene + S. mormonia, and S. callippe + S. edwardsii + S. egleis. The S. coronis carolae lineage was strongly supported as sister to the S. callippe lineage in the Bayesian analysis, but this was weakly supported in the maximum likelihood and parsimony analyses.

Among the more derived lineages of Speyeria, five of the ten species with multiple taxa sampled were recovered as monophyletic with high support in all analyses, with the other five paraphyletic or polyphyletic (Fig. 2, Figures S2, S3). Well-supported monophyletic species included S. aphrodite, S. atlantis, S. cybele, S. hesperis and S. hydaspe. Species that were not monophyletic included: S. coronis, S. egleis, S. mormonia and S. zerene. The diverse S. callippe complex was paraphyletic or polyphyletic, depending on analytical method. In all analyses the S. callippe complex included S. edwardsii, S. egleis egleis, S. egleis albrighti, and S. zerene picta, with S. callippe chilcotinensis sister to S. coronis + the S. callippe complex in the maximum likelihood and parsimony analyses. Speyeria coronis was paraphyletic, with the sister relationship between the two S. coronis subspecies not supported, and S. carolae sister to S. coronis coronis. The two S. egleis subspecies were never recovered as monophyletic, and instead were found within different lineages of the S. callippe complex. Speyeria zerene was polyphyletic, with S. mormonia closely related to a Great Basin S. zerene lineage, and S. egleis picta arising from within the S. callippe complex. The two S. mormonia subspecies were only weakly supported as monophyletic.

Our divergence time analysis indicated that the split between Argytnis and Speyeria + Fabriciana occurred 9.10 Ma [95% highest posterior density interval (HPD): 6.27–11.14]. The Argytnis s.s. radiation then diversified beginning 7.48 Ma (95% HPD: 5.12–9.64). The Fabriciana lineage diverged from Speyeria + Mesoacidalia 7.59 Ma (95% HPD: 4.79–9.01), with extant species diversifying beginning 4.47 Ma (95% HPD: 2.50–5.64). The lineage comprising Speyeria + Mesoacidalia began its radiation 6.60 Ma (95% HPD: 4.21–8.06), with the split between A. clara and Speyeria dated at 6.14 Ma (95% HPD: 3.93–7.55), and the genus Speyeria diversifying in North America starting 5.18 Ma (95% HPD: 3.19–6.29). The tree file containing mean ages and 95% HPD for all divergence points is included as a supplementary file (File S1).

Discussion

Systematics and classification

This study has clarified the higher relationships among the lineages in this complex group and points the way to clarifying lower-level relationships. The phylogenetic analyses conducted here all found strong support for three major monophyletic lineages (Fig. 2, Figures S1, S2): (i) Argytnis s.s., containing all Palearctic subgenera except Mesoacidalia and Fabriciana; (ii) Argytnis subgenus Fabriciana; and (iii) Speyeria + Mesoacidalia. Speyeria was nested within Argytnis s.l. as in previous analyses (Simonsen et al., 2006), and our results conclusively demonstrate the monophyly of North American Speyeria. The strong support for these higher-level relationships and corroboration of relationships within the subtribe Argytnina that includes Issoria, Brenthis and Argytnis s.l. (Simonsen et al., 2006) mean these relationships are most likely stable and robust phylogenetic hypotheses. However, there is clearly work to be done to clarify the lower-level relationships in these lineages. Future studies incorporating several more genes, or using many more markers with methods such as random amplified DNA sequencing (RAD), may help to understand the patterns of divergence within these genera.

The results of our phylogenetic analyses have strong implications for the classification of these butterflies. In our opinion, there are only two ways to interpret the topology for a robust classification: either the entire clade is considered to be the genus Argytnis or the three strongly supported clades can be considered as the genera Argytnis, Fabriciana and Speyeria. When considering these two alternatives, we need to take into account issues of stability. The current recommendation is for the entire clade to be called Argytnis (Simonsen et al., 2006), yet in North America Speyeria continues to be widely used, especially in matters related to conservation. We thus feel that retaining the name Speyeria is necessary for stability and consistent usage. This would mean that the name Argytnis would be applied to Argytnis s.s., that is, all traditionally recognized Palearctic subgenera except Mesoacidalia and Fabriciana. In addition, we recommend that Fabriciana (rev. stat.) be elevated to generic status. Lastly, we recommend that Mesoacidalia be
synonymized with Speyeria. This last change is likely to face the greatest opposition, but is warranted for the following reasons. The name Speyeria takes precedence over Mesoacidalia based on the year each was described. This arrangement causes the fewest problems with historic usage of species nomenclature, and best reflects phylogenetic patterns. Finally, this arrangement makes sense based on the position of A. clara in our analyses, which was either sister to North American Speyeria or unresolved (Fig. 2; Figures S1, S2). Our revised classification is given in Table 3.

The revised genera resulting from our molecular analyses are strongly associated with characters of morphology and genitalia. At one level this is not surprising, given the number of historically described subgenera that were based on such traits. However, the molecular analyses here provide a robust framework for interpreting the morphological variation, some of which is discussed here. All members of Argyynnis s.s. species have on the dorsal hindwings net-like patterns, lines or two-toned colouration, rather than the heavy spotting found in Fabriciana and Speyeria (Fig. 1). Members of the Argyynnis s.s. lineage also have relatively large, broadly shaped hindwings, with extended scalling along the margin (Tuzov, 2003). The hindwing shape in Speyeria and Fabriciana is generally relatively smaller, with less scalling on the margins (Tuzov, 2003). The male genitalia of Argytnnis s.s. are variable with respect to each other, and therefore difficult to summarize. They are, however, distinctive from the other two major lineages in having a relatively smooth and thin, generally elongate uncus ending in a sharp hook, or with elaborate projections on the uncus, and the ampulla is highly variable among species with elaborate projections, strong teeth, or is elongate and relatively thin (Tuzov, 2003; Simonsen, 2006a). Simonsen’s (2006a) morphological analysis did not recover Argyynnis s.s. as monophyletic and so further study is needed to identify potential synapomorphies for this genus. Fabriciana and Speyeria can be distinguished by differences in the male genitalia. All members of Fabriciana have a sigmoid or elongate ampulla (Tuzov, 2003; Simonsen, 2006a), and three additional unique apomorphies (see characters 33:1, 35:1 and 93:1 of Simonsen, 2006a). In addition, members of Fabriciana, except for F. kamala, possess a crest at the dorsal aspect of the uncus. In contrast, members of the Speyeria lineage (including Mesoacidalia) have a relatively broad ampulla that overlaps the proximal end of the valve, and a relatively smooth and recurved uncus (Simonsen, 2006a). The Speyeria + Mesoacidalia relationship was supported by one unique autapomorphy (character 64:1) in Simonsen (2006a).

Although the higher-level relationships among genera were strongly supported, relationships within each of the genera were less clear. Within Argyynnis s.s. for example, several species pairs were identified, but the relationships among species groups were not well supported. Previous systematic work has recognized the same species pairs, *zenobia* + *childreni*, *ruslana* + *laodice*, *paphia* + *sagana* (Simonsen, 2006a; Simonsen et al., 2006), and historically the first two pairs have been placed in their own subgenera (Childrena and Argynnone, respectively). The ambiguity among species and species groups observed in this study is consistent with the challenges expressed by dos Passos & Grey (1945): ‘A formal subdivision of the extreme genitalic heterogeneity into series of anything like comparable value would entail recognition of several small or monotypical genera or subgenera’. The relationships within Argyynnis thus represent a continued and interesting problem, with evolutionary implications that will be discussed further below.

The resulting relationships within Fabriciana corroborate previous work, and highlight the transitional morphology in F. kamala. Our analysis is consistent with previous phylogenetic work identifying F. kamala as sister to the other species in this lineage (Simonsen, 2006a; Simonsen et al., 2006). Our results for relationships within Fabriciana are also consistent with the morphological work of Simonsen (2006a), in that both analyses resulted in weakly supported internal relationships. Fabriciana kamala appears morphologically somewhat transitional compared with its congeners. Fabriciana kamala lacks the dorsal crest on the uncus, and on the ventral hindwings the marginal and sub-marginal spots are fused, resembling the vertical banding seen in some members of Argyynnis s.s. The other members of Fabriciana are more uniform in these characters. In this way the Fabriciana lineage matches the other two genera, Argyynnis and Speyeria, in that the first species to diverge are somewhat distinct from their congeners.

The recognized Argyynnis s.l. species missing from this analysis, *A. argyrospilata*, is probably a member of the Fabriciana lineage and its classification should be revised to reflect this (Table 3). Agrpynnis argyrospilata shares an elongate sigmoid ampulla in the male genitalia, which is found in all Fabriciana species (Tuzov, 2003; Simonsen, 2006a). The members of Fabriciana, other than F. kamala, also have a pronounced crest on the dorsal aspect of the uncus (Simonsen, 2006a), and have a more conspicuous ventral spotted colour pattern on the hindwings. Agrpynnis argyrospilata has the pronounced crest (Tuzov, 2003) and conspicuous ventral hindwing spotted pattern. Together, these traits indicate that *F. argyrospilata* belongs among the derived lineages within Fabriciana.

Our results conclusively demonstrate the monophyly of North American Speyeria and its close relationship with species of subgenus Mesoacidalia, and indicate that much additional work is needed to provide clarity for this group. The ambiguous position of S. clara found here should be further studied and may be clarified with additional data. Adding to the ambiguity surrounding S. clara is the fact that Simonsen’s (2006a) analysis placed S. clara as the sister species to a clade containing Speyeria and Mesoacidalia. The paraphyly revealed by our increased taxonomic sampling in Speyeria, coupled with previous research, suggests that future studies should focus on clarifying species boundaries in this group. Previous phylogenetic work sampling exemplars from multiple subspecies of Speyeria species has uncovered similar levels of paraphyly as found in our study (McHugh et al., 2013). McHugh et al. (2013) showed some of the same relationships as observed here, with one lineage of S. zerene closely related to S. mormonia and another related to S. callippe. In both our study and McHugh’s, paraphyly was not limited to S. zerene. Both studies exhibited paraphyly in S. egleis, S. callippe and S. coronis. Unlike the results of our study, the analysis of McHugh...
Table 3. Revised classification and distribution of species in this study.

| Genus    | Generic synonyms          | Species                      | Distribution                                                                 |
|----------|---------------------------|------------------------------|------------------------------------------------------------------------------|
| Argynnis Fabricius |                           |                              |                                                                              |
|          | SYN Nephargynnis Shirozu  | paphia (Linnaeus)            | Across the Palearctic from Ireland to Japan                                   |
|          | SYN Pandoriana Warren     | zenobia Leech                | Southeast Russia, China, Tibet, Korea                                         |
|          | SYN Childrena Hemming     | childreni Gray               | China, Burma, Thailand, Laos, Vietnam, Korea                                  |
|          | SYN Argyreus Scopoli      | hyperbia (Linnaeus)          | Afghanistan, Pakistan, India, China, Korea, Japan                            |
|          | SYN Damora Nordmann       | anadomyene Felder & Felder   | Southeast Russia, China, Korea, Japan                                         |
|          | SYN Argyronome Hübner     | pandora (Denis & Schiffermüller) | Southeast Europe to Asia Minor and western China                           |
|          |                           | sagana Doubleday             | Southeast Russia, Mongolia, China, Korea, Japan                              |
|          |                           | laodice (Pallas)             | Eastern Europe, northern India, China, Japan                                 |
|          |                           | raslana Motschulsky          | Southeast Russia, eastern China, Korea, Japan                                |
|          |                           |                              |                                                                              |
| Fabriciana Reuss |                           | kamala (Moore)               | Western Himalayas                                                           |
|          |                           | jainadeva (Moore)            | Pakistan and northern India, western Himalayas                               |
|          |                           | auresiana (Fruhstorfer)     | Algeria, Morocco                                                            |
|          |                           | argyrospilata Kotsch         | Afghanistan and Tajikistan                                                   |
|          |                           | elisa (Godart)               | Corsica, Sardinia                                                           |
|          |                           | neriipe (Felder & Felder)    | Southeast Russia, eastern China, Korea, Japan                               |
|          |                           | nioie (Linnaeus)             | Wide distribution across Europe to western China                              |
|          |                           | xipe (Grum-Grshimailo)       | Southeast Russia, Mongolia, China, Korea, Japan                              |
|          |                           | SYN coredippe Leech          | Southeast Russia, China, Korea, Tibet, Japan                                 |
|          |                           | vorax (Butler)               | Wide distribution from Ireland to Japan                                      |
|          |                           | adipe (Denis & Schiffermüller) |                                                                              |
| Speyeria Scudder | SYN Semnopsyche Scudder  | aklaja (Linnaeus)            | Wide distribution from Ireland to Japan                                      |
|          | SYN Mesoacidia Reuss      | alexandra (Méntrés)          | Azerbaijan, Turkmenistan, Iran                                               |
|          |                           | clara (Blanchard)            | Northern India, Tibet, West China                                           |
|          |                           | diana (Cramer)               | East coast U.S.A extending west to Tennessee                                 |
|          |                           | cybele (Fabricius)           | Northern distribution across North America                                  |
|          |                           | aphrodite (Fabricius)        | Northern distribution across North America                                  |
|          |                           | idalia (Drury)               | Segregated populations across central U.S.A.                                |
|          |                           | nokonis (W.H. Edwards)       | Segregated populations across southwestern U.S.A.                          |
|          |                           | edwardsii (Reakirt)          | Primarily in north central U.S.A.                                           |
|          |                           | coronis (Behr)               | Broadly across western North American mountains                             |
|          |                           | carolae dos Passos & Grey    | Spring Mountains, Nevada, U.S.A.                                            |
|          |                           | zerene (Boisduval)           | Broadly across western North American mountains                             |
|          |                           | callippe (Boisduval)         | Broadly across western North American mountains                             |
|          |                           | egleis (Behr)                | Coastal mountain ranges, California, U.S.A.                                 |
|          |                           | adiaste (W.H. Edwards)       | Distributed across northeastern North America                               |
|          |                           | atlantis (W.H. Edwards)      | Distributed across northwestern North America                               |
|          |                           | hesperis (W.H. Edwards)      | Found across western North American mountains                              |
|          |                           | hydaspe (Boisduval)          | Found across western North American mountains                              |
|          |                           | mormonia (Boisduval)         |                                                                              |

et al. (2013) identified two lineages of S. hesperis (= S. atlantis hesperis, S. atlantis cornelia, S. atlantis dodgei in that paper), one nested within a lineage of S. zerene, and the other within a lineage containing S. egleis and S. callippe. It should be noted that Argynnis and Fabriciana are not exempt from these problems, and that here we are focused on Speyeria because of the increased sampling of subspecies in that group. Similar levels of difficulty in resolving relationships among Argynnis and Fabriciana species may occur with increased sampling of subspecies in those groups.

These results are clearly not satisfactory with regard to molecular markers helping to delimit species in this challenging group. The differences between McHugh et al. (2013) and our results may be that this is the result of their use of COI coupled...
with missing data for many exemplars, compared with our near complete dataset and use of COI. Perhaps these patterns could be clarified with additional sampling of individual genes, or many more markers from recent analytical methods (e.g. RADSeq). However, the fact that this group presents challenges in consistently delimiting species with morphology as well as genetic data requires us to consider explanations other than amount of data and molecular markers.

Explanations for the ambiguous relationships among Speyeria species include issues related to taxonomic organization and/or evolutionary processes. Assuming the resulting trees reflect true evolutionary relationships among species, taxonomic issues could include: (A) some of the subspecies are placed in the incorrect species; and (B) the species-level diversity is currently under-recognized. Assuming the taxonomy is correct, evolutionary processes leading to the observed patterns could include: (C) historical and recent hybridization may be obscuring monophyly; and (D) the radiation has happened quickly and gene trees and species trees are incongruent because of incomplete lineage sorting.

It is likely that each of the aforementioned taxonomic and evolutionary explanations are playing a part in the observed relationships. Speyeria taxa have been moved between species (A), good examples being S. carolae (Emmel & Austin, 1998) and S. z. gunderi (Grey, 1975, 1989). With additional work and data this type of reorganization may help to make congruent with phylogeny the regional similarity present among species phenotypes (e.g. Great Basin S. zerene gunderiplatina, S. callippe nevadensis/harmonia, S. egleis toyabetreidi, S. coronis snyderi). Elevation of subspecific taxa to species level (B) has occurred in the recent past (e.g. S. adiaste, Grey, 1989), is not uncommon in the literature (e.g. S. hollandi used in McHugh et al., 2013), and workers differ in their recognition of species such as S. atlantis and S. hesperis (i.e. Scott et al., 1998; Pyle, 2002). Hybridization is also likely given recent work indicating that most species within North America are interfertile (Hammond et al., 2013). Finally, given the pattern of relationships seen in the trees here, coupled with our dating analysis indicating that the majority of Fabriciana and Speyeria species originated < 2 Ma, it seems likely that Argynnis s.l. is a radiation within which there has been repeated, rapid diversification (D), which is still in the process of coalescing to single ancestral alleles that differ among species.

Evolutionary implications and diversification

The tree topologies resulting from our analyses provide a window into the patterns of diversification in Argynnis, Fabriciana and Speyeria, and are indicative of rapid and repeated radiation. Viewing each lineage from base to the tips, each is characterized by long basal branches, with relatively short branches and ambiguous intermediate relationships among species/species groups, followed by longer branches leading to species (Fig. 2; Figures S1, S2). This repeated pattern in the trees indicates that diversification occurred early and relatively rapidly in each of the three major lineages, and that rapid diversification is a hallmark of Argynnis s.l. evolution. Considering the weak support for many internal relationships, additional data might help to clarify internal relationships within genera, but are not likely to increase branch lengths and alter this interpretation. Assuming rates of evolution have not changed across the tree, the long basal branches indicate either single species that are long-lived or extant species whose stem relatives have gone extinct.

Our analyses have implications for the biogeographic history of Argynnis s.l. Argynnis and Fabriciana have their highest species richness in Central and East Asia, and both genera appear to have begun diversifying across Europe and Asia before the Bering Strait was first open (between 4.8 and 7.3–7.4 Ma; Marincovich & Gladenkov, 1999). However, the weak support for intermediate nodes makes it difficult to infer more detailed biogeographic patterns (Fig. 2, Figures S1, S2). In these two genera, the sister taxa indicated by our analyses generally show a partial overlap in their ranges. Argynnis, Fabriciana, as well as Speyeria species inhabit mountainous regions that no doubt have helped to influence levels of genetic differentiation between populations, and are probably responsible for the numerous geographic variants and species. The present-day overlap in distributions in Argynnis and Fabriciana sister taxa may be explained by allopatric speciation in different mountainous regions, followed by overlap via range expansion.

Based on our results, the origins and radiation of Speyeria have a clear biogeographical signal. Within Speyeria the first species to diverge (subgenus Mesoaclidalia) are found in Asia, with North American species monophyletic and with origins dated approximately 6 Ma. Thus, the Speyeria radiation began in Asia and a single lineage moved into North America between approximately 6 and 5 Ma, giving rise to the radiation there (Fig. 3). This timing coincides with the first opening of the Bering Strait, which occurred between 4.8 and 7.3–7.4 Ma (Marincovich & Gladenkov, 1999). This coincident timing indicates that ancestors of North American Speyeria crossed at a time when the land bridge may have been disappearing, and that formation of the strait reduced gene flow relatively soon after their arrival.

In contrast to the single dispersal event of Speyeria, other butterfly genera exhibit many independent dispersal events from the Palearctic to the Nearctic during this same general time frame (Simonsen et al., 2010; Vila et al., 2011). Analysis of Boloria, another group of argynnine butterflies, indicates at least nine independent dispersal events occurred between the Palearctic and Nearctic, and that the B. bellona group crossed into North America from Asia at approximately the same time as Speyeria (Simonsen et al., 2010). In addition, Polyommatus butterflies show repeated invasions across the Bering land bridge into North America from 11 to 1 Ma (Vila et al., 2011).

Furthermore, given that the first diverging lineage leading to the North American species, S. idalia, is distributed in eastern North America, it may be that the North American Speyeria radiation began on the eastern side of the continent. The fact that another eastern species, S. diana, is the second lineage to diverge in our Bayesian analysis corroborates this hypothesis, although S. diana’s position fluctuated with analytical method. This east to west diversification is similar to the pattern observed
Argynnis and Speyeria diversification

Fig. 3. Divergence time estimates for Argyunnis and Speyeria butterflies. Estimates of mean divergence times are indicated for select nodes. Nodes with ages all have posterior probabilities of 1.0, except for the sister relationship of Argyunnis clara and Speyeria (posterior probability = 0.86). The scale below the figure and error bars on each node indicating 95% highest posterior density are in millions of years. Specific error values for key nodes are indicated in the text. Ma, millions of years ago; ‘M’ denotes subgenus Mesoacidalia. [Colour figure can be viewed at wileyonlinelibrary.com].

for butterflies in the Boloria bellona group as well (Simonsen et al., 2010). Range disjunctions between eastern Asia and eastern North America, in which eastern North American species are more similar to Asian species than to western North American species, are also well known for many plants (Xiang et al., 1998, 2000, and references therein). Analyses of these disjunctions indicate that the disjunct species are not necessarily close relatives, but rather parts of additional diversification events (Wen, 1999), similar to what we have described for Speyeria. Thus, this widespread pattern lends further, albeit indirect, support to the hypothesis that North American Speyeria diversified from east to west. It should also be noted that although Speyeria origins in North America predate the Pleistocene, the cyclical glacialation of that epoch and associated colonization/isolation of boreal and mountain habitats are almost certainly involved with speciation and the many subspecies in North America.

It is common for food plants to be implicated in the co-evolutionary diversification of herbivorous insects (Janz et al., 2006) but the details of the relationships are not always clear (Futuyma & Agrawal, 2009). For example, species of plants and insects may co-evolve by reciprocal changes with short time lags (Zangerl & Berenbaum, 2003, 2005; Nielsen & de Jong, 2005; Toju & Sota, 2006). Alternatively, insects may radiate onto existing plant diversity which itself diversified by escaping from herbivory as suggested by Ehrlich & Raven (1964), or for other reasons unrelated to pressure from herbivores. Argynnis s.l. appear to be a clear example of insects that radiated onto existing plant diversity. Species in these three genera all feed exclusively on Viola as larvae (Simonsen, 2006a; Dunford, 2009). Only Speyeria aglaia is known to feed on a non-Viola host in addition to Viola (Fric et al., 2005).

Centres of diversity in Argyunnis, Fabriciana (Central/East Asia)
and _Speyeria_ (North America) are clearly correlated with centres of diversity in _Viola_ sections _Chamaemelanium_ (western North America), _Plagiostigma_ (East Asia) and _Melanium/Viola_ (western Eurasia) (Marcussen _et al._, 2012), but the timing refutes co-speciation. Research on _Viola_ suggests that the genus originated 31 Ma in South America (Marcussen _et al._, 2015) and arrived in North America as early as 18 Ma (Marcussen _et al._, 2012, and references therein), with the oldest Eurasian fossils dated at ~19.5 Ma (Marcussen _et al._, 2012). Given these dates and biogeographic scenario, the diversification of _Viola_ clearly preceded the North American _Speyeria_ radiation, indicating that once _Speyeria_ arrived in North America they probably encountered extant _Viola_ diversity, which facilitated diversification. The arrival of _Viola_ in Asia also probably preceded the Europe/Asian _Argynnis_ and _Fabriciana_ radiations. _Argynnis_ originated approximately 9.1 Ma and radiated 7.5 Ma, with _Fabriciana_ diverging from _Speyeria_ 7.6 Ma and radiating 4.5 Ma (see Results for 95% HPD intervals). Given the relatively recent diversification of both _Argynnis_ and _Fabriciana_, it is not likely that they co-speciated with _Viola_, but rather diversified onto extant _Viola_ food plant species across Asia and Europe.

**Conclusions**

In conclusion we have demonstrated that there is strong evidence for three natural lineages in _Argynnis_ s.l. that should be recognized as genera: _Argynnis_, _Fabriciana_ and _Speyeria_. The tree topologies strongly suggest that this group of butterflies has undergone repeated, rapid radiations. Evidence from the dating analyses for this radiation and its _Viola_ host plants suggests that _Argynnis_ and _Fabriciana_ probably radiated onto existing _Viola_ diversity, but depending on dating error they may have co-evolved with _Viola_ as it arrived in Asia from North America. The monophyly of North American _Speyeria_ species and divergence dating strongly indicate that _Speyeria_ arrived into North America via the Bering land bridge between 6 and 5 Ma and radiated onto already diversified _Viola_ food plants.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12236

**Figure S1.** Results of maximum likelihood analysis without partitions.

**Figure S2.** Results of parsimony analysis.

**Figure S3.** Results of MRBAYES analysis of the dataset partitioned by gene.

**Table S1.** Information and GenBank accession numbers for individual samples included in this study.

**File S1.** Results of BEAST analysis containing mean ages and 95% HPD for all divergence points.

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