Is Sexual Conflict a Driver of Speciation? A Case Study With a Tribe of Brush-footed Butterflies

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Abstract.—Understanding the evolutionary mechanisms governing the uneven distribution of species richness across the tree of life is a great challenge in biology. Scientists have long argued that sexual conflict is a key driver of speciation. This hypothesis, however, has been highly debated in light of empirical evidence. Recent advances in the study of macroevolution make it possible to test this hypothesis with more data and increased accuracy. In the present study, we use phylogenomics combined with four different diversification rate analytical approaches to test whether sexual conflict is a driver of speciation in brush-footed butterflies of the tribe Acraeini. The presence of a sphragis, an external mating plug found in most species among Acraeini, was used as a proxy for sexual conflict. Diversification analyses statistically rejected the hypothesis that sexual conflict is associated with shifts in diversification rates in Acraeini. This result contrasts with earlier studies and suggests that the underlying mechanisms driving diversification are more complex than previously considered. In the case of butterflies, natural history traits acting in concert with abiotic factors possibly play a stronger role in triggering speciation than does sexual conflict. [Acraeini butterflies; arms race; exon capture phylogenomics; Lepidoptera macroevolution; sexual selection; sphragis.]

Species diversity varies drastically across the tree of life, and this disparity has long fascinated biologists (Farrell 1998; Mayhew 2007). For instance, insects represent 73% of all species of animals on Earth, with nearly a million species described thus far (Chapman 2009), while their sister group Diplura (Misof et al. 2014), is only represented by 800 living species (Chapman 2009). This disparity in species richness among groups is thought to result from uneven diversification rates (i.e., the balance between speciation and extinction) (Condamine et al. 2012; Wiens 2017). The underlying mechanisms responsible for differences in diversification rates are often complex to isolate, although many studies have analyzed whether or not different traits (Wiens 2017), including those pertaining to either morphology, ecology, or distribution (Fordyce 2010; Condamine et al. 2012; Wagner et al. 2012; Hardy and Otto 2014; Kozak and Wiens 2016), contribute to diversification rate differences either among or within clades.

Darwin (1871) considered sexual selection to be an important driving force of speciation and, consequently, diversity. Variation among populations in female preference and on the male trait that is being selected for, would drive reproductive isolation and thereby speciation. In accordance with these views, several authors have suggested that sexual selection could be associated with higher diversity (Barraclough et al. 1995; Owens et al. 1999; Katzourakis et al. 2001; Stuart-Fox and Owens 2003; Janicke et al. 2018). Within the array of sexual selection processes, sexual conflict is of particular interest.

Sexual conflict results from unbalanced reproductive interests of the sexes, where male reproductive success is usually constrained by the number of receptive mates, whilst female reproductive success is limited by egg production (Bateman 1948; Parker 1979; Thornhill and Alcock 1983; Holland and Rice 1998). This inequality can generate sexually antagonistic coevolution, where traits that increase reproductive success may evolve in one sex, while concurrently resulting in a direct decrease of reproductive success in the opposite sex. Counter adaptations may evolve in the "losing" sex in order to overcome the detrimental strategies of the sex that was previously "winning". Sexual conflict could generate alternative evolutionary trajectories due to dissimilarities in the interactions between males and females either among or within populations, working as an "engine for speciation" by triggering rapid evolutionary change and reproductive incompatibility (for a diagram, see Supplementary Fig. S1 available on Dryad http://dx.doi.org/10.5061/dryad.kwh70rz18) (Rice and Hostert 1993; Pierre 1985; Parker and Partridge 1998; Gavrilets 2000, 2014; Gavrilets and Waxman 2002; Martin and Hosken 2014).

Different aspects of sexual conflict, such as the difference in mating frequency, have been suggested to be linked with variation in diversity. Arnqvist et al. (2000) analyzed closely related genera within five insect orders and found that polyandry is associated with speciation rates four times higher than in related monandrous groups. Studies on birds have also shown

FIGURE 1. Female of *Acraea issoria formosana*. Sphragis is attached to the female genitalia on the venter of the terminal abdominal segments. Source: Hsing-Han Li in "The etymology of scientific names of Formosa butterflies (in Chinese)", 2018. Copyrights owned by Hsing-Han Li. Used with permission.

that mating systems play a role in diversification and that promiscuity fuels diversity (Mitra et al. 1996; Iglesias et al. 2019). However, these conclusions using insects and birds contrast with results from other studies rejecting the hypothesis that sexual selection is related to higher diversity (Gage et al. 2002; Huang and Rabosky 2014).

In species with sexual conflict, multiple mating events can be beneficial for females, but disadvantageous for males in species with last male precedence (Drummond 1984). Thus, mating conflict can lead to males evolving strategies that increase certainty of paternity (Rice 1996) by reducing or preventing female remating and sperm competition (Parker 1970). One such strategy is the mating plug, which occurs in several animal groups such as spiders, nematodes, snakes, primates, and butterflies (Devine 1975; Dixson and Anderson 2002; Uhl et al. 2010; Matsumoto et al. 2018; Canales-Lazcano et al. 2019). Some butterflies have externalized plugs called sphragis, that are species-specific and morphologically complex (Pierre 1985) (Fig. 1). The sphragis has been detected in at least 280 species of Nymphalidae and Papilionidae butterflies (Carvalho et al. 2017, 2019). Much like mating plugs of other animals, males produce and place the sphragis after sperm transfer to the female, physically blocking her copulatory opening (Matsumoto 1987; Orr 1988). The evolution of the sphragis is thought to be a result of the conflict between males and females over female mating frequency, triggering the coevolution of plugging behavior (male) and anti-plugging (female) adaptations (Orr 1988; Carvalho et al. 2019). Other studies have linked genitalia morphologies to sexual conflict and sexually antagonistic coevolution (Arnqvist and Rowe 2002; Chapman et al. 2003), but research on how intersexual conflict in sphragis-bearing species relates to shifts in diversification rates is still needed.

Most sphragis-bearing species belong to the primarily tropical butterfly tribe Acraeini (Nymphalidae, Heliconiinae) (Carvalho et al. 2017). This diverse tribe is comprised of 307 species (Desmier de Chenon et al. 2002; Lamas 2004; Küppers 2006; Müller and Beheregaray

2010; Pierre and Bernaud 2014; Willmott et al. 2017; Freitas et al. 2018) distributed in the genera *Acraea* (291 species) and *Cethosia* (16 species), that are typically unpalatable and aposematically colored, serving as models of many diurnal Lepidoptera mimics (Brown 1992; Larsen 2005). This is an ideal system to test whether sexual conflict is correlated with diversification processes because Acraeini is the most speciose sphragis-bearing lineage in butterflies, and the sphragis is clearly the by-product of the different reproductive interests of males and females (Orr 1988). In this study, we test the hypothesis that sexual conflict, identified by the presence or absence of the sphragis, impacts diversification rates and species diversity. To do so, we reconstruct a robust and comprehensive dated phylogeny of Acraeini and use macroevolutionary analyses to investigate diversification dynamics and trait-dependent diversification.

MATERIALS AND METHODS

Taxon Sampling, Library Preparation, and Hybrid Enrichment Sequencing

The data set was composed of 160 Acraeini species (52% of the described diversity of the tribe) and 48 outgroups for a total of 208 species. The included species represented two valid Acraeini genera, *Acraea* and *Cethosia*, and all *Acraea* species-groups (*sensu* Pierre and Bernaud (2014)). Specimens for sequencing were gathered from natural history collections and were either dry- or ethanol-preserved. One hundred specimens were sequenced *de novo* for this study, including 97 Acraeini (Supplementary Table S1 available on Dryad). Genomic DNA was extracted using the OmniPrep™DNA extraction kit (G-Biosciences) following Espeland et al. (2018) and Kawahara et al. (2018). DNA extracts underwent library preparation for Anchored Hybrid Enrichment using the BUTTERFLY2.0 probe set (Kawahara et al. 2018) at RAPiD Genomics (Gainesville, FL, United States). The BUTTERFLY2.0 probe set captures up to 13 loci including the barcoding mitochondrial gene cytochrome c oxidase subunit 1 (COI; Supplementary Table S1 available on Dryad). Enriched libraries were multiplexed for paired-end sequencing on an Illumina HiSeq 3000 with 100 cycles.

To broaden taxon sampling, we included 108 species (63 ingroups and 45 outgroups) already sequenced for any of the 13 BUTTERFLY2.0 loci and available on GenBank or from Espeland et al. (2018) (Supplementary Table S1 available on Dryad).

Phylogenetic Analysis, Divergence Time Estimation, and Ancestral State Reconstruction

Each of the 13 single locus data sets were concatenated using FASconCAT-G 1.0.4 (Kück and Meusemann 2010). Data were partitioned *a priori* by locus and codon positions, and the best partitioning scheme and substitution models were selected using the "greedy" algorithm in PartitionFinder 2.1.1 (Lanfear et al. 2017)

based on the Bayesian Information Criterion. We ran maximum likelihood (ML) phylogenetic analyses in IQ-TREE 1.6.9 (Nguyen et al. 2015) with 100 independent tree searches. Nodal support was assessed using 1000 SH-aLRT (Guindon et al. 2010) and 1000 ultrafast bootstrap (UFBoot) replicates (Minh et al. 2013; Hoang et al. 2018). In order to overcome model violations inherent to UFBoot calculations (Hoang et al. 2018), we used the "-bnni" command. Divergence times and ancestral state reconstruction were estimated using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) v.1.10.1 (Drummond et al. 2012), with four secondary calibration points derived from Espeland et al. (2018) (additional information can be found in Supplementary File S1 available on Dryad).

Diversification Analyses

To examine shifts in diversification rates between clades where sphragis is largely present and another where it is entirely absent, we used Bayesian Analysis of Macroevolutionary Mixtures (BAMM) v.1.10.4 (Rabosky 2014). We compare *Acraea* to *Cethosia*, and more broadly, Acraeini to its sister clade Heliconiini. Given recent criticisms of BAMM and its inability to identify diversification rate shifts in extinct lineages, we also performed a Lineage-Specific Birth-Death-Shift (LSBDS) analysis in RevBayes (Höhna et al. 2019) on Acraeini + Heliconiini.

We complemented our set of macroevolutionary analyses with the R package RPANDA (Phylogenetic Analyses of Diversification (Morlon et al. 2016)), to directly test different diversification models on clades of interest. To study the effect of sexual conflict (with sphragis as a proxy) on diversity, we used HiSSE (Hidden State Speciation and Extinction) and a BiSSE-like (Binary State Speciation and Extinction) implementation of HiSSE using the R package hisse (Beaulieu and O'Meara 2016). Additional information on methodology and data utilized in this study can be found in Supplementary Files S1–S4 (available on Dryad).

RESULTS AND DISCUSSION

Acraeini Phylogeny

Our phylogenetic reconstruction of Acraeini is the most robust and comprehensive to date. The ML tree strongly supported backbone relationships in the tribe (SH-aLRT > 85 and UFBoot > 95) (Supplementary Fig. S2 available on Dryad). As in other studies (Espeland et al. 2018; Chazot et al. 2019), the sister group relationship of Acraeini and Heliconiini is robustly supported, and our analysis suggests that *Pardopsis*, a genus previously placed in Acraeini (Penz and Peggie 2003) belongs in the Argynnini, corroborating results of Rice and Hostert (1993).

Within Acraeini, six well-supported clades were recovered, one supporting the monophyly of *Cethosia*

and five major clades within *Acraea*, which represent previously valid genera or subgenera of *Acraea* (*sensu lato*): *Acraea* (*sensu stricto*), *Actinote*, *Bematistes*, *Rubraea/Stephenia*, and *Telchinia* (Supplementary Fig. S2 available on Dryad). Within each of these clades, however, nodal support varied significantly, and support was generally higher in groups for which more loci were present.

Divergence Time Estimation and Ancestral State Reconstruction

Our dating analysis recovered the split between Old World and Tropical American *Acraea* (*Actinote*) to be in the Miocene *ca.* 23.3 Ma (95% credibility interval CI = 19.4-26.8 Ma, Supplementary Fig. S3 available on Dryad). Crown ages for the five main clades of *Acraea* were dated to the Oligocene or Miocene (24-17 Ma; Fig. 2, Supplementary Fig. S3 available on Dryad) and the origin of the sphragis in Acraeini appears to be due to a single event, sometime in the Eocene or Oligocene, *ca.* 32 Ma (95% CI = 28.1-35.3 Ma; Fig. 2), with the presence of the sphragis being the plesiomorphic condition in *Acraea.* The trait was lost six times in this genus and later regained three times in *A. oncaea*, *A. pharsalus*, and *A. quirinalis*.

The Impact of Sphragis on Diversification of Acraeini Butterflies

Recently, the efficacy of using extant timetrees to infer diversification rates has been called into question (Louca and Pennell 2020). However, Morlon et al. (2020) show that these methodologies are still useful for hypothesis testing of diversification dynamics, especially when considering biological data, as we have done here. Furthermore, we found consistent results; all four methods of diversification analyses reject a positive relationship between diversification and sexual conflict in Acraeini. Changes in the sphragis state do not promote shifts in diversification rates, with the best BAMM model presenting zero shifts in Acraeini (when only including *Cethosia* and *Acraea* posterior probability (PP) = 0.91, Fig. 2; and when including Acraeini and Heliconiini PP = 0.9). Previous studies have suggested that a lack of correlation between sexual selection and diversity would be due to extinction rates being as high as speciation rates, which would keep the net diversification rate constant (Morrow et al. 2003). Our data do not support the hypothesis that this lack of correlation is associated with extinction, since variation in both speciation and extinction rates were negligible throughout the phylogeny (Supplementary Figs. S4a and b available on Dryad).

The LSBDS analysis was the only analysis performed that suggested any variation in speciation rates across our phylogeny (Supplementary Fig. S5 available on Dryad). A key difference between this analysis and the
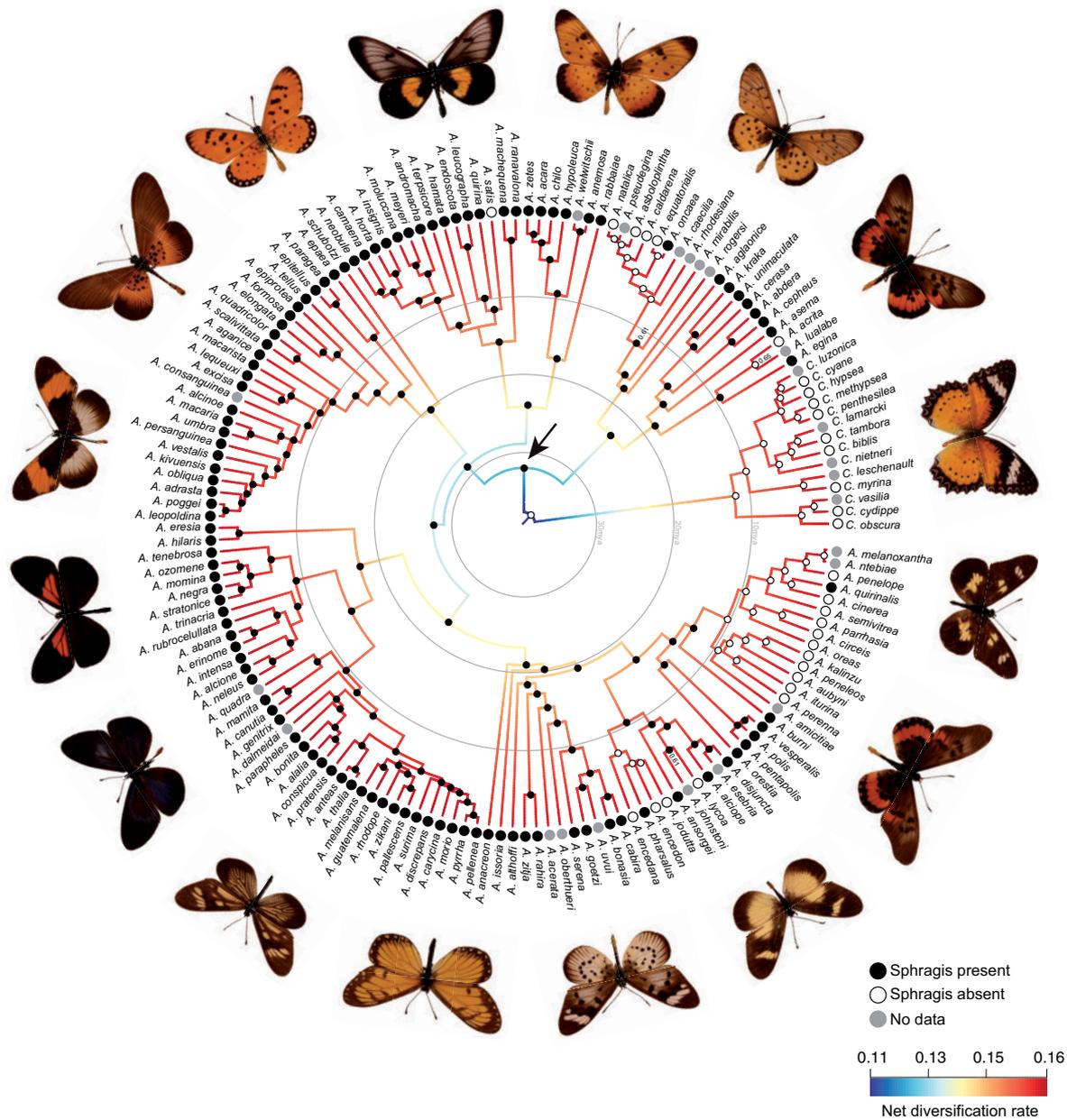
FIGURE 2. Bayesian diversification rate analysis (net diversification shown) from Bayesian Analysis of Macroevolutionary Mixtures (BAMM) with Bayesian ancestral state reconstruction of the Acraeini (*Cethosia* and *Acraea*) sphragis conducted in Bayesian Evolutionary Analysis Sampling Trees (BEAST). Topology was fixed to the best Maximum Likelihood tree of 100 independent IQ-TREE runs. Node fill refers to the sphragis state (presence or absence) that has the highest posterior probability (PP) for that node. All sphragis states are PP > 0.75 unless specified. Black arrow signifies the origin of the sphragis in the tribe.

one performed with BAMM is that LSBDS takes into consideration diversification rates in extinct lineages. Even though we detected shifts in speciation rates across the Acraeini tree, they do not seem to be linked to the presence or absence of the sphragis. For example, the highest speciation rates are found in clades that both have (American members of subgenus *Actinote*) and do not have (*Cethosia*) the sphragis, and the opposite is also found in these results. We believe that there are other

traits of natural history (perhaps those related to habitat or distribution) that could better explain this pattern. In the above example where we observe higher speciation rates with LSBDS, there are well-defined geographic patterns that may be related with the observed speciation regimes. American *Actinote* are the only Acraeini found in the New World and *Cethosia* are exclusively Asian, with the vast majority of other Acraeini being African.

TABLE 1. Results of the RPANDA analysis of diversification dynamics for the clades *Acraea* and *Cethosia*

Models	Parameters				Results		
	λ starting	μ starting	λ estimated	μ estimated	AICc	ΔAICc	AIC weight
Acraea							
Birth constant	0.10	NA	0.17	NA	887.54	0	0.37
Birth exponential	0.01, 0.17	NA	0.18, −0.0089	NA	889.06	1.53	0.17
Birth linear	0.18, −0.0089	NA	0.18, −0.0016	NA	889.04	1.50	0.17
Birth exponential, death constant	0.18, −0.0089	0.01	0.18, −0.0089	−1.90E-07	891.15	3.61	0.06
Birth constant, death constant	0.18	−1.90E-07	0.18	0.0057	889.58	2.04	0.13
Birth constant, death exponential	0.18	.0057, 0.01	0.18	−0.0014, 0.16	890.93	3.39	0.07
Birth exponential, death exponential	0.18, −0.0089	−0.0014, 0.16	0.18, −0.0041	9.0E-07, 0.43	892.86	5.32	0.03
Cethosia							
Birth constant	0.10	NA	0.20	NA	67.99	0	0.54
Birth exponential	0.01, 0.20	NA	0.22, −0.025	NA	70.67	2.68	0.14
Birth linear	0.22, −0.025	NA	0.23, −0.0073	NA	70.63	2.64	0.14
Birth exponential, death constant	0.22, −0.025	0.01	0.22, −0.025	−7.20E-08	73.98	5.99	0.027
Birth constant, death constant	0.20	−7.20E-08	0.20	−1.80E-07	70.75	2.76	0.14
Birth constant, death exponential	0.20	−1.80E-07,0	0.18	−0.0014, 0.16	890.93	822.94	1.08E-179
Birth exponential, death exponential	0.22, −0.025	−0.0014, 0.16	0.12, 0.53	−0.15, 0.51	75.97	7.98	0.01

TABLE 2. Results of the HiSSE analyses of diversification dynamics in Acraeini

Model	Free parameters for both Turnover rate (τ) and Extinction fraction (ε)	Transition rate matrix (dual transitions disallowed)	Hidden states	logL	AIC	ΔAIC
1	0=1	BiSSE one rate	0	−529.6707	1065.341	10.281
2	0=1	BiSSE two rates	0	−530.6769	1069.354	14.294
3	0≠1	BiSSE one rate	0	−524.16	1058.32	3.26
4	0≠1	BiSSE two rates	0	−524.9271	1061.854	6.794
5	0A=1A, 0B=1B	All rates free	2	−520.4455	1064.891	9.831
6	0A=1A, 0B=1B	One rate for sphragis, two rates for hidden	2	−522.0576	1058.115	3.055
7	0A, 0B, 0C, 0D, 1A, 1B, 1C, 1D all free	All rates equal	4	−520.1294	1058.259	3.199
8	**0A, 0B, 0C, 0D, 1A, 1B, 1C, 1D all free**	**One rate for sphragis, two rates for hidden**	**4**	**−516.9773**	**1055.955**	**0.895**
9	0A, 1A, 0B, 1B all free	All rates free	2	−517.1396	1066.279	11.219
10	0A, 1A, 0B, 1B all free	All rates equal	2	−519.32	1056.64	1.58
11	0A, 1A, 0B, 1B all free	One rate for sphragis, all hidden rates free	2	−517.5921	1061.184	6.124
12	0A, 1A, 0B, 1B all free	One rate for sphragis, two rates for hidden	2	−518.7026	1059.405	4.345
13	0A=0B, 1A and 1B free	All rates equal	2	−522.1004	1058.201	3.141
14	**0A=0B, 1A and 1B free**	**One rate for sphragis, two rates for hidden**	**2**	**−518.5302**	**1055.06**	**0**
15	0A=0B, 1A and 1B free	One rate for sphragis, all hidden rates free	2	−518.9541	1059.908	4.848
16	0A=0B, 1A and 1B free	All rates free	2	−518.6792	1065.358	10.298
17	0A and 0B free, 1A=1B	One rate for sphragis, two rates for hidden	2	−525.7151	1069.43	14.37
18	0A and 0B free, 1A=1B	All rates equal	2	−526.3087	1066.617	11.557
19	0A and 0B free, 1A=1B	All rates equal	2	−524.9504	1071.901	16.841
20	0A and 0B free, 1A=1B	All rates free	2	−521.0043	1070.009	14.949

Notes: Twenty models tested in the HiSSE and their parameters, transition rate matrices, number of hidden states, log-likelihood, AIC scores, and ΔAIC. Free parameters for Turnover rate (τ) and Extinction fraction (ε) are given for observed sphragis state (0 = absent, 1 = present) and hidden state (A = hidden state absent or B = hidden state present; additional letters C and D are used for four hidden state models). Parameter designations are always equal for both τ and ε for any single model. The best two models according to AIC score are in bold.

Our results do not support significant differences in diversification regimes between *Acraea* and *Cethosia*. The RPANDA estimations of λ (speciation rate) are very similar for both sphragis-bearing *Acraea* and sphragis-lacking *Cethosia* when considering the best fit model for each clade ($\lambda_{Acraea} = 0.17, \lambda_{Cethosia} = 0.20$) (Table 1). The best fit model for *Acraea* is a Yule constant birth (no extinction) model (AICc = 887.5), and in *Cethosia* the best model was also constant birth (AICc = 68.0).

Moreover, sphragis-bearing in *Acraea* does not appear to be significantly coupled with diversification dynamics. No diversification rate shifts specifically associated with the presence or absence of the sphragis in Acraeini were detected with the HiSSE analyses. The two best models are models 8 (AIC = 1055.96) and 14 (AIC = 1055.06), with a maximum ΔAIC=1.58 from the third best model (Table 2, Supplementary File S1 available on Dryad). There was no support for the BiSSE-like model which assumes that all diversification rate differences result from an observed character (presence/absence of the sphragis) and not a hidden state (model 4, AIC = 1061.85; ΔAIC = 6.79). Although no model is significantly preferred over the others in our HiSSE analyses, the two models with the best fit reject the hypothesis of state-dependent diversification solely linked to the sphragis in Acraeini, with a potential impact of hidden characters in the diversification dynamics of the group. Likewise, the state-dependent BiSSE-like model is rejected.

Sexual conflict has long been implied to be a driver of speciation (Rice and Hostert 1993; Rice 1996; Parker and Partridge 1998; Arnqvist et al. 2000; Gavrilets 2000, 2014; Gavrilets and Waxman 2002; Martin and Hosken 2014), considering that the association between sexual selection and speciation would cause rapid evolution of sexually-selected traits and result in reproductive isolation. Different methods have been used to test this hypothesis, usually through the use of pairwise comparisons of species richness between sister clades or closely related groups with variation in sexual traits for several vertebrate and invertebrate groups (Barraclough et al. 1995; Mitra et al. 1996; Arnqvist et al. 2000; Panhuis et al. 2001). Butterflies, among other insect lineages, were used in pairwise species richness comparisons of closely related groups presenting disparities in the presence or absence of sexual conflict (Arnqvist et al. 2000). The genera *Acraea* and *Cethosia* represent such a model, and a first glance at species richness patterns in these two genera would lend support to a positive correlation between diversity and sexual conflict. However, recent studies have challenged the efficacy of these methods for drawing such far-reaching conclusions (Panhuis et al. 2001).

The decoupling of diversification rates and proxies of sexual selection in Acraeini may appear surprising, yet this finding is in line with the results of a few additional recent studies. For instance, Huang and Rabosky (2014) analyzed diversification rate shifts with respect to ultraviolet reflectance (as a measurement of sexual selection) in birds, and found no link of this trait with speciation rates, confirming that the establishment of such complex behavioral and evolutionary processes may in fact be uncorrelated or indirectly related to diversification dynamics.

CONCLUSION

Our macroevolutionary analyses reject a significant correlation between the presence/absence of the sphragis and diversification dynamics, suggesting that speciation is not significantly influenced by sexual conflict. Our results suggest that the mechanisms driving diversity in organisms that experience intense sexual conflict are possibly much more complex than previously proposed. This new corpus of evidence represents a shift in our understanding of sexual selection evolution and paves the way for a more in-depth and systematic reappraisal of underlying patterns and processes across the tree of life. In order to understand the evolution of mating systems and of diversification dynamics, the impact of alternative behavioral, ecological, or morphological traits should be explored using state-dependent models. We suggest that the traditional view of sexual conflict as a driver of diversification should be revisited with methodologies such as the one utilized here to shed a new light on macroevolutionary trends in groups with a complex suite of biological features.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.kwh70rz18.

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