

Intersexual ‘Arms Race’ and the Evolution of the Sphragis in *Pteronymia* Butterflies

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Abstract

Sexual reproduction is often associated with intra- and intersexual conflict, especially in species where females mate multiple times. A strategy that has evolved in males to ensure offspring paternity is the ability to produce a complex, external mating plug called a sphragis. The sphragis has been found in 273 butterfly species; however, little is known about the sphragides of the butterflies in the nymphalid genus *Pteronymia*. In this study, we describe the sphragides of all sphragis-bearing species in *Pteronymia*, including the newly discovered sphragides of *P. alissa* (Hewitson), *P. andreas* (Weeks), *P. ozia* (Hewitson), and *P. zerlina* (Hewitson). Three additional species, *P. fulvimargo* Butler & Druce, *P. oneida* (Hewitson), and *P. ticida* (Hewitson), are found to bear an irregular sphragis-like structure. We use molecular and morphological data from a recent study to construct a phylogeny of species in the genus and examine the number of independent origins of the sphragis. Our ancestral state reconstruction using Bayesian inference suggests that the sphragis evolved three times in *Pteronymia*, whereas parsimony character optimization performed on a maximum likelihood tree suggests only one origin of this structure. Our data on ancestral state patterns, frequency of incomplete sphragides, and morphology of female external genitalia suggest that sphragis-bearing *Pteronymia* may be in active intersexual conflict, where females develop strategies to prevent male plugging.

Key words: coevolution, Lepidoptera, monandry, phylogeny, reproduction

Sexual reproduction is a dynamic and intimate interaction between two or more organisms, in which individuals often have partially or completely opposing reproductive interests, triggering intra- and intersexual conflict (Parker 1970). Male–female conflict is largely a consequence of differential investment in the offspring, known as anisogamy (Scudo 1967, Parker 1979, Chapman et al. 2003). In the absence of male parental care, male investment in their offspring is often limited to providing sperm. Female investment in each individual offspring is generally more substantial (Trivers 1972), not only because of the size of their gametes, but also because the female is usually responsible for embryo nourishment and, frequently, parental care (Wong et al. 2013). In groups where there is no obvious parental care, the female often must still find an appropriate site to deposit her eggs.

In several animal groups, males increase their fitness by mating as many times as possible with as many females as possible (Bateman 1948, Arnqvist and Nilsson 2000). Female fitness, however, is not necessarily maximized by the absolute number of matings, but

mostly by the nutritional and genetic quality of the ejaculate received from males.

Variance in mating success is typically much higher among males than females. This imbalance can trigger intense male–male competition for access to receptive females (Bateman 1948). Female insects, which can store sperm for long periods, often mate more than once during their lifetime due either to a need for more sperm, better or different genes, or nutritional substances transferred by the male to the female during mating (Ehrlich and Ehrlich 1978, Walker 1980, Thornhill and Alcock 1983, Drummond 1984, Gwynne 1984). In several insects, the sperm of the last male to mate with a female will be used to fertilize the eggs before the sperm obtained from previous matings. Therefore, the final male’s sperm will fertilize the majority, if not all, of the ova (Labine 1966, Boggs and Watt 1981).

Sperm competition is a form of male–male competition, but since the sperm of two or more males compete to fertilize the ova, it can also have direct implications in intrasexual interactions (Parker, 1970). Males have evolved an array of strategies to establish final

precedence of their sperm by enforcing female monogamy (Gillies 1956, Gilbert 1976, Ehrlich and Ehrlich 1978, Sillén-Tullberg 1981, Thornhill and Alcock 1983, Svard and Wiklund 1988, Dickinson and Rutowski 1989, Poole 1989, Mclain 1989, Matsumoto and Suzuki 1995, Orr 1995, Orr 1999, Sauter et al. 2001, Dixson and Anderson 2002, Schulz et al. 2008, Timmermeyer et al. 2010, Uhl et al. 2010, Carvalho et al. 2017, Malouines 2017), and any male that prevents a female from remating will be in direct conflict with her if the female would otherwise benefit from remating. This intersexual conflict can trigger sexually antagonistic coevolution (Rice 1996), where adaptations evolve in one sex in order to increase its fitness, which will result in lower fitness for the opposite sex. Subsequently, such antagonism will be followed by adaptations to overcome loss of fitness. Examples of traits evolved due to sexually antagonistic coevolution include male grasping and female anti-grasping strategies in water striders (Hemiptera: Gerridae) (Arnqvist and Rowe 1995) and in diving beetles (Coleoptera: Dytiscidae) (Bergsten et al. 2001), and toxic substances in the seminal fluids of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Rice 1996). In the case of female mating frequency, antagonistic coevolution will be in place when males try to suppress frequency of female matings to ensure their paternity, while females attempt to overcome male attempts to enforce monogamy.

One male strategy that enforces monogamy onto a female is the mating plug, which has arisen in several unrelated vertebrate and invertebrate groups (Gillies 1956, Ehrlich and Ehrlich 1978, Dickinson and Rutowski 1989, Matsumoto and Suzuki 1995, Dixson and Anderson 2002, Timmermeyer et al. 2010, Uhl et al. 2010). Mating plugs are often temporary, mostly used as a form of delaying female remating. In most insects, females have only one reproductive orifice that serves both to receive sperm and release eggs. Female butterflies, however, have a ditrysian system of reproductive ducts and openings, where the copulatory orifice and the egg laying opening are widely separated (Stitz 1902, Kristensen 2003). The ditrysian reproductive system therefore allows for the existence of permanent mating plugs, which do not impede oviposition. In some cases, the permanent mating plug is large, and is wholly or partly external, in which case it is called a sphragis.

Sphragides and other sphragis-like structures (protosphragides and vestigial sphragides) are morphologically diverse in shape and size (Orr 1988, Matsumoto and Suzuki 1995, Carvalho et al. 2017), and have been found in 273 butterfly species (Carvalho et al. 2017). Sphragis-bearing species (SBS) share morphological and behavioral similarities, even in phylogenetically unrelated taxa. In SBS, the sexual opening (ostium bursae) of the female genitalia is regularly externalized (Orr 1988, Matsumoto et al. 2018). By contrast, in the majority of Ditrysia, which do not bear a sphragis, the ostium bursae is usually located inside a pouch-like sterigma. The sphragis itself is species-specific, but males may produce incomplete sphragides when copulations occur successively, thus depleting the male's reserves of material necessary to produce the sphragis (Orr 1988, 1995, 2002). The sphragis (and sphragis-like structures) appears to have evolved independently at least twelve times in Papilionidae and Nymphalidae (Carvalho et al. 2017), including in the widely distributed and diverse genera *Parnassius* Latreille (Lepidoptera: Papilionidae) and *Acraea* (Lepidoptera: Nymphalidae) Fabricius. However, more clade-specific phylogenetic studies focused on SBS are necessary to provide a clearer picture on how many times this structure evolved.

The Neotropical genus *Pteronymia* Butler & Druce (Lepidoptera: Nymphalidae) is the most speciose genus of the Ithomiini (De-Silva et al. 2017), with 48 described species plus some that are yet unnamed (De-Silva et al. 2017). This genus is thought to have originated and

diversified in the Northern Andes and later colonized other areas in the Neotropics (De-Silva et al. 2017). Many *Pteronymia* species belong to Müllerian mimicry rings (Poole 1970, Beccaloni 1997) and are thought to be unpalatable (Beccaloni et al. 2008). The presence of the sphragis in *Pteronymia* was first noted by De-Silva et al. (2017), and later by Carvalho et al. (2017). Because these structures have only recently been documented, most of their morphological variability are yet to be described. Here, we 1) identify *Pteronymia* species that bear a sphragis after copulation by examining mated females of the available species in the genus, 2) describe and characterize the female external genital morphology of the SBS in *Pteronymia*, 3) investigate the number of origins of the sphragis in *Pteronymia*, and 4) discuss the processes of sexually antagonistic coevolution involved in the evolution of the sphragis in this genus and others.

Material and Methods

Taxon Sampling and Morphological Description

Of the 48 species of *Pteronymia* described (De-Silva et al. 2017), 41 were inspected for the presence of a sphragis (see [Supp. Table S1](#) for a list of these species). We examined 629 dried, pinned, female museum specimens in the McGuire Center for Lepidoptera and Biodiversity (MGCL), Florida Museum of Natural History, Gainesville, FL; and 590 from the Smithsonian National Museum of Natural History (USNM), Washington, DC. These numbers include only specimens that were fully intact—specimens that lacked an abdomen or had damaged genitalia were not examined. Each museum specimen was studied using a dissecting microscope.

For each SBS, we took photographs of at least one specimen with and another specimen without a sphragis. These images were taken with a Canon EOS 6D camera with an infinity Model K2 Long-Distance Microscope with a CF-4 objective. Photographs were taken of lateral and ventral views of the posterior segments of the abdomen. For each image, 20 to 40 image layers were taken across a series of close-spaced focal planes using the Automated Macro Rail for Focus Stacking StackShot (Cognisys Inc., Traverse City, MI). These were later stacked using the software Helicon Focus (Helicon Soft, Ukraine) on a PC computer. Figures were edited and assembled using Adobe Photoshop CS4.

We found that the external sterigmata of SB *Pteronymia* species are quite conserved morphologically. We therefore describe the female external genitalia of SB *Pteronymia* all at once rather than on a species by species basis. The sphragides of different *Pteronymia* species, however, are extremely variable, thus we describe them separately for each species. In addition to complete sphragides, we provide the number of specimens that we found with incomplete or amorphous sphragides. We also calculate the ratio of the width of the sterigma to the length of the abdomen for some species. These measurements were made using the Wild Heerbrugg M5 microscope with a scale set at 50× (sterigma width) and 6× (abdomen length). We chose specimens with abdomens in perfect or near-perfect condition and avoided specimens where abdomens were bent, crushed, twisted, or had deformed sterigma.

Taxonomic names follow Lamas (2004) with the updates of De-Silva et al. (2017). Terminology for general genitalia morphology follows Klots (1970), and terms for the sphragis follow Carvalho et al. (2017). In our sphragis descriptions, we define incomplete sphragides as structures that clearly had a reduced amount of sphragidal material forming the sphragis, and that were contained within the area of the sterigma. We also define ordered scales as those that are derived from the male that were probably intentionally

placed on the sphragis. Not ordered scales refer to those that are not arranged in a particular manner and appear to have been inadvertently attached to the sphragis.

Phylogenetic Analyses

Molecular and Morphological Data

Molecular and morphological data were obtained from De-Silva et al. (2017) for all described taxa available, including outgroups. Some taxa, however, only had one or the other data type available. The molecular matrix was split into each of the five loci so that individual alignments could be examined in AliView (Larsson 2014). Using this program, we trimmed the ends of all loci separately and concatenated them into a single, 3,960 bp alignment. Individual genes and their lengths were: COI (1,506 bp), COII (657 bp), EF-1a (1,017 bp), tRNA (63 bp), and TKY (717 bp). Molecular data were partitioned by gene and by codon position (except for the noncoding tRNA). Substitution models were selected using the greedy algorithm in PartitionFinder2 (Lanfear et al. 2017). The best-fit model for each partition was selected using the Bayesian Information Criterion (BIC). All molecular phylogenetic analyses used the same partitions and models selected by PartitionFinder2 (Supp. File S1). We downloaded the morphological data as a supplementary file from De-Silva et al. (2017) and removed the character for sphragis presence/absence (Character 87) in order to avoid redundancy in subsequent analyses (see below).

We initially created three datasets: Dataset 1 (molecular + morphology), Dataset 2 (molecular only), and Dataset 3 (morphology only) (Table 1). We assigned a Jukes-Cantor like model (MK + FQ) for the morphology portion of Datasets 1 and 3, and these regions were treated as a single partition. In all datasets, we removed four species that were in the study of De-Silva et al. (2017) (*P. sp. nov. 1*, *P. sp. nov. 2*, *P. sp. nov. 3*, and *P. sp. nov. 4*), because they lack formal names and we could not assign a character state of the sphragis to these species.

We also created a fourth dataset, consisting of only a single sphragis character derived from morphological Character 87 of De-Silva et al. (2017) (Supp. File S2). De-Silva et al. (2017) included two states (absence and presence of sphragis) for this character but our results from the present study revealed that sphragis morphology is complex, and should be coded into three states. We therefore appropriately re-coded the states as: sphragis absent (0), protosphragis/vestigial sphragis present (1), and sphragis present (2). Missing data, as well as ambiguity about the state, were treated as uncertain (given a '?' in the matrix). All outgroups were coded as 0 (sphragis absent), except for *Amauris ellioti* Butler and *Dircenna* sp., which were coded as 1 (protosphragis/vestigial sphragis) in both Bayesian inferences (BI) and ML. This coding scheme is based on Carvalho et al. (2017) (Supp. File S2).

Analyses

We performed three different maximum likelihood (ML) analyses in IQ-TREE v.1.5.3 (Nguyen et al. 2015) (Table 1). Initially, we performed ML analysis on the molecular plus morphology data (Dataset 1) (ML1, see Table 1). For this analysis, we conducted a tree search in IQ-TREE, while utilizing both SH-aLRT support and ultrafast bootstraps (Minh et al. 2013) (UFBoot), both with 1,000 replicates (ML 1). IQ-TREE commands for tree search were '-sp', and for SH-aLRT and UFBoot were '-alrt 1000' and '-bb 1000' respectively. Additionally, we performed two different ML analysis for molecular (ML2) and morphological (ML3) datasets separately, in order to evaluate potential conflicts between the two datasets. For both analyses, tree searches were performed using the command '-s', and nodal supports were calculated using UFBoot and SH-aLRT as mentioned above for ML1.

BI was performed on Dataset 2 in BEAST v. 1.8.4 (Drummond et al. 2012) using a command file generated in BEAUTi v.1.8.4 (Drummond et al. 2012) with the Markov chain Monte Carlo set to 200 million generations, sampling every 10,000 trees to calculate posterior probabilities (PP). The tree root age was set to a 95% credibility interval of 45–59 mya following Wahlberg et al. (2009). We provided a starting tree to BEAST in order to guarantee the recovery of the dated node. This initial tree was constructed using FastTree (Price et al. 2009) implemented in Geneious R.11 (Biomatters, United States), and was exclusively used as a BEAST starting tree. We ran Bayesian analyses implementing one universal relaxed clock to all partitions or seven relaxed clocks, one for each partition, and a Yule process or Birth–Death tree prior. Each combination of clock number and tree prior was performed twice. In summary, we ran eight analyses: 1 Relaxed clock + Yule Process (twice); 1 Relaxed clock + Birth–Death Process (twice); 7 relaxed clocks + Yule Process (twice); and 7 relaxed clocks + Birth–Death Process (twice). We applied the option 'path sampling/stepping-stone sampling' in order to be able to access the log marginal likelihood for each run. The log marginal likelihood for each analysis is listed in Table 1. We chose the combination that had the highest log marginal likelihood score, which was one of the 7 relaxed clocks + Birth–Death Process runs. We combined the two runs of 7 relaxed clocks + Birth–Death Process using LogCombiner v.1.8.4 (Drummond et al. 2012), discarding 10% (20 million) of the trees of each individual analysis as burn-ins. We analyzed the Effective Sample Size (ESS) of all parameters using Tracer v.1.6 (Rambaut et al. 2018). We concatenated the tree files of both 7 relaxed clocks + Birth–Death Process analysis using the command 'cat', and then generated a maximum clade credibility (MCC) consensus tree in Tree Annotator v.1.8.4 (Drummond et al. 2012), selecting the options 'Maximum clade credibility tree' and 'Median heights'. All BI analyses were performed in the University of Florida's cluster HiperGator2.

Table 1. Datasets and analyses performed

Dataset name	Dataset type	Analysis conducted	Partitions	Log likelihood	
Dataset 1	Molecules + Morphology	ML1	8	–45646.57	
Dataset 2	Molecules only	ML2	7	–44657.04	
		BI (Birth–Death + 7 clocks)	7	–45475.62	–45513.33
		BI (Birth–Death + 1 clock)	7	–45567.28	–45567.84
		BI (Yule + 7 clocks)	7	–45510.82	–45507.56
		BI (Yule + 1 clock)	7	–45569.43	–45566.25
Dataset 3	Morphology only	ML3	1	–1690.99	

The number of partitions was one for morphological analyses and seven for molecular analyses. ML1 thus included eight partitions. BI were performed twice for each analysis.

In order to study the evolution of the sphragis in *Pteronymia*, we performed ancestral state reconstruction in BEAST on the BI tree. This method allows us to assign states for the sphragis trait (Dataset 4) and uses this information to reconstruct the evolutionary history of the trait on the ancestral nodes. We input the sphragis trait matrix (Dataset 4) in the tab 'Traits' in BEAUTi and created a partition for Dataset 4. We selected the option 'Reconstruct state at all ancestors' and calculated the posterior probability of a given state at a given node in BEAST by taking into consideration the uncertainty of the tree. We also performed character state optimization through parsimony ancestral state reconstruction on the ML1 tree in Mesquite v. 3.5 (Maddison and Maddison 2018). For this method, we also assign states for the sphragis trait (Dataset 4), but Mesquite does not provide probabilities of the state at the nodes as BEAST does. Mesquite does, however, allow us to use a phylogenetic tree that was reconstructed with the combination of morphological and molecular data (ML1). We treated the states (Dataset 4) as ordered to reflect the hypothesis of previous studies that protosphragides/vestigial sphragides are intermediate states between the absence and presence of a sphragis. Tree files and sequence alignments are deposited in DRYAD (doi:10.5061/dryad.6qm33c6).

Results

Of 1,219 female specimens and 41 species of *Pteronymia* examined (Supp. Table S1) true sphragides were present in 11 species (27% of described species in the genus). The 11 SBS were: *P. alissa* (Hewitson), *P. andreas* (Weeks), *P. calgiria* Schaus, *P. latilla* (Hewitson), *P. obscuratus* (Fabricius), *P. ozia* (Hewitson), *P. picta* (Salvin), *P. sao* (Hübner), *P. simplex* (Salvin), *P. tucuna* (H. Bates), and *P. zerlina* (Hewitson) (Figs. 1–16). Within almost all species bearing true sphragides, we found females with complete and incomplete structures (broken or incompletely produced), except in *P. alissa* (only incomplete sphragides found) and *P. calgiria* (only one specimen examined, bearing a complete sphragis). In all SBS, except *P. calgiria* and *P. tucuna*, we found specimens that did not bear any sphragidal material on the sterigma (Table 2), but we could not confirm whether this was because the female was virgin, because the sphragis was removed/lost, or because she mated with a male depleted of sphragidal material.

We found irregular sphragis-like structures, that could be considered protosphragides or vestigial sphragides, in three species: *Pteronymia fulvimargo* Butler & Druce, *Pteronymia oneida* (Hewitson), and *Pteronymia ticida* (Hewitson).

Female External Genitalia

SBS of *Pteronymia* have a strongly sclerotized sterigma. The degree of fusion of the lamella ante- and postvaginales is quite variable, but together they form a bowl-like plate, which is noticeably broader than in non-SB *Pteronymia* (Fig. 17). *Pteronymia sao* displays one of the most extreme examples of this condition, the diameter of the sterigma bowl-like region in this species is wider than the abdomen itself (Fig. 8A and Fig. 17C), with an abdomen length to sterigma width ratio of 1.3 ($n = 10$). In comparison, the non-SBS *Pteronymia cotytto* (Guérin-Ménéville) (Fig. 17A) and *Pteronymia aletta* (Hewitson) have a much narrower sterigma relative to abdomen width (ratios of 0.14 and 0.21, respectively; $n = 10$ in both cases).

There is some variation in female external genitalia among SB *Pteronymia* species. *Pteronymia sao* has the lamella postvaginalis projected ventrally (Fig. 8C), which differs from other *Pteronymia* SBS. The sterigma of *P. picta* is relatively deep, but its ventral margins are more constricted, forming an opening that is smaller than in other *Pteronymia* SBS. The sterigma of *Pteronymia veia* (Hewitson), a species that was previously mentioned in the literature as having a true sphragis, and *P. picta* (ratio 0.41, $n = 10$), are more cylindrical than the bowl-like shape of some SBS in the genus, such as *P. sao* and *P. simplex* (ratio 0.54, $n = 10$). Finally, the sterigma of *P. obscuratus* is the most distinctive among the *Pteronymia* SBS. A ridge originating from the posterior margin of the sterigma, apparently from the ninth segment, extends outwards towards the center of the sterigma, which can be seen in virgin females (Fig. 4A). The same structure can also be found in *P. alissa*, *P. calgiria*, *P. latilla*, and *P. sao*, but is much less developed in these species.

Scales completely surround the margins of the bowl-like sterigma of *P. latilla* and *P. picta*. In other species, scales are sparse, but this could be a consequence of lost scales during the female's life. In *P. sao* and *P. simplex*, long thin setae extend from the margin of the sterigma, pointing either toward the center of the sterigma, or

Table 2. Sphragis-bearing species examined in the present study with the number specimens examined in each

Species	Sphragis type	Females examined	Complete sphragis	Incomplete or amorphous sphragis	Sphragis undetected ^a	Complexity category
<i>P. alissa</i>	True	31	0	19	12	?
<i>P. andreas</i>	True	22	1	12	9	2
<i>P. calgiria</i>	True	1	1	0	0	2?
<i>P. latilla</i>	True	134	28	8	98	2
<i>P. obscuratus</i>	True	15	11	3	1	3
<i>P. ozia</i>	True	12	1	2	9	2?
<i>P. picta</i>	True	108	3	41	64	2
<i>P. sao</i>	True	56	15	12	29	3
<i>P. simplex</i>	True	94	39	41	14	2
<i>P. tucuna</i>	True	3	1	2	0	3
<i>P. zerlina</i>	True	13	5	3	5	2
<i>P. fulvimargo</i>	Proto-/vestigial	9	3	NA	6	1
<i>P. oneida</i>	Proto-/vestigial	15	4	NA	11	1
<i>P. ticida</i>	Proto-/vestigial	9	2	NA	7	1
<i>P. veia</i>	Proto-/vestigial	38	10	NA	28	1

"?" indicates uncertainty regarding sphragis complexity category.

^aValues could be false negatives of sphragis absence if the female was a virgin.

ventrally. In *P. obscuratus*, these hair-like setae also extend from the central ridge of the sterigma (Fig. 8A).

Sphragis

Sphragides of *Pteronymia* differ considerably between species as described below and, although the sphragides of all species meet the definition of a true species-specific sphragis (Sensu Carvalho et al. 2017), we also found some level of morphological variation within species. This variation is likely a result of different amounts of male material investment in each sphragis and the fact that the position of the sphragis on the female differs between individuals. For this reason, the following descriptions refer to the most complete and well-formed sphragides that could be found for each species. We also include descriptions of intraspecific variation when applicable. The total numbers for incomplete sphragides per species should be considered as potential underestimations. Incomplete sphragides were often located entirely or nearly entirely inside the bowl-like sterigma, and sometimes the shape of the sterigma did not allow confident determination if there was sphragidal material covering the ostium bursae.

Below we describe the sphragis of the 11 *Pteronymia* species for which we have observed a true sphragis (Sensu Carvalho et al. 2017). See Table 2 for values of complete and incomplete sphragis presence, as well as the number of specimens in which we were unable to detect a sphragis.

Descriptions of *Pteronymia* sphragides

Pteronymia alissa (Hewitson, 1869)

Figure 1

Females examined: 31. 19 bore incomplete or amorphous sphragides; none had a complete sphragis.

Description: Coloration—varied from whitish yellow to dark brown. Shape—varied from plate-like to a small amorphous structure. Scales—absent. Complexity category—could not be assigned.

Comments: The specimen figured here (Fig. 1) has the largest sphragis we could find in this species, but this specimen likely bears an incomplete sphragis, as it is similar in shape to incomplete sphragides of other *Pteronymia* species described below, such as *P. andreas*.

Pteronymia andreas (Weeks, 1901)

Figure 2

Females examined: 22. 1 with a complete sphragis; 12 bearing incomplete or amorphous sphragides.

Description: Coloration—golden-brown to yellowish off-white. Shape—plate-like. Structure—mostly hollow. Scales—found in some specimens, but not ordered, probably accidentally attached. Complexity category—2.

Comments: The complete sphragis that we observed was likely deformed due to the way it was attached to the sterigma. Nearly all (12/13, 92%) of the sphragides observed for this species were incomplete. Incomplete sphragides were in the form of a plate, and always completely internalized inside the bowl-like sterigma. The amount of sphragidal material was variable, but often it only covered the ostium bursae and immediate surroundings.

Pteronymia calgiria Schaus, 1902

Figure 3

Females examined: 1 bearing complete sphragis.



Fig. 1. The sphragis of *Pteronymia alissa*.

Description: Coloration—golden-brown. Shape—round, cupped, extremities curled inward. Structure—mostly solid. Scales—absent. Complexity category—likely 2, but more specimens are needed in order to accurately assign a category.

Pteronymia latilla (Hewitson [1855])

Figure 5

Females examined: 134. 28 with complete sphragides; 8 bearing incomplete or amorphous sphragides.

Description: Coloration—golden-brown to cream. Shape—Anteriorly, the sphragis is rounded and nested mostly within the bowl-like sterigma. Medially, the sphragis has a cavity. The posterior half of the sphragis is external to the sterigma, and wider than the anterior half and rectangular in shape. The posterior region is not wider than the anterior region in several specimens examined. Structure—mostly solid. Scales—sometimes present, but when present not ordered.

Comments: Among the 28 specimens that had a sphragis, one had two sphragides with a spermatophore (almost certainly belonging to the second male to mate with the female) attached to the outside of the female body. Females bearing more than one sphragis, as found here and in other species mentioned below, are unusual in SBS, but have been seen in previous studies. When the second sphragis is accompanied by a spermatophore, it shows that the sphragis of the first male works in preventing the transfer of the spermatophore of more than one male.

Pteronymia obscuratus (Fabricius, 1793)

Figure 4

Females examined: 15. 11 with complete sphragides; 3 bearing incomplete or amorphous sphragides.

Description: Coloration—golden-brown. Shape—rounded anteriorly with two symmetrical lateral cavities, an elevated projection medially, and the sphragis curves upward and over the sterigma posteriorly, overhanging the sterigma. The tip of the sphragis is pointed and nearly touches the papillae anales. Structure—mostly solid. Scales—present, ordered, hair-like. Attached to leading edge of the overhanging posterior half of sphragis.

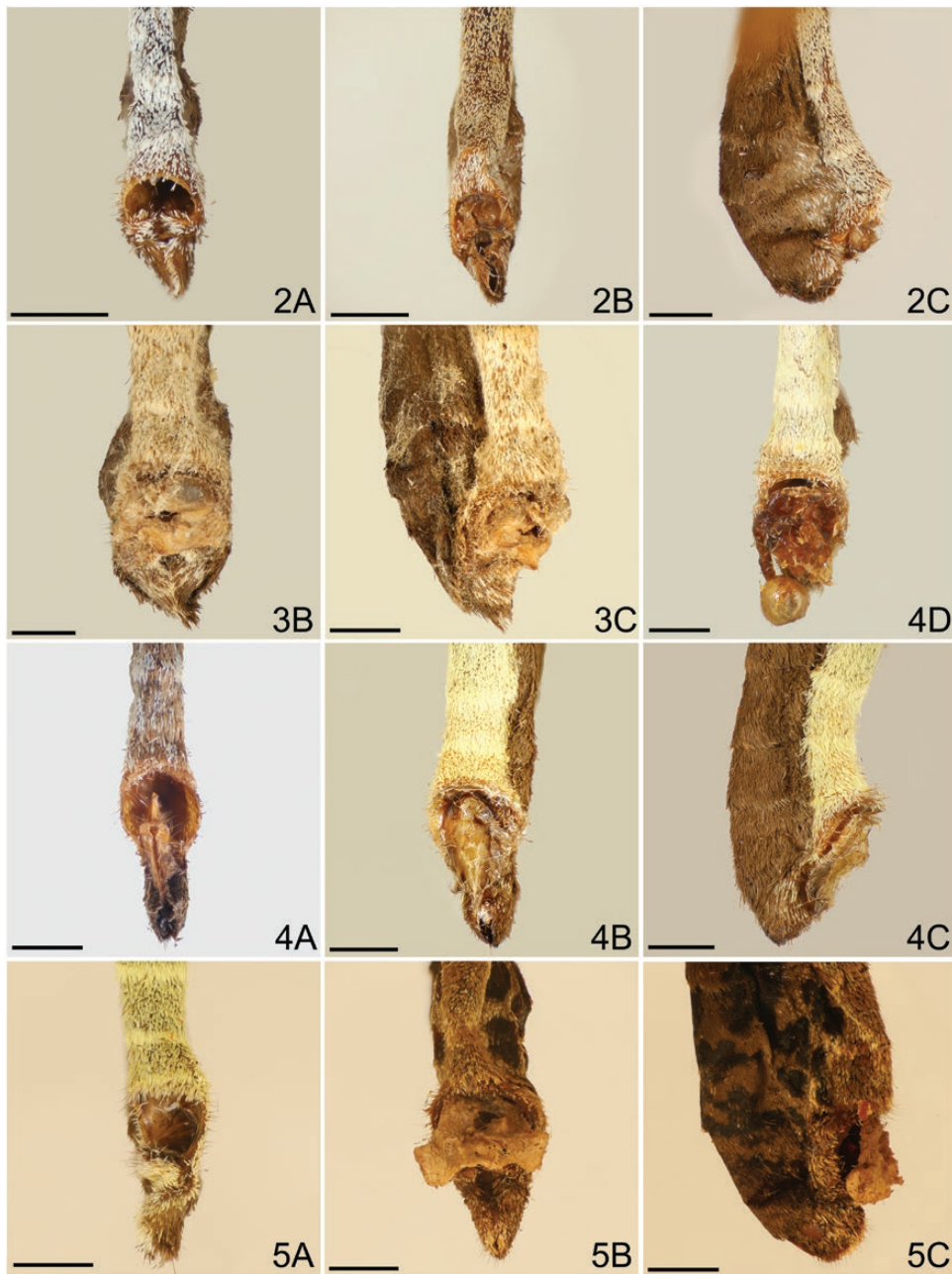


Fig. 2-5. The sterigma and sphragis of *P. andreas* (2), *P. calgiria* (3), *P. obscuratus* (4), and *P. latilla* (5). A: ventral, sphragis absent; B: ventral, sphragis present; C: lateral, sphragis present; D: *P. obscuratus* with a spermatophore externally connected to the sphragis.

Comments: We observed one female with a spermatophore attached to the sphragis outside of the female body (Fig. 4D). It is likely that there was already a sphragis in place on the female's sterigma, and that the second male was unsuccessful in depositing his spermatophore inside the female's reproductive tract, but still produced a complete sphragis on top of the first one.

Pteronymia ozia (Hewitson, 1870)

Figure 6

Females examined: 12. 1 with a complete sphragis; 2 bearing incomplete or amorphous sphragides.

Description: Coloration—dark chocolate-brown. Shape—plate-like. Structure—mostly hollow. Scales—absent. Complexity category—likely 2, but more specimens are needed to properly assign a complexity category.

Comments: The sphragis figured here is the only one that was complete among the sphragides examined. It is placed laterally in the sterigma, and we suspect it was not properly deposited by the male or that it was displaced after attachment.

Pteronymia picta (Salvin, 1869)

Figure 7

Females examined: 108. 3 with complete sphragides; 41 bearing incomplete or amorphous sphragides.

Description: Coloration—yellowish off-white. Shape—bowl-like. Structure—mostly hollow. Ventral margin curled inward. Scales—few, not ordered.

Comments: Most of the sphragides that we found attached to the sterigma were incomplete, and fully within the bowl-like sterigma. Much like *P. alissa*, incomplete sphragides were in the

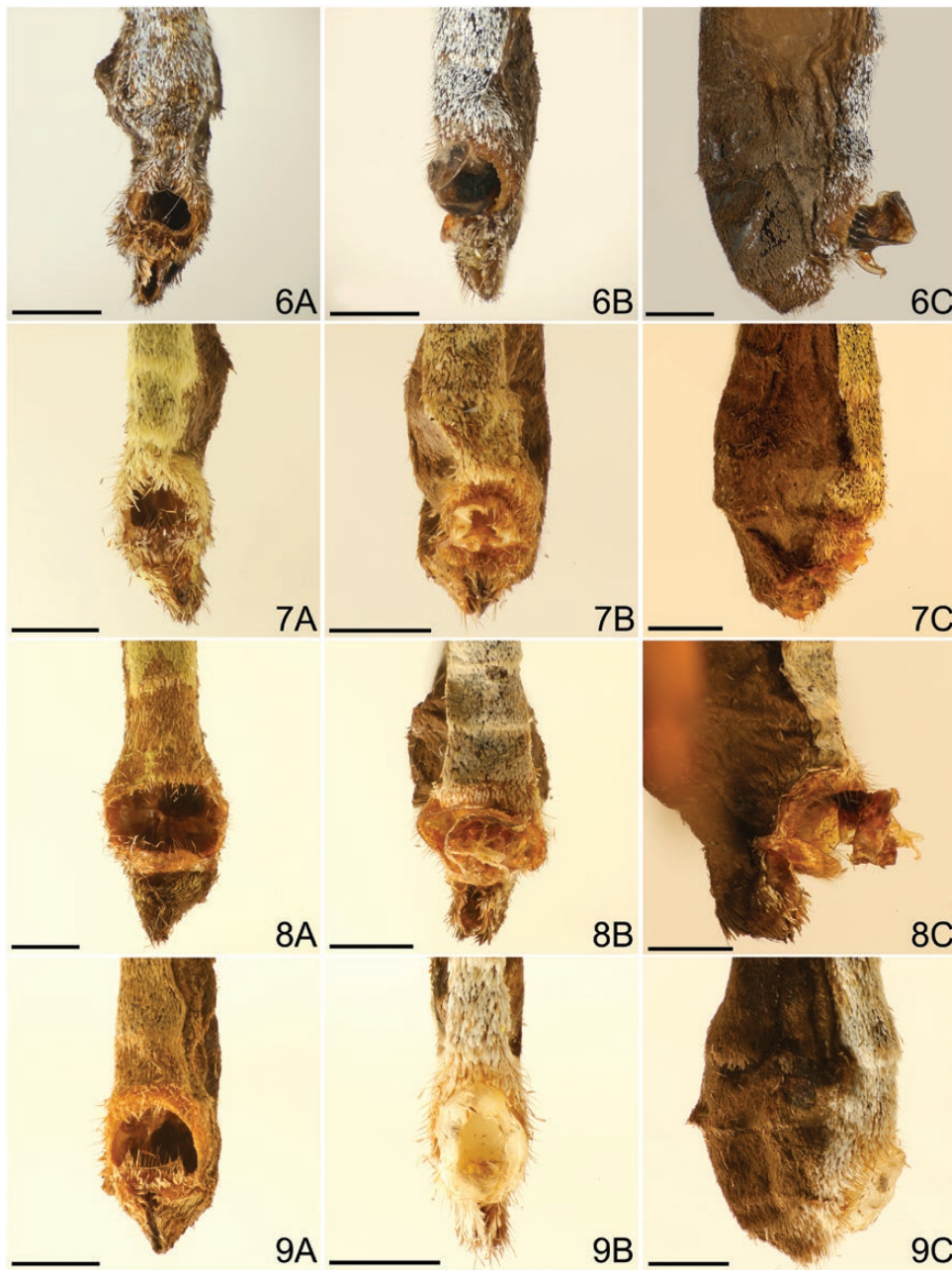


Fig. 6–9. The sterigma and sphragis of *P. ozia* (6), *P. picta* (7), *P. sao* (8), and *P. simplex* (9). A: ventral, sphragis absent; B: ventral, sphragis present; C: lateral, sphragis present.

shape of a plate, and the amount of sphragidal material was highly variable.

Pteronymia sao (Hübner, [1813])

Figure 8

Females examined: 56. 15 with complete sphragides, 12 bearing incomplete or amorphous sphragides.

Description: Coloration—golden-brown. Shape—circular shape when viewed ventrally, irregular in shape with several lacunae when viewed laterally. Structure—mostly hollow. Scales—few scales present, not ordered.

Pteronymia simplex (Salvin, 1869)

Figure 9

Females examined: 94. 39 with complete sphragides; 41 bearing incomplete or amorphous sphragides.

Description: Coloration—white to golden yellowish brown. Shape—bowl-like. Margin of the sphragis curled. Structure—mostly hollow. Scales—few, not ordered.

Comments: We found five females bearing two sphragides each (e.g., Fig. 16), but we could not locate the spermatophore of the second male outside of the body of any of these females, and they were likely broken.

Similar to *P. alissa* and *P. picta*, the level of sphragis completeness of *P. simplex* was highly variable. The shape of these incomplete sphragides was comparable to those that had complete sphragides, differing only in its smaller size or placement, which was entirely within the bowl-like sterigma. We also observed

some structures that resembled a complete sphragis, but which were barely externalized beyond the margins of the bowl-like sterigma. It was occasionally difficult to determine whether the sphragis was complete, and this is due to the fact that the amount of sphragidal material produced by males in SB *Pteronymia* is extremely inconsistent.

Pteronymia tucuna (H. Bates, 1862)

Figure 10

Females examined: 3. 1 with complete sphragis; 2 bearing incomplete or amorphous sphragides.

Description: Coloration—golden-brown. Structure—mostly solid. Shape—somewhat posteriorly bilobed roughly following the bilobed shape of sterigma. Surface textured with a medial cavity.

Scales—male scales orderly and attached to the anterior edge of the sphragis. Complexity category—3.

Comments: The anterior portion of the sphragis is protruding to the outside of the sterigma. We are uncertain if the protrusion of the anterior part of the sphragis is a common feature of this sphragis, but it is likely that this sphragis was dislocated from its original position.

Pteronymia zerlina (Hewitson, [1856])

Figure 11

Females examined: 13. 5 with complete sphragides; 3 bearing incomplete or amorphous sphragides.

Description: Coloration—golden-brown to grayish white. Shape—mostly closed bowl with extremities converging inward,

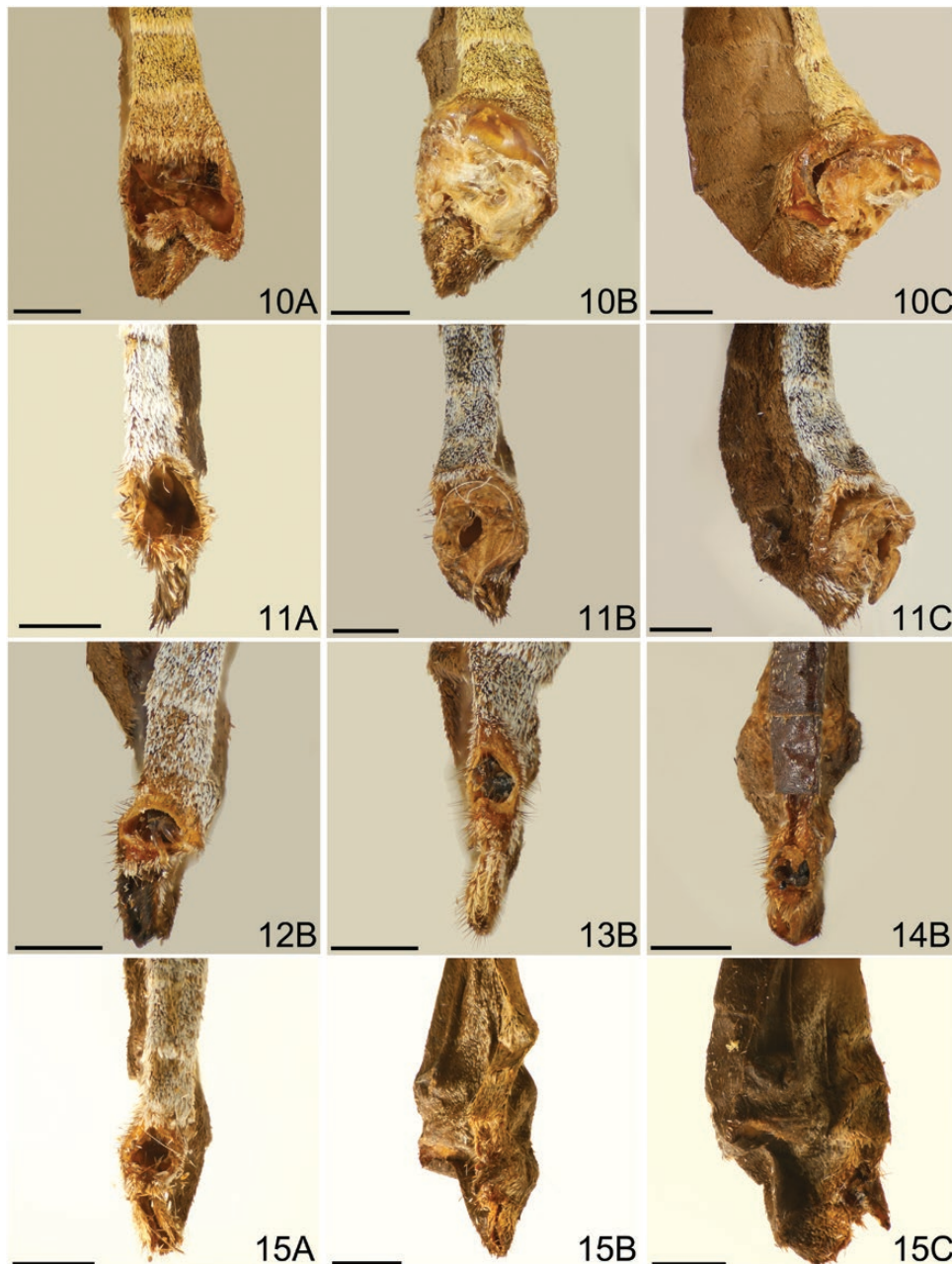


Fig. 10–15. The sterigma and sphragis of *P. tucuna* (10), *P. zerlina* (11), *P. fulvimargo* (12), *P. oneida* (13), *P. ticida* (14), and *P. veia* (15). A: ventral, sphragis absent; B: ventral, sphragis present; C: lateral, sphragis present.

forming a small central hole. Structure—mostly hollow. Scales—few scales present, not ordered. Complexity category—2.

Sphragis-Like Structures in *Pteronymia*

In addition to true sphragides, we also found in *P. fulvimargo* (Fig. 12B), *P. oneida* (Fig. 13B), and *P. ticida* (Fig. 14B) sphragidal material that should be treated as a protosphragis or vestigial sphragis (Sensu Carvalho et al. 2017). *Pteronymia alina* Haensch, *Pteronymia medellina* Haensch, and *Pteronymia parva* (Salvin) also had similar structures, but additional specimens would be needed in order to confirm the nature of the structure in these species. For the ASR analyses, we coded these taxa as ‘?’ (uncertain) for the sphragis state.

Carvalho et al. (2017) treated *P. veia* as having a true sphragis, but after further examination, we believe it is instead amorphous accumulation of sphragidal material. This structure was composed of dark, amorphous, outwardly pointed/triangular sphragidal material deposited in the sterigma (Figs. 15B and C), resembling more a protosphragis/vestigial sphragis than a true sphragis. The fact that we could not find a female of *P. veia* with a true sphragis does not indicate that this lacks one. We believe that additional specimens are needed for confirmation.

Phylogeny of *Pteronymia* and the Evolution of its Sphragis

We first conducted phylogenetic analyses on the datasets of De-Silva et al. (2017) using ML and BI optimality criteria. Comparing our

ML tree based on molecules only (ML2 analysis; Supp. Fig. S4), with the molecular only ML analysis of De-Silva et al. (2017), we found that clade A (shown in Supp. Fig. S4) was recovered similarly to the study of De-Silva et al. (2017), except without the species *P. euritea*. Clade C in our analysis was largely congruent with De-Silva et al. (2017), but with the addition of the clade containing *P. rufocincta* + *P. cotytto* ((*P. alissa* + *P. laura*) + (*P. andreas* + *P. sexpunctata*)).

When the molecular ML analysis (Dataset 2, ML2) is compared with the morphological analysis (Dataset 3, ML3), clade A was recovered in both ML2 (Supp. Fig. S4) and ML3 (Supp. Fig. S5), but without (*P. sao* + *P. obscuratus*), which was nested in clade B in the ML3 tree. Another difference between the ML2 and ML3 trees is that clade C was recovered as a monophyletic group in the tree derived from molecular only (ML2) but not in the tree morphology only tree (ML3).

PP (BI) for most nodes in the *Pteronymia* phylogeny were >0.8, but there were exceptions (Supp. Fig. S1). ML1 and ML2 had mostly moderate (50 < SH-aLRT < 80; 50 < UFBoot < 95) to high (SH-aLRT > 80; UFBoot > 95) backbone support values (Supp. Fig. S4), whereas ML3 support values were mostly moderate.

The ancestral state reconstruction in BEAST suggests that the sphragis originated three times in *Pteronymia* (Fig. 18). Most of the PP were ≥ 0.8 , with the only exceptions being in the clade (*P. cotytto* + *P. rufocincta*) + ((*P. alissa* + *P. laura*) + (*P. andreas* + *P. sexpunctata*)) (PP = 0.56), and in clade A (PP = 0.79). PP of the branches in the tree are provided in the Supporting Information (Supp. Fig. S1).

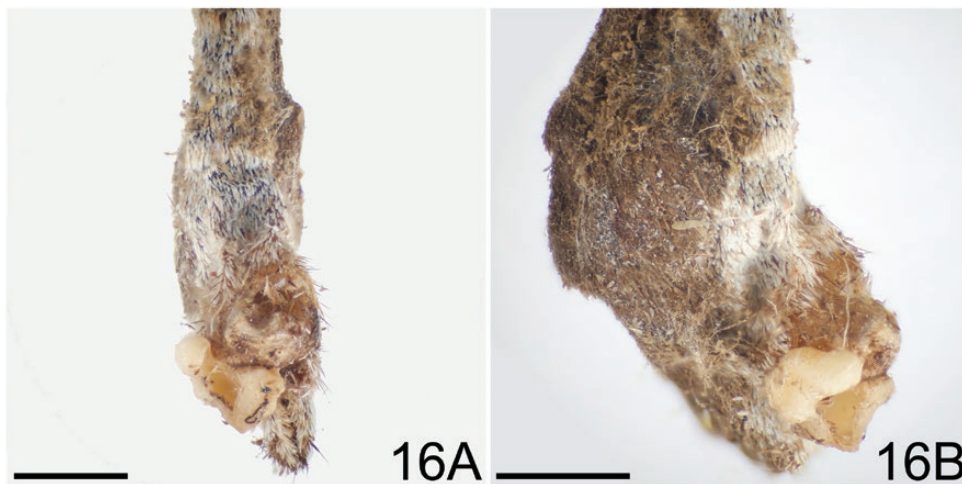


Fig. 16. Double sphragis in *P. simplex*. A: ventral; B: lateral.

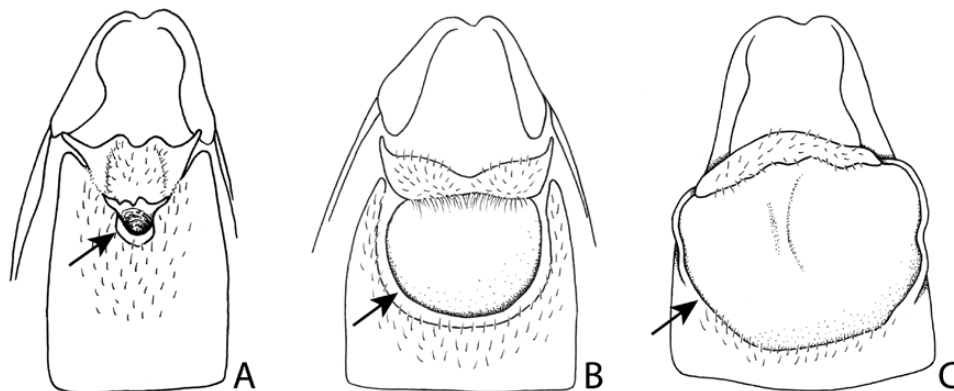


Fig. 17. The sterigma of *P. cotytto* (A), *P. simplex* (B), and *P. sao* (C). Image shows illustration of ventral side of the terminal segments of female abdomen. Illustrations not to scale.

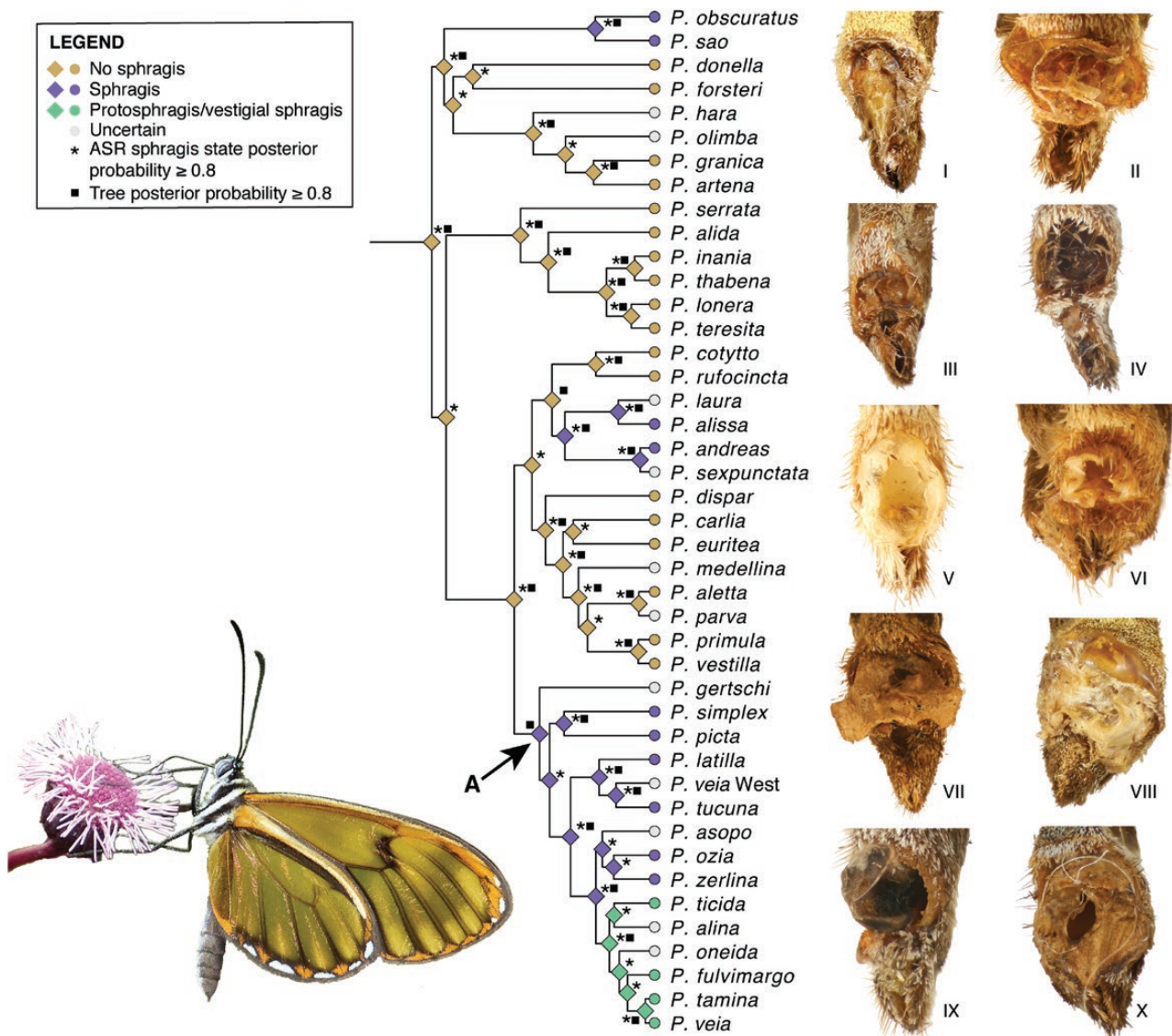


Fig. 18. Bayesian ancestral state reconstruction (ASR) of the sphragis in *Pteronymia* conducted in BEAST. This tree is a re-analysis of the molecular data of De-Silva et al. (2017). Node colors refer to the sphragis state that has the highest PP for that node. Asterisks: PP ≥ 0.8 for the sphragis state in the ASR. Black squares: tree nodal PP support ≥ 0.8 . Node A designates a specific clade referenced in the text. I: *P. obscuratus*, II: *P. sao*, III: *P. andreas*, IV: *P. alissa*, V: *P. simplex*, VI: *P. picta*, VII: *P. latilla*, VIII: *P. tucuna*, IX: *P. ozia*, X: *P. zerlina*.

The ML character state optimization (Supp. Fig. S2) suggests that the sphragis originated only once in *Pteronymia*, in the ancestor of clade A, and that it was later lost in clade C. Clade B, which is sister to clade A, does not contain any confirmed SBS. In both ML and Bayesian analyses, all species bearing protosphragides/vestigial sphragides (state 1) were nested within an SB clade and had an SB ancestor. However, only in the ML ancestral state analysis, the true-SBS *P. ozia* and *P. zerlina* have protosphragis/vestigial sphragis-bearing species as ancestors. The same was not found in the BI ancestral state reconstruction due to differences in topology.

Discussion

Describing the morphology of the sphragis and tracing the evolutionary history of this trait on a phylogeny of *Pteronymia* provides an opportunity to hypothesize the pattern of evolution of sexually

antagonistic traits in butterflies. The current prevailing theory that explains sphragis evolution was proposed by Orr (1988), who focused his argument on the presence of externalized genitalia in females of SBS, which could be associated with plugging avoidance. He hypothesized that 1) males developed mating plugs to prevent female remating and to ensure paternity; 2) females developed externalized genitalia to facilitate plug loss or removal by other males by making the plug more exposed; and 3) males invested more resources in the production of the plug, resulting in larger structures, which, at some point, reached the size and complexity of a true sphragis. Considering this male–female evolutionary arms race, a possible ‘female wins’ scenario is postulated for species in which the genitalia are externalized, the sphragis is lacking and females mate many times. However, because the sphragis has originated multiple independent times (Orr 1995, Carvalho et al. 2017), it is possible that this trait may have originated by different forces of selection. Our results

show that the genitalia of SB *Pteronymia* are not externalized in the same way as in other SB butterfly groups, but the morphology of the sterigma in these species could be equivalent to a form of externalization which would make plugging less efficient, especially in species where the ostium and ductus bursae are also unusually broad (e.g., *P. latilla*, *P. sao* and *P. simplex*; De-Silva et al. 2017), which might facilitate males bypassing the sphragis. Females of sphragis-bearing *Pteronymia* have a rigid, very wide, bowl-like sterigma—somewhat similar to the condition observed in some *Acraea* species (Orr 1988). This might facilitate sphragis loss and removal considering that 1) a wide bowl-like sterigma would require larger amounts of sphragidal material to fill this cavity and effectively plug the female, preventing a second male from accessing the ostium bursae with his aedeagus; or 2) a small sphragis that is not adequately attached to the sterigma would be more susceptible to being lost by the female during flight, oviposition, or from removal by a subsequent male. Despite the almost unique sterigma morphology of *Pteronymia*, sphragides found in this genus are comparable in complexity with sphragides of female SBS that have a wholly externalized ostium bursae (Carvalho et al. 2017).

If the complete sphragides in *Pteronymia* are truly less common than incomplete ones, and mated females do not always bear sphragides, this would be a strong indication that anti-plugging strategies that evolved in females are somewhat effective in *Pteronymia*. This would imply that the strategy in *Pteronymia* could be closer to a ‘female wins’ scenario than in other SB groups, such as most parnassiines, acraeines, and in the few SB troidines, where female specimens lacking a sphragis in wild-caught specimens are very rare (Bryk 1919, Ackery 1975, Orr 1988, Matsumoto and Suzuki 1995; A.P.S.C., personal observation).

Pteronymia laura (Staudinger) is a possible example of the ‘female wins’ scenario. This species lacks a sphragis, but is nested in a predominantly SB clade (Fig. 18). Therefore, the sphragis may have been lost through evolutionary time, but the females of the remaining SBS in this clade are still ‘fighting’. However, we were only able to examine five female *P. laura* specimens and we therefore acknowledge the need for further examination of specimens of this species.

The presence of incomplete sphragides has been detected in most SB groups (Orr 1988, 2002; Matsumoto et al. 2018) due to depleted males’ inability to produce fully formed sphragides. In *Pteronymia*, the incidence of incomplete sphragides is far more extreme than in other SB groups—in *P. picta*, for example, only three of the 44 females with some material deposited on the sterigma had a complete sphragis. The SB Heteronympha *penelope* Waterhouse (Lepidoptera: Nymphalidae) appears to have the most similar pattern of male investment to SB *Pteronymia* (Orr 2002). In this species, males can probably produce only one complete sphragis during their lifetime.

However, it is important to point out that the total number of incomplete and complete sphragides might be an underestimation because a sphragis can be lost or removed after mating, especially if they are incomplete (Orr 2002). Additionally, specimens in collections could have been reared in captivity, and therefore the females would likely be virgins. For example, of the 90 *P. latilla* examined at USNM, 20 were reared and, potentially, never mated.

Mating behavior in *Pteronymia* may explain why we were unable to find many complete sphragides. Although specific data on *Pteronymia* behavior is lacking, it has been suggested that male ithomiines lek (Haber 1978), a behavior which would give females control over matings. This female control appears to be absent in other SB lineages, where forced copulation is the mating norm (Epstein 1987, Matsumoto 1987, Orr 1988, Sourakov and Emmel 1997, Francini et al. 2005). Another characteristic that is unique in

SB *Pteronymia* is the presence of a large bursa with an appendix (De-Silva et al. 2017), which could be an indication of a larger male investment via a spermatophore.

Remating is often advantageous for the females. However, the sphragis is probably a beneficial adaptation for females of other SB butterflies, where multiple matings are disadvantageous. The sphragis may benefit the female by preventing unwanted copulations, given that she no longer receives nutrients from the male and loses time for oviposition as well as risking injury during violent mating attempts through forced copulation (Orr 1988; A.P.S.C., personal observation) in which the sphragis is virtually impossible to remove if normally formed. Initially, the ridge in the sterigma of some species (e.g., *P. obscuratus*) could be interpreted as an indication of male–female conflict if this structure has evolved to make plugging difficult for the males. Conversely, this structure could help hold the sphragis in place considering that it increases the area of attachment of the plug to the female’s body. The latter hypothesis is supported by the fact that we have found complete sphragides in 73% of the specimens examined.

Ancestral state reconstruction of the sphragis revealed that this trait is a derived condition in *Pteronymia*, and that all species bearing irregular sphragis-like structures have an ancestor that produced a true sphragis. This suggests that these irregular structures are likely vestigial sphragides and not protosphragides, since vestigial sphragides represent a sphragis state associated with groups in early stages of sphragis loss, potential cases where females are ‘fighting back’ the arms race with males.

The number of evolutionary origins differed between our analyses. The BI analysis suggests that the sphragis originated three times in the genus, while the ML1 analysis, which includes both molecular and morphological data, indicated only one evolutionary origin of the sphragis in the genus. In order to confirm that the difference in the number of sphragis origins in *Pteronymia* was not related to the optimality criterion (BI vs ML), but to the presence of morphological data in the ML1 analysis, we performed character optimization on the ML2 tree (molecular only, not figured here). This analysis also resulted in three evolutionary origins of the sphragis. Results from these analyses suggest that morphological data in this study are impacting the ancestral state reconstructions of the sphragis.

Considering the morphology of the sphragis and, especially, the strong similarities of the sterigma of SBS in *Pteronymia*, it seems likely that the sphragis has evolved only once in this genus. A careful analysis of male genitalia could further clarify this since we only focused on females on the present study.

The discrepancy in the number of evolutionary origins of the sphragis between different analyses appears to be a consequence of the conflict between the morphological and molecular data in ML1. The main instance of this is in the placement of *P. sao* + *P. obscuratus*, two SBS. This clade is recovered in a different topological placement in the trees based on molecules (Supp. Fig. S4) and morphology (Supp. Fig. S5). The morphology only tree (ML3) places these two species within clade B (Supp. Fig. S5), a group of SBS. On the other hand, this sister pair is placed in clade A in the molecules only analysis of ML2. Therefore, the inclusion of two conflicting datasets appears to be affecting the number of origins of the sphragis in *Pteronymia*.

The ML1 tree suggests that the sphragis was secondarily lost in *Pteronymia alcmena* and in clade C (Supp. Fig. S2). If intrasexual conflict in *Pteronymia* can be inferred on the basis of sphragis presence alone, females of species in this clade may be ‘winning’ in the sexual arms race. The true number of sphragis gains and losses is also unclear because some species had none or a very small number of specimens

available for evaluation to confidently infer the sphragis state. These species include *Pteronymia asopo* (Felder & Felder), *Pteronymia gertschi* Fox, and *P. alina*, *P. laura*, *Pteronymia sexpunctata* (Bryk), and *Pteronymia tamina* Haensch (Fig. 18 and Supp. Fig. S2).

Branch support values in the ML1 tree seem to have been negatively affected by the inclusion of morphological data, as noted by De-Silva et al. (2017). Adding morphological data can improve phylogeny estimation (Wahlberg et al. 2005, Ylla et al. 2005, Simonsen et al. 2012), but in this case, there were species that were scored only for morphology, resulting in a large amount of missing data. Missing data can have a negative impact on branch support, especially when they constitute a majority of the characters (Lemmon et al. 2009).

Rowe and Day (2006) highlighted the need for additional studies that examine the natural history of intersexual conflict and traits involved in sexually antagonistic coevolution. For example, data on mating frequency, male investment directed to the sphragis, material investment in each copulation, paternity analysis, and confirmation of the occurrence of lekking, would provide valuable information to analyze male-female interactions in *Pteronymia*.

Little is known about potential adaptations in the internal male and female genitalia relative to the presence of the sphragis in *Pteronymia*. Illustrations of the corpus bursae of sphragis-bearing species, such as *P. simplex* and *P. oneida* in De-Silva et al. (2017, Supp. Fig. S8, plate 4) show that they are somewhat reduced and with a thick membrane, very common traits in other SB butterflies (Orr 1988). An unusual trait for SBS though, is the presence of an appendix bursae in species such as *P. sao* and *P. simplex*. The illustrations also show that males of SBS such as *P. fulvimargo* and *P. obscuratus* have larger uncus than the uncus of non-SBS, and that SBS males do not have a gnathos. We postulate that the large uncus implies greater ability for the male to remove sphragides from mated females, but further direct evidence is needed.

Due to differences in ASR results, we were unable to confidently confirm the number of evolutionary origins of the sphragis in this genus. The choice of outgroups and subsequent morphological coding of the outgroups could also influence ASR results. Additional data on sphragis presence in clades sister to *Pteronymia*, such as in *Episcada* Godman & Salvin (Lepidoptera: Nymphalidae), would be worth investigating in a future study as this was not possible here due to the relatively few outgroups used. Inclusion of more outgroups, broadly sampling Ithomiinae, could provide more informative results regarding the evolutionary history of the sphragis in the subfamily, and provide more robust results in *Pteronymia*. Future studies should include more molecular data, seek additional informative morphological traits, and examine specimens that may remain hidden in museum collections.

Finally, while the number of sphragis-bearing butterfly species now appears to be at least 280, we predict that the true number is higher, especially if additional *Pteronymia* specimens are examined. The intersexual arms race found in SB *Pteronymia* could be a novel strategy that portrays sexual antagonistic coevolution and may have led *Pteronymia* to become the most diverse ithomiine genus.

Supplementary Data

Supplementary data are available at *Insect Systematics and Diversity* online.

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