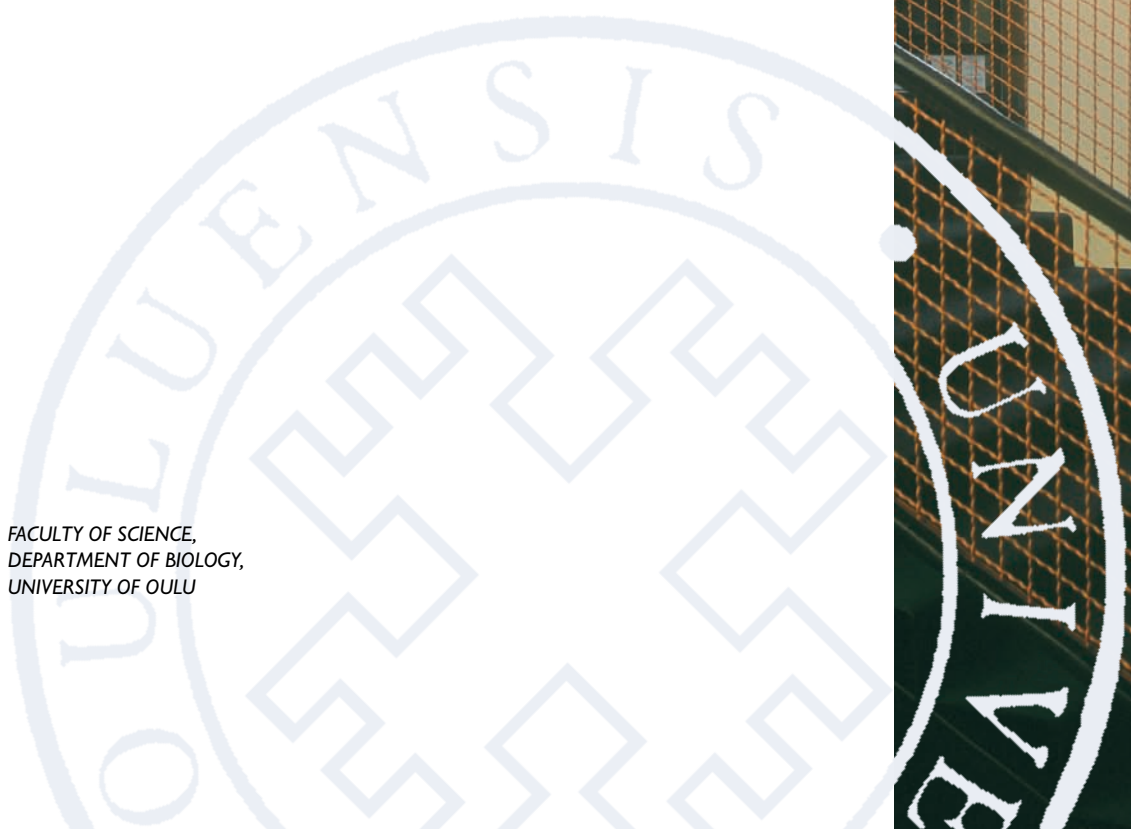*ACTA* **UNIVERSITATIS OULUENSIS**

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GENITAL VARIATION IN MOTHS— EVOLUTIONARY AND SYSTEMATIC PERSPECTIVES

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Academic Dissertation to be presented with the assent of the Faculty of Science, University of Oulu, for public discussion in Kuusamonsali (Auditorium YB210), Linnanmaa, on May 20th, 2006, at 12 noon

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Abstract

Insect genital characteristics have peculiar morphological patterns. They show great divergence between species. At the same time, they are assumed to vary little within species by taxonomists who frequently use genital features to delimit species. I studied moth (Lepidoptera) genital size and shape variation within and between species. I also tested hypotheses proposed to explain rapid genital diversification among species. Studies were carried out using traditional distance measurement morphometrics and modern geometric morphometrics. Several moth species were analyzed.

I found that genital structures show variation that in closely related species may show structural overlap. More surprisingly, the amount of variation in internal genitalia was equal to non-genital traits. These and some other findings are against the predictions of the lock-and-key hypothesis, which suggests that genital differences form a mechanical isolation system between species. Meanwhile, the findings are in good accordance with the various mechanisms of the sexual selection hypothesis. I found that external genital traits express varying amounts of variability. However, both external and internal genitalia consistently show small variation in size so that large individuals have disproportionately small genitalia and vice versa. This finding is consistent with the lock-and-key theory, but also with the cryptic female choice hypothesis. In conclusion, the results suggest that sexual selection plays a major role in genital diversification, but the exact mechanism remained unclear in this study. Some structures in moth genitalia strongly suggest that sexual conflict is present as well. It is possible that several mechanisms of sexual selection are in action simultaneously.

My findings have implications to insect taxonomy. Genital characters, although often useful, should not be considered superior to other characters because they may vary considerably within species. I have shown that subjective visual evaluation of genital characteristics and a priori assumption of their low variability may easily lead to unsound taxonomic conclusions. Sophisticated morphometric tools are very useful and objective in delimiting sibling species. Geometric morphometrics is particularly useful since it helps to evaluate limits of variation. There are, however, no theoretical grounds to assume that genitalia are not subject to intraspecific geographic variation. Such variation was detected in this study as well. Geographic relationships should therefore be taken into consideration more frequently when delimiting populations into different species.

To my family,

Acknowledgements

I formally began working on this thesis in 2001, but the whole story is much longer and actually originates in 1987, when I started to collect butterflies and moths. In 1992, I conducted my first genital dissection (to a moth!). At the beginning of my "lepidopterist career", Juhani "Jussi" Itämies was simply a necessity. His in-depth knowledge of Lepidoptera and all kinds of "bugs" has no equal. Most importantly, he has always generously shared his endless knowledge, which is no doubt a major reason that there are, and have been, so many entomologists in Oulu. I am deeply indebted to Jussi for leading me to the wonderful world of insects. At the beginning, also Reima Leinonen greatly helped me in becoming familiar with collecting butterflies and moths. I am vary grateful for my parents, Marja-Leena and Tapani, for their supportive attitude concerning my odd enthusiasm and for allowing me to run after butterflies all summers long, daily and nightly. Two of my brothers, Tomi and Teppo, have shared this enthusiasm with me. I wish to thank them particularly for numerous exciting field trips.

My supervisor Arja Kaitala was the one who introduced me to the more scientific questions concerning the genital evolution of insects. Throughout the entire thesis, her support and keenness has been a driving force. Not the least, to a man with a family, she has greatly helped me of funding. There is no doubt that, much because of Arja's help, I have been lucky to be continuously funded. I have enjoyed belonging to Arja's group and feel that working there has deepened my scientific thinking. I thus wish express my deep gratitude to Arja.

Working on a thesis is not, and never should be, lonely work. There have been a number of colleagues around me, most importantly my roommate at the museum, Panu Välimäki. The number of discussions around scientific questions (and all other aspects of life) are infinite, or at least very near to that. These discussions have, hopefully, been mutually fruitful. In any case, I am sure that my own thinking has evolved much because of these discussions. I am grateful to my co-authors Jari Lindén, Mikko Mönkkönen, Resia Pretorius, Seppo Rytkönen and Janne Sinkkonen for numerous discussions and crucial help in preparing papers. A number of colleagues have had discussions with me, commented on my papers, and helped in many other ways during my thesis. I wish to mention Jouni Aspi, Folmer Bokma, Åsa Borg, David Carrasco, Maarit Haataja, Esa

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Marko Mutanen

List of original papers

The thesis is based on the following papers, which will be referred to by their Roman numerals.

I   Mutanen M, Kaitala A & Mönkkönen M (2006) Genital variation within and between three closely related *Euxoa* moth species: testing the lock-and-key hypothesis. Journal of Zoology 268: 109–119.

II Mutanen M & Kaitala A (2006) Genital variation in a dimorphic moth *Selenia tetralunaria* (Lepidoptera, Geometridae). Biological Journal of the Linnean Society 87: 297-307.

III Mutanen M (2005) Delimitation difficulties in species splits: a morphometric case study on the *Euxoa tritici* complex (Lepidoptera, Noctuidae). Systematic Entomology 30: 632–643.

IV Mutanen M, Rytkönen S, Lindén J & Sinkkonen J (2006) Male genital variation and asymmetry in a moth *Pammene luedersiana* (Lepidoptera: Tortricidae). Submitted manuscript.

V   Mutanen M & Pretorius E (2006) Subjective visual evaluation versus traditional and geometric morphometrics in species delimitation: a comparison on moth genitalia. Submitted manuscript.

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Introduction

1.1 General background

"Authors sometimes argue in a circle when they state that important organs never vary; for these same authors practically rank that character as important which does not vary; and, under this point of view, no instance of an important part varying will ever be found: but under any other point of view many instances assuredly can be given." Charles Darwin, 1859

When these words were written a hundred and fifty years ago, the prevailing species concept was the one adopted a hundred years earlier by another famous naturalist, Carolus Linnaeus and his contemporaries. They assumed species to be more or less invariant, i.e. to stay constant in space and time. Such a definition of a species is now referred to as the typological species concept (Mayr 1942, 1963). Since Darwin's theory of evolution, the typological concept was doomed to pass into oblivion, and be replaced by more relevant concepts (though there is still no consensus on that issue, see Wheeler & Meier 2000). Currently, it is known that, as a result of random mutations and both natural and sexual selection, all morphological characters show variation. Conversely, no character is superior in defining the limits of species. Right?

Not exactly; there is an exception. Although practically all widely distributed species show geographic, and most of them also local variability in almost all features, this variation does not seem to extend to insect genital morphology. Roughly one million insect species have been described, but only a few species with polymorphism in genitalia are known (II). Observations of species showing considerable variation in genitalia are seldom reported. To my knowledge, there are no moth species with notable differences in genitalia between isolated populations. Regarding the non-genital characters, an almost infinite number of such cases have been reported. Considering that genital characters are significantly complex and vary more than any other morphological features *across the species* (Rentz 1972, Eberhard 1985, Shapiro & Porter 1989), genital invariability within species appears a very peculiar phenomenon. One possible explanation is that presented by Darwin: invariability is an artifact and is simply due to

circular reasoning. There is no variability in genitalia, because species are defined by them (Darwin 1859, Porter & Shapiro 1990). However, there is also another possibility, to which we return later.

Hence, there are two interesting phenomena. First, genital evolution seems to be both rapid and divergent between species. Second, once diverged, genital morphology remains stable. Much attention has been paid to the first phenomenon (e.g., Eberhard 1985). There is practically no disagreement that the observation itself is true (but see Huber 2003). The first to pay attention to the extent of the phenomenon was Dufour (1844). In a purely evolutionary context, the topic had been relatively little discussed (but see Mayr 1963) until Eberhard (1985) widely examined the generality of the phenomenon, reviewed the earlier hypotheses, and presented cryptic female choice as a potential mechanism behind the phenomenon. He also presented a large number of examples of rapid divergent genital evolution in a wide variety of taxa, and since this work, further support has been presented (e.g., Proctor *et al.* 1995). Actually, the extensive use of genital characteristics in taxonomy *per se* indicates their value in species delimitation and that they show great divergence between species. While the generality of the phenomenon itself is little disputed, its evolutionary causes are controversial. Sexual selection may also affect genital morphology through sperm competition (Waage 1979, 1984, Parker 1984) or sexual conflict (Parker 1979, Thornhill 1984, Arnqvist 1989, Arnqvist & Rowe 1995, Lessells 1999). Several examinations and tests have been performed to distinguish between the hypotheses. Most recently the topic was reviewed by Hosken and Stockley (2004).

Meanwhile, the second phenomenon concerning the limited intraspecific variability of genital characters is little studied. The reality of the phenomenon itself, as well as its generality, is insufficiently investigated. In-depth studies applying sophisticated, objective and quantitative methodology are particularly scarce. Similarly, providing that the phenomenon is true, its evolutionary causes are rarely discussed.

1.2 Hypotheses of genital evolution

1.2.1 The lock-and-key hypothesis

"If I see a number of keys, of very minute and elaborate workmanship, all different, I cannot doubt that every one is intended to fit some special lock…and this conviction is the stronger, the more varied and the more complex are the keys." P. H. Gosse, 1882

The high interspecific diversity of insect genitalia and the value of genitalia in classification were noticed well before the theory of evolution was presented. In pre-Darwinian time, genital characters had great value as they were seen to be close to the "essence" of an organism. For example Linné based his plant taxonomy exclusively on flower traits. Similarly, the first explanation for high genital diversity, the lock-and-key theory, was not presented in an evolutionary context. Dufour (1844) spoke about genital differences as "a guarantee of the conservation of Types, and a safeguard for the legitimacy of species". The lock-and-key hypothesis formed the theoretical justification

for taxonomical practices implying that species with different genitalia are reproductively isolated. The evolutionary version of the lock-and-key hypothesis was reviewed by Eberhard (1985) and Shapiro and Porter (1989). According to it, females develop complicated genitalia so that only males of their own species, those having corresponding genitalia, can fertilize their eggs. Thus, the lock-and-key hypothesis explains that the species-specific genitalia evolve to prevent interspecific copulation and fertilization. In time, the diversification is caused by disruptive selection and morphological character displacement in genitalia in situations where the distributions of two biologically distinct species, initially not having isolation mechanisms between them, come into contact (Shapiro & Porter 1989). In such a situation, rapid genital diversification occurs in hybrid areas to fill the lack of a safe mechanical isolation barrier. Presently, the lock-and-key hypothesis is not favored by evolutionary biologists, but in practice it is still largely applied in insect taxonomy, particularly in delimiting species. Most theoretical and empirical evidence is against it (Hosken & Stockley 2004), but there are also supporting observations (Sota & Kubota 1998, Kawano 2004, Usami *et al.* 2006).

1.2.2 The pleiotropy hypothesis

The pleiotropy hypothesis was presented quite early (Mayr 1963). It suggests that genitalia as internal organs are not subject to strong selection, and that their structure is pleiotropically (indirectly) determined by genes affecting traits that are under direct selection. It has been considered unlikely that structures acting in such an important role as sperm transfer and reproduction could be weakly selected both naturally and sexually (Eberhard 1985). Its disfavor is probably due to the fact that it is difficult to test, but particularly because it has been considered as a less plausible explanation (Eberhard 1985).

1.2.3 The sexual selection hypotheses

There are at least three possible mechanisms attempting to explain how sexual selection may drive genital divergence. Sperm competition was the one noticed first (Waage 1979). In polyandrous species, in which a female mates multiple times and several males compete over fertilization of her eggs, the male-male competition over fertilization may cause rapid evolution of genital structures (Birkhead & Hunter 1990, Birkhead & Møller 1998). This has been shown to be the case in odonates, in which males have developed structures that enable removal of sperm from the female's genital tracts (Waage 1979, 1984, Robinson & Novak 1997). Although frequently documented in odonates, there are few additional examples in other insects (e.g., Haubruge *et al.* 1999), and it is not probable that physical mechanisms enabling sperm removal are common, whereas some other mechanisms may be more common (Cordoba-Aguilar 1999).

The cryptic female choice hypothesis was presented by Eberhard (1985), and since then many supporting observations have been reported (e.g., Eberhard 2001, 1997, Tallamy *et al.* 2002). According to the hypothesis, polyandrous females are able to

control the sperm that fertilize their eggs, and males with best ability to stimulate females during copulation are favored (Eberhard 1985, 1996). There are two potential selection mechanisms by which genital evolution by cryptic female choice may be driven (Hosken & Stockley 2004): 1. by selection for sexy son (Fisherian Runaway process), and 2. by selection for good genes. These selection pressures are not easily distinguishable, and are a subject of ongoing theoretical debate (e.g., Kirkpatrick 1985, Møller & Jennions 2001, Cameron *et al.* 2003, Cordero & Eberhard 2003). In both alternatives female preference is favored indirectly (Chapman *et al.* 2003).

Sexual conflict was the last hypothesis to attract wider attention, but since then strong empirical evidence has been presented for it in various taxa (see Arnqvist & Rowe 2005 and references therein). It suggests that conflicts over mating or reproductive decisions between males and females drive rapid antagonistic genital co-evolution between the sexes (Thornhill 1984, Arnqvist 1989, 1997, Arnqvist & Rowe 2002a, 2002b, 2005, Rowe & Arnqvist 2002). The conflict is inherently present wherever there is not strict genetic monogamy and when reproduction is costly, and hence female preference is under direct natural selection (Parker 1979, Rice & Holland 1997, Chapman *et al.* 2003, Hosken & Stockley 2004). Neither sex can eventually win the battle (Chapman *et al.* 2003), hence the ongoing rapid antagonistic genital evolution.

The acting sexual selection mechanism is a subject of vigorous debate among evolutionary biologists (e.g., Cordero & Eberhard 2003, Arnqvist & Rowe 2005, Hosken & Stockley 2004). Particularly, this concerns the cryptic female choice and the sexual conflict hypotheses. Since both frequently give similar predictions concerning morphological effects (see below), these hypotheses have proven to be difficult to distinguish using morphological methods alone (Arnqvist 1997). Moreover, as pointed out by Arnqvist (1997) and Hosken and Stockley (2004), they are not mutually exclusive, but may act simultaneously in various combinations.

The sexual selection hypothesis, in a broad sense, is strongly supported (Arnqvist 1998, Hosken & Stockley 2004). Few evolutionary biologists now doubt that sexual selection is largely responsible for rapid genital diversification. Nevertheless, this change in point of view has had a weak effect on systematics. Morphology-based taxonomy is still largely based on the lock-and-key idea and on the assumption that genitalia show minor intraspecific variation, although this choice is possibly often unconscious.

1.3 Predictions of genital evolution hypotheses on morphology

The genital evolution hypotheses make several predictions about morphological patterns between and within species (reviewed by Shapiro & Porter 1989, Arnqvist 1997), several of which were examined in this study as well.

First, the lock-and-key hypothesis predicts that species differ in their genitalia so that no structural overlap is present. This is important, because genitalia form a mechanical isolation system between species or a backup system when the primary isolation mechanism fails (Eberhard 1985, Mikkola 1992). Sexual selection and pleiotropy hypotheses also predict rapid divergence, but as a by-product rather than as a result of selection acting upon divergence itself, as is the case in the lock-and-key concept.

Therefore, providing that sexual selection or pleiotropy is responsible for genital evolution, there may or may not be genital differences between species. In closely related species, genital morphology may show structural overlap.

Second, the lock-and-key hypothesis predicts that both male and female genital evolution is rapid, and that there is tight structural correspondence between them. Such a pattern is not predicted by the pleiotropy hypothesis, and not necessarily by sexual selection either. However, some mechanisms of sexual selection, particularly the sexual conflict hypothesis, also predict correlated evolution between the sexes (Arnqvist & Rowe 2002a, 2002b). Therefore, a structural correspondence between sexes alone is not evidence for the lock-and-key hypothesis, contrary to arguments in Mikkola (1992).

Third, geographic patterns suggesting character displacement are predicted by the lock-and-key hypothesis. Such patterns are particularly assumed in hybrid zones, where closely related species occur in sympatry (Shapiro & Porter 1989, Ware & Opell 1989). Overall, closely related species should exhibit more rapid divergence in sympatry than in allopatry. Neither sexual selection nor pleiotropy predicts geographic patterns otherwise than by chance. According to these hypotheses, there should be equal rates of divergence regardless of geographic relationships between species.

Fourth, since the lock-and-key hypothesis assumes genital divergence as a result of natural selection against heterospecific mating, genitalia of geographically isolated species should evolve slower and exhibit larger intraspecific variability. The pleiotropy hypothesis does not suggest such a pattern. Neither does sexual selection, which however, predicts more rapid divergence in polyandrous than in monandrous species. This pattern, on the contrary, is not predicted by the lock-and-key hypothesis (see Arnqvist 1997).

Fifth, the lock-and-key hypothesis assumes stabilizing selection acting on genitalia, which therefore should exhibit small intraspecific variability both in size and shape, in particular on a local scale. Providing that size variation is small in genitalia, genital size should express negative allometry in relation to body size. This means that in large individuals the genitalia should be disproportionately small when compared to small individuals. Sexual traits usually show relatively high variance in shape (Andersson 1994, Pomiankowski & Møller 1995, Arnqvist 1997). Whether or not the same concerns size variability is under dispute and depends on the acting selection mechanism (Eberhard *et al.* 1998, Bonduriansky & Day 2003). Since the pleiotropy hypothesis assumes no selection on genitalia, there should be shape and size variation equal to or larger than that in other traits.

Finally, the lock-and-key hypothesis assumes genital size and shape to be highly canalized (in local scale) and hence not greatly affected by varying juvenile conditions. Genitalia are usually bilaterally symmetric structures, and providing that their development is highly canalized, they should express very little fluctuating asymmetry. Whether this is true in terms of sexual selection depends on the operating sexual selection mechanism. The pleiotropy hypothesis assumes a relatively high degree of condition dependence.

1.4 Aims of the study

I aimed at testing genital evolution hypotheses as well as testing the relevance of morphology-based taxonomy, which largely relies on the assumption that genital characters vary less than non-genital characters both locally and geographically. These issues are very poorly studied and still less with moths. The study was based on morphological comparisons and was performed using sophisticated morphometric tools. The morphometric approach provides effective tools for distinguishing between the lock-and-key and the sexual selection hypotheses, but it is less efficient in discriminating between sexual selection mechanisms. Most taxonomic studies do not apply quantitative morphometric methods, and even fewer use modern geometric morphometrics in quantitatively exploring the extent of inter- or intraspecific variation. In this thesis, I

1. explored the extent of interspecific variation and overlap of genital variation between closely related moth species resembling each other in genitalia (I, V).
2. explored the amount of intraspecific variation, allometric patterns and asymmetry within and between external genitalia, internal genitalia and non-genitalic traits of moths (I, II, III, IV).
3. explored the relevance of the taxonomic practice that genital characters are superior in species delimitation and that they exhibit small variation in a local and geographic scale (I, III, IV).
4. explored the power of taxonomy based on a subjective non-quantitative approach as well as traditional and geometric morphometric approaches in species differentiation (V).

2 Methods

2.1 Study species

Since I focused on questions concerning genital morphology and variability in general rather than specifically, I have used several species for comparisons. For consistency, the study was, however, restricted to Lepidoptera, excluding the day-active butterflies (the rest referred to as moths). The material was composed partly of samples that were specifically collected for this study, but mostly they were gathered from museums and private collections. Although butterflies are possibly a natural, monophyletic group, moths almost certainly are not (Kristensen & Skalski 1999). Moths are simply Lepidoptera other than butterflies and are therefore paraphyletic. Lepidoptera, with about 150 000-160 000 described species and several hundreds of thousands not yet described ones (Heppner 1998, Kristensen & Skalski 1999), is one of the most prominent and species-rich insect orders. In this study, members from three significant families, noctuids (Noctuidae), geometrids (Geometridae) and tortricids (Tortricidae) were used. These three families share one important feature in respect to this study: in each group genital characteristics play a major role in their classification both in specific and higher taxonomic levels as well as in morphology-based cladistic phylogenies. Although these taxa also include genitalically similar species, the extensive use of genital morphology indicates that, in general, genital evolution in these groups is both rapid and divergent. In papers I and III, three closely related species of the genus *Euxoa* (*E. cursoria* (Hufnagel, 1766), *E. obelisca* (Denis & Schiffermüller, 1775) and *E. tritici* (Linnaeus, 1775)) were used. These moths are known to be polyandrous (Byers 1978). This group of noctuids has recently been subjected to taxonomic revision (Fibiger 1990, 1997), and the taxonomy is not wholly stabilized. Two studies were conducted with geometrids, one on the temporally dimorphic *Selenia tetralunaria* (Hufnagel, 1767) (II), and the other using species groups from four genera (*Dysstroma* spp., *Lampropteryx* spp., *Operophtera* spp. and *Pasiphila* spp.) (V). Tortricoids were applied in two studies (IV and V) as well. In the former, a single species, *Pammene luedersiana* (Sorhagen, 1885) was used while three close relatives from the genus *Ancylis* were analyzed in the latter.

2.2 Genital morphology of moths

Moths are excellent organisms for examining the questions presented in this thesis. Their genitalia are generally complex and very diverse. Many parts of external male genitalia are originally modifications of dorsal and ventral plates of segments A9 and A10 (Scoble 1995). The parts analyzed in this study, the valvae, phallus (also penis or aedeagus) and endophallus are, however, all derivations of primary phallic lobes (Scoble 1995). Valvae are external structures that function in clasping the female during copulation. There are frequently notable appendages in valvae, the function of which are inadequately known in moths. There are a number of observations of rhythmical movements during copulation in such parts in many invertebrates, and many observations suggest that they have titillating or stimulating functions (Eberhard 1991, 1993a, 1993b, 1994, 2001). Male genital structures other than phallus are not intromittent organs but couple with the female abdomen externally (though the tip of phallus may penetrate into the female) and are in this thesis referred to as external genitalia. The phallus is an intromittent organ, and the endophallus is its invaginated caudal end (Callahan & Chapin 1960, Scoble 1995, Kristensen 2003). The endophallus is eversible and often a large and complex structure, and its evolution seems to be particularly rapid since they usually have good diagnostic characters (Kristensen 2003, for instances, see e.g., Fibiger 1990, 1997, Roig-Alsina 1993, Sihvonen 2001, Yela 2002). The endophallus is mostly membranous, but there are normally sclerotized spines (cornuti) on it. These cornuti may be remarkably large and harsh-looking. Cornuti have traditionally been thought to aid in gripping the female during copulation (Scoble 1995, Kristensen 2003), but they might well be a result of evolutionary sexual conflict (see Arnqvist 1989, Arnqvist & Rowe 2005). Phallus and endophallus couple with female genitalia internally (though in many cases the phallus penetrates only partially into the female) and are therefore referred to as internal genitalia in this thesis.

The most external part of the female genitalia, papillae anales (ovipositor) are derivations of the ventral appendages of segments A8 and A9 (Scoble 1995). Papillae anales are paired, short, broad and usually setose in moths. The most prominent parts of the female genitalia are ductus bursae and corpus bursae. Ductus bursae is usually well sclerotized, at least in some parts, and in close contact with the male phallus and endophallus during copulation. The male spermatophore is stored in the large, sack-like and usually weakly sclerotized corpus bursae. There is usually a sclerotized plate, a signum, in the corpus bursae. Most other parts of female genitalia, like the oviduct, are membranous, transparent tubes usually bearing less taxonomic information. The male phallus is inserted via the ostium bursae, which is often surrounded by one or more sclerotized plates.

2.3 Genital dissection

To obtain morphometric data from small objects like insect genitalia, they have to be dissected. Principally the same preparation procedure was followed throughout the study. The dissection of moth genitalia is explained in detail in Robinson (1976). In short, the

rigid genitalia were separated from the body tissues by boiling the abdomen in 10% KOH. Similarly, the other examined body parts were boiled prior to mounting. The genitalia were cleaned, stained, and dehydrated with ethanol, and then mounted on Euparal fixative on microscope glass. The structures that are not flat were not mounted, but preserved in liquid Euparal in Eppendorf vials. The male endophalli, which are less rigid structures and everted in copulation only (otherwise they rest folded inside the phallus), were artificially everted predominantly following a procedure described in Hardwick (1950) and Dang (1993).

After preparation, the examined body parts were photographed using a digital camera. All the morphometric measurements were carried out using these photographs.

2.4 Morphometric analyses

This study was entirely based on morphometric data. Literally, morphometrics means measurement (from Greek: "metron") of shape (from Greek: "morph"). There are several ways to gather morphometric data, two of which are applied here.

2.4.1 Traditional morphometrics

The conventional way, hereafter referred to as traditional morphometrics (see Marcus 1990), is an application of multivariate statistical methods to a set of size and shape variables. Usually, these variables are distance measurements, but sometimes ratios or angles are applied as well. Distance measurements are always correlated with the size of the measured structures, and for that reason the method is not optimal for shape quantification. There have been attempts to quantify and separate shape properties from those of size, such as principal component analysis, but no traditional morphometric method can completely separate them from each other (Rohlf & Marcus 1993, Zelditch *et al.* 2004). For definitions of size and shape, see next chapter. On the other hand, traditional morphometrics is not very powerful for collecting information about shape either (Bookstein *et al.* 1985, Marcus 1990, Zelditch *et al.* 2004). This is mostly because linear distance measurements are, by definition, usually highly correlated with size.

As shown in this study, genital size is of great importance both for evolutionary biologists and systematists. Still, studies on genital morphology based solely on traditional morphometric methods easily remain ambiguous. This is because, as explained in the introduction, rapid genital evolution concerns shape rather than size properties of genital structures, and consequently because particularly systematists and taxonomists mostly apply shape features. In addition, traditional morphometrics does not enable visualizations of shape changes or shape differences between taxa.

Traditional morphometrics (see below) were used to investigate whether the three closely related *Euxoa* species show morphological overlap in genitalia (I). Similarly, this approach was used to study whether genital traits vary less than non-genital ones (I) as well as in studies of allometry (I, II). Moreover, the traditional and geometric methods were used combined with exploration of genital dimorphism (II) and exploring the genital

variation in the *Euxoa tritici* species complex (III). In the last study, I compared the usefulness of traditional and geometric morphometrics in species differentiation by their genital morphologies (V). In these studies, a set of distance measurements from the external and internal structures of male and female genitalia were taken using the ImageJ 1.34 Java image processing program.

2.4.2 Geometric morphometrics

Many attempts have been performed to mathematically differentiate the size and the shape in morphometric data (Zelditch *et al.* 2004). Although much progress was achieved, these attempts failed to completely eliminate the effects of size from those of shape. A theoretical breakthrough was achieved by Kendall (1977, 1981, 1984). Kendall (1977) defined shape as all the geometric information of configuration of points that is invariant to changes in translation, rotation, and scale. Almost all modern geometric morphometric applications are based on Kendall's theory of shape spaces (Zelditch *et al.* 2004). In that space, the smallest distance between two optimally superimposed (variation in size, location and rotation mathematically eliminated) configurations is called the Procrustes distance. There are several ways to perform superimposition (Rohlf & Slice 1990, Slice 1996, Zelditch *et al.* 2004). In this thesis, Generalized Procrustes analysis (GPA) (Rohlf & Slice 1990) was applied.

Kendall's shape space is non-Euclidean, and therefore conventional linear multivariate statistics cannot be applied to it. However, if variation between objects is small (as it usually is), Euclidean distances in the tangent space can be used to approximate Procrustes distances in Kendall's shape space (Bookstein 1996, Rohlf 1999, Slice 2001). The approximated Procrustes distances can be used in conventional statistical analyses. Furthermore, the individual centroid size, which is calculated in geometric morphometrics for each specimen to eliminate the effects of size from the shape data, is a very reliable indicator of a specimen's overall size (Zelditch *et al.* 2004).

A fundamental advantage in geometric morphometrics is that it enables the visualization of shape changes between configurations (Bookstein 1989, 1991). For that purpose, so called partial warp scores are calculated. Partial warps are an auxiliary structure for the interpretation of shape changes in sets of landmarks. Partial warp scores are quantities that characterize the location of each specimen in the space of the partial warps (Bookstein 1991). Partial warp scores are calculated to construct thin-plate spline deformation grid visualizations of shape changes between configurations. A complete set of partial warp scores can be used for conventional multivariate statistical analyses. Partial warp scores can therefore be interpreted as shape variables, but usually only a complete set of partial warp scores should be applied (Rohlf 1998). This is because a single partial warp score alone does not have any biological relevance. Geometric morphometrics is nowadays widely and increasingly used in different fields of biological sciences (Adams *et al.* 2004).

Genital shape is usually complex. Yet, there is typically a lack of easily detectable landmarks in moth genitalia. This is due to the lack of intersecting lines, tight angles etc. clear-cut and easily identifiable structures. Many genital structures, like the clasping

organs and valvae, are flat. Their outline can therefore be clearly and unambiguously defined. Similarly, the intromittent male phallus and its extension, the endophallus, were analyzed. Methods that enable outline shape quantification and combine both landmarks and semilandmarks were used. There are many outline methods; the one applied here uses a sliding semilandmark approach (Bookstein 1997). Semilandmarks are points situated on the curve between the endpoints (which must be "true" landmarks). Sliding semilandmarks are allowed to slide along the approximated outline curve to a position that minimizes its distance to a certain reference point (Bookstein 1997, Adams *et al.* 2004, Zelditch *et al.* 2004). The slid landmarks are then treated as usual landmarks.

Geometric morphometrics (see below) were used to explore genital shape dimorphism in *S. tetralunaria* (II). In a study of genital variation in the *Euxoa tritici* species complex, the method was used together with traditional morphometric tools. Studies of geographic variation, asymmetry and size-shape correlation of *P. luedersiana* were carried out using geometric methods only. Finally, the efficiency of the method was compared with traditional and non-analytic visual differentiation (V). Geometric morphometric analyses were carried out using the programs of the TPS series. The landmark digitations were done with TPSDIG 1.40 (Rohlf 2004a). For various geometric morphometric analyses the following programs were used: TPSUTIL 1.26 (Rohlf 2004b), TPSRELW 1.35 (Rohlf 2003a), TPSREGR 1.17 (Rohlf, 2003b).

2.5 Measurement error

All morphometric data is affected by at least some inaccuracy in measurements. Measurement error makes real patterns more obscure and consequently more difficult to detect. Measurement error cannot be completely eliminated, but its effect can be reduced and evaluated. Evaluating measurement error is important for two reasons. First, it enables evaluation of the relative magnitude of measurement inaccuracy. Second, it increases the statistical power of the analyses (Yezerinac *et al.* 1992, Arnqvist & Mårtensson 1998). The measurement error consists of methodological inaccuracy (distortion in specimen preparation), instrumental error (optical distortions) and inaccuracy in the measurement itself or landmark digitations (Arnqvist & Mårtensson 1998).

Some optical distortion is inherently present in all optical systems, but this distortion can be diminished using complex lens systems. Therefore, the effect of instrumental error is best reduced by using optically good equipment. With small objects such as insect genitalia, the morphometric examination usually requires the objects to be permanently mounted in a fixative between microscope slides, which makes the evaluation of preparation error practically impossible. In this study, I evaluated the measurement error by repeating measurements or digitations twice and by taking this uncertainty into account in statistical analyses. In traditional morphometric analyses, I evaluated the extent of measurement error by calculating the repeatability of measurements (I). In geometric morphometric analyses (V), the procedure of Klingenberg and McIntyre (1998) was mostly followed.

2.6 Visual subjective differentiation

Morphological species-level taxonomy is still predominantly based on non-quantitative visual categorization, though morphometric methods are increasingly used particularly to support taxonomy in difficult species groups. In two studies (III, V), a subjective visual method was used, and in V its efficiency was compared with morphometric approaches. In both studies, photographs of the genitalia of study moths were "blindly" classified by experienced entomologists.

3 Results and discussion

3.1 Interspecific genital variation

I tested whether structures that could enable safe mechanical discrimination exist in three closely related sympatric *Euxoa* species. I found that in most cases these structures were statistically different between species, but regularly with overlap (I). I tested the species-specificity of genitalia as a whole by using ordination statistics, more specifically, discriminant function analysis. This revealed that internal genitalia, that is, those genital parts that conduct sperm transfer and are inserted into a female during copulation, are more species-specific than are external genitalia (I). External genitalia have a clasping function at the start of the copulation, but there are also structures of which the function is not precisely known.

Interspecific genital variability is seldom investigated using tools that enable quantitative evaluation. Although moths usually show rapid genital diversification, there are many species complexes showing minor genital divergence between species in moths, possibly because of recent divergence of the species. One such complex is the large noctuid genus *Euxoa* (Lafontaine 1987, Fibiger 1990, 1997). The lock-and-key hypothesis suggests that spatially isolated species, that after speciation come into secondary contact, show rapid character displacement in genital morphology (Shapiro & Porter 1989). Thus, in terms of the lock-and-key hypothesis, there should be structures permitting mechanical discrimination against copulation or sperm transfer in the case of interspecific copulation.

The function of many structures in *Euxoa* genitalia is not known. For example, while a straight duct from a male into a female is all that is needed for successful sperm transfer, there are regularly complex structures present. In *Euxoa*, as well as in other noctuids in the subfamily, the male endophallus is not a simple straight and smooth duct, but consists of many large appendages, spines and cornuti. Since the male endophallus is in close contact with the female genital tract, such structures might potentially act as "key lobes" in terms of the lock-and-key hypothesis. Therefore, those structures should be species-specific as well. A unique combination of those structures would be necessary.

I examined the interspecific variability of external genitalia by using several methods in five other species complexes as well (V). The external genitalia in many of those groups, although showing some indicative differences, do not fully assure safe discrimination between species. This implies that there is intraspecific variation in external genitalia, and that this variation is sometimes so extensive that there is morphological overlap between species. It is therefore not probable that external genitalia in general might act in mechanical isolation. On the other hand, external genitalia do not couple intimately and internally with the genitalia of females and the presence of the locks and keys in these parts should be considered improbable for that reason as well.

Euxoa internal genitalia might form a relatively safe mechanical isolation system between the three study species, but experimental evidence does not support this possibility. Byers and Hinks (1978), similarly using a close complex of North American *Euxoa* moths, tested whether morphological differences in internal genitalia might prevent successful interspecific fertilization. They found that despite genital differences, sperm transfer was frequently successful. Therefore, providing that sexual selection is responsible for genital divergence, it appears possible that this selection is stronger in internal than in external genitalia, resulting in more prominent species-specificity in internal genitalia. In that case, species-specificity *per se* would be a by-product of rapid evolution driven by sexual selection. If so, uniqueness of genitalia should not be interpreted as evidence for the lock-and-key hypothesis. Meanwhile, the lack of species-specificity can be seen as evidence against the lock-and-key hypothesis. In terms of the pleiotropy hypothesis, there is hardly any reason to assume observed patterns, but on the other hand, this hypothesis does not reject the observed patterns either. It is possible that internal genitalia are less subject to natural selection than external genitalia, leading to less rapid diversification and hence less species-specificity there.

My findings emphasize the importance of internal genital structures in morphological studies of genital evolution (cf., Mikkola 1992). Unfortunately, internal genitalia are neglected in many studies. This is apparently because the most significant internal genital structure in moths and many other insects, the endophallus, while bearing much valuable information is a cryptic organ that is inverted and folded inside the phallus while at rest. It is everted into the female's corpus bursae during copulation only. A technique for its artificial eversion has been developed relatively recently (Hardwick 1950), and has been commonly used in taxonomy for only a few decades.

3.2 Intraspecific genital variation

I examined intraspecific genital variation in several species, and performed a quantitative comparison of non-genital body parts with males of three *Euxoa* moths (I). In this study, genitalia were divided into external and internal genitalia (see Materials and methods). There are usually projections in external genitalia which possibly have titillating or stimulating functions.

I found external genitalia to vary the least (I). This pattern was similar in all three analyzed species. Meanwhile, internal genitalia, those showing the most divergence between the species, seem to vary considerably within species. Variation in internal

genitalia was equal to that of non-genitalia. The same was true in *S. tetralunaria*, which also indicated more variability in internal than in external genitalia (II). However, although not compared with non-genitalia, the investigation of the external genitalia of *P. luedersiana* revealed considerable variation there (IV). Furthermore, overlap between species in many groups of closely related species indicates at least some intraspecific variation in the external genitalia (V).

Studies investigating genital variability have observed a varying amount of variation (Goulson 1993, Eberhard *et al.* 1998, Teder 1998, Baminger & Haase 2000, House & Simmons 2002, Itämies *et al.* 2003, Kawano 2004). Arnqvist and Thornhill (1998) quantitatively explored and compared genital variation with that of non-genitalia variation in the water strider. They detected that genitalia vary as much as non-genital traits and therefore did not find support for the lock-and-key hypothesis. Meanwhile, their results were in accordance with the sexual selection hypothesis, which assumes relatively high variation in sexually selected traits (Alatalo *et al.* 1998, Pomiankowski & Møller 1995, Arnqvist 1997). Nevertheless, there are studies revealing opposite patterns as well. Eberhard and colleagues (1998) investigated coefficients of variation in twenty species of insects and spiders. This comparison revealed a trend of limited variation in genitalia since in 13 out of 20 species genital variation was smaller than that of non-genitalia. This was the case in species where genitalia were rigid rather than soft. Accordingly, I found the rigid external genital parts of *Euxoa* moths to vary little, but the softer internal structures were more variable (I).

Eberhard and colleagues (1998) correctly pointed out that the small values of coefficients of variation in genitalia may be solely caused by negative allometry. Moth genitalia usually show negative allometry with body size (I, II) (see below). It is therefore possible that my observation of low variation in external genitalia of *Euxoa* moths might be due to size allometry. In that case, stabilizing selection would be directed to size, but not necessarily to shape features, which are those evolving most rapidly. Alternatively, the same artificial pattern may emerge if there is positive allometry in a part assigned as a body size indicator. The latter possibility is less likely in my study, because femur length, a part used to indicate body size, was found to reliably describe the overall body size. These potential problems could be avoided using geometric morphometric tools, because this method eliminates all size variation from the measurement data (e.g., Zelditch et al 2004). Since there was clear negative allometry in internal genitalia as well, it is possible that, for the reason mentioned above, the shape of those characters varies more than that of non-genitalia. On the other hand, internal genitalia as less rigid structures may be more susceptible to preparation error. Since insect genitalia are permanently mounted on a fixative, the preparation cannot be repeated and consequently the preparation error cannot be measured. This problem may prove to be difficult to avoid when working with insect genitalia (V).

Taken together, despite small variability in *Euxoa* external genitalia, my observations of intraspecific variation cannot be interpreted as clear support of the lock-and-key hypothesis. Rather, I have shown that internal genitalia generally vary greatly. Low variability in external genitalia suggests that pleiotropic affects are weak there. The results are in best agreement with the sexual selection hypothesis. In many cases typological interpretation of genital differences would lead to unjustified splits of genitalically variable species (II, III, and IV). To answer whether external genitalia in

general vary less and whether they are under stabilizing selection demands further studies concentrating separately on their size and shape.

   I found discontinuous variation in male and female external and internal genitalia between the genetically identical but life-historically differing forms of *S. tetralunaria* (II). The finding is among the few reported cases of dimorphism in genitalia (Schrag *et al.* 1994, Mound *et al.* 1998, Hausmann 1999, Huber & Gonzalez 2001, Jocque 2002). In spiders, the polymorphism is presumably caused by sexual selection (Jocque 2002), but in *S. tetralunaria* polymorphism is probably an indirect consequence of variation in number of larval instars between the forms; the first generation larvae usually having one instar more than the second generation. Both forms may however occur contemporarily as well (Mikkola *et al.* 1989). My finding suggests that genital morphology is not tightly canalized, as predicted by the lock-and-key hypothesis. Providing that sexual selection would be responsible for genital evolution, this phenomenon is quite expected. The low number of genital polymorphism may be an artifact, i.e. because such forms may readily become described as different species. Particularly this is the case on a larger geographic scale, where populations differing in genitalia are automatically treated as different, reproductively isolated species (Porter & Shapiro 1990).

3.3 Allometry between genital and body size

I investigated the correlation and allometry between genital and body size in males of three *Euxoa* moths (I), and allometric patterns in both sexes of *S. tetralunaria* (II). The sizes of separate genital traits were in general found to correlate positively with body size. However, in the internal genitalia of *E. cursoria*, only one out of five traits showed a positive correlation. Despite frequent positive correlations, both external and internal genital traits usually showed clear negative allometry to body size (I). This suggests that small individuals tend to have disproportionately large genitalia and vice versa. These patterns were not likely due to positive allometry between femur length (used to indicate overall body size) and overall body size, because of its isometric relation to other non-genital traits. Negative allometry was more pronounced in internal genitalia, though only indicatively (I). The allometry investigation in *S. tetralunaria* revealed consistent patterns in both sexes. Similarly, the negative allometry was more prominent in internal genitalia (II).

   The relationship between genital and body size has been studied on several occasions recently (Eberhard *et al.* 1998, Palestrini *et al.* 2000, Schmitz *et al.* 2000, Uhl & Vollrath 2000, Tatsuta *et al.* 2001, Bernstein & Bernstein 2002, Eberhard 2002, Ohno *et al.* 2003, Kawano 2004, Hosken *et al.* 2005) and the evolutionary causes and consequences of various allometric patterns are currently under a debate (Bonduriansky & Day 2003). Traditionally, sexually selected traits have been assumed to be under directional selection, and because size of sexual characters is thought to express male fitness, genital traits should show positive allometric relationships (genitalia disproportionately large in large individuals) to body size (Green 1992, Petrie 1992). In terms of cryptic female choice and good viability genes models, females might be able to evaluate the overall size of males by their genital size, leading to a situation where males being able to grow

disproportionately large genitalia are favored by females (Eberhard *et al.* 1998). The same pattern is expected by the sexual conflict hypothesis since it assumes that males with large genitalia would be more capable of forcing females to copulate with them (Alexander *et al.* 1997, Fairbairn *et al.* 2003). There is also empirical evidence that traits (other than genitalia) used in sexual interactions indeed tend to show positive allometric slopes (Petrie 1988, Alatalo *et al.* 1988, Møller 1991, Green 1992, Burkhardt *et al.* 1994, Simmons & Tomkins 1996, Teder & Tammary 2005). On the basis of a couple of earlier investigations, Arnqvist (1997) concluded that genital size generally correlates positively with body size. The allometric patterns, however, remained poorly studied.

Eberhard and colleagues (1998) reported, in a number of insects and spiders, that against all their presumptions, genital traits tended to show negative rather than positive allometric slopes with body size. In other words, they found large individuals to have disproportionately small genitalia. This finding was so far in good agreement with the lock-and-key, but not with the sexual selection hypothesis. They, however, defined a new theory consistent with the cryptic female choice hypothesis. The one-size-fits-all theory suggests that sexually selected traits that operate in the tactile rather than visual channel may imply different selection. Therefore, in that case intermediate genital size should be favored by females. Similarly, selection by females should favor standard genital size. Eberhard and colleagues (1998) divided sexual traits into those that are designed to stimulate females tactilely, those designed to force females into mating and those designed to visually or aurally affect female choice. Eberhard and colleagues' (1998) conclusions were questioned on methodological grounds (Green 1999, also see Eberhard *et al.* 1999), but as indicated by Bernstein and Bernstein (2002), the regression methodology does not significantly affect the eventual results.

The results I obtained are in a good agreement with Eberhard and his colleagues' (1998) theory, but are not fully inconsistent with the lock-and-key hypothesis either. Several recent studies have further supported the generality of negative allometry in genitalia in diverse arthropod species (Palestrini *et al.* 2000, Schmitz *et al.* 2000, Uhl & Vollrath 2000, Tatsuta *et al.* 2001, Bernstein & Bernstein 2002, Eberhard 2002, Ohno *et al.* 2003, Kawano 2004, Hosken *et al.* 2005). It therefore seems justified to conclude that the pattern is common, at least in arthropods, but its evolutionary causes remain unclear. Additional notes to the discussion on allometric patterns were given recently by Bonduriansky and Day (2003), who showed by modeling that sexual selection may actually produce any pattern of allometric or isometric relationships depending on selective regimes and the precise nature of net selection. The generality of negative allometry suggests that there is a common cause behind the phenomenon. Although rarely shown empirically (but see Tadler 1999), it seems reasonable to assume that genital size is under stabilizing selection (see also Hosken *et al.* 2005). There is also empirical evidence in beetles that difference in genital size may create costs and reduce gene flow between species (Sota & Kubota 1998, Usami *et al.* 2006). It cannot therefore be rejected that a somewhat stable size of genitalia may be necessary for successful mating for mechanical reasons, hence the relatively low size variance in genitalia compared to other sexually selected traits (House & Simmons 2002). It is worth noting that this observation alone should not be interpreted as direct support for the lock-and-key hypothesis.

In any case, the finding of negative allometry in genitalia is in poorest agreement with the predictions of the pleiotropy hypothesis.

3.4 Fluctuating and directional asymmetry in genitalia

Male moth genitalia are usually bilaterally symmetrical, even though there are exceptions among many taxa (IV). In this study, I measured the degrees of fluctuating and directional asymmetry in male valvae of *P. luedersiana* (IV). The analysis indicated significant directional and fluctuating asymmetry both in size and shape, which is not artificially caused by systematic or random measurement error (see Merilä & Björklund 1995). In centroid size, the amount of fluctuating asymmetry was much lower than in superimposed landmarks, possibly indicating that there is either stabilizing selection on genital size or that genital shape expresses unusually large variability.

   Fluctuating asymmetry between genital sides is a little studied issue, although Arnqvist (1997) emphasized its potential in distinguishing between hypotheses by revealing the relative degree of canalization versus condition dependence in genital traits. So far only one study has concentrated on the subject (Arnqvist & Thornhill 1998). Even though they found condition dependence in genital size, genital traits showed lower levels of condition dependence and fluctuating asymmetry than non-genital traits. There was a notable amount of measurement error involved, which possibly blurred their results. I did not compare the amount of genital fluctuating asymmetry with that of non-genital traits, but a comparison was made between size and shape asymmetry of genitalia (IV). Measurement error was relatively small, and the results suggest that size effects may be more tightly regulated than shape effects, which is in agreement with the amount of intraspecific variation in those features. The results support the possibility that genital size is under stabilizing selection (Hosken *et al.* 2005), but it is difficult to evaluate whether shape variables are more sensitive to preparation error than is genital size. As mentioned above, even though the effects of digitizing error were taken into consideration, preparation error was not, because of difficulties in repeating dissection and mounting.

   Directional asymmetry was, although statistically significant, regularly small (IV). I found directional asymmetry both in shape and size features of *P. luedersiana*. Since female internal genitalia are not symmetric in moths, including the study species, the occurrence of directional asymmetry in male clasping organs is not a totally unexpected phenomenon. It is possible that this asymmetry is due to the nervous system of the female abdomen. It is, however, not known why some taxa have evolved substantially asymmetric male external genitalia while others show no significant, or only slight differences between genital sides. Presumably, male genital asymmetry is evolved either to affect cryptic female choice or as a result of sexual conflict. Causes for strong genital asymmetry have apparently not been studied in an evolutionary context, but should be.

3.5 Geographic variation of genitalia

Geographic variation in genital characters is an issue of both evolutionary and systematic importance. Unfortunately, it is also a very poorly investigated topic which was investigated to a limited extent in this thesis as well, partly because of a general lack of suitable material. Geographic variation in size and shape features was studied in *P.*

luedersiana in a restricted area of its whole geographic range. No effects were found in shape properties, but overall male genital size was significantly larger in one population than in other populations (IV). It was not examined whether specimens of this population are larger than those of other populations.

Occurrence of morphological character displacement in genitalia in contact zones of closely related species, a pattern suggested by the lock-and-key hypothesis, has apparently been rarely studied (Ware & Opell 1989, Kawano 2002, 2003). The previous authors did not find any character displacement in spiders, while the latter did, namely in several beetle species. Hribar (1994) observed genital variation between populations of *Anopheles* mosquitoes. It seems that due to too few tests, not much can be said about the extent of geographic variation in genital morphology. This study shows that in moths at least size may vary, but this observation cannot distinguish between genital evolution hypotheses. That is because neither the sexual selection nor the lock-and-key hypotheses actually reject gradual geographic change in genital traits. Even if there would be a working mechanical isolation system in genitalia, there still might be gene flow between adjacent populations. Thus, a taxonomic practice that delimits species regardless of geographic patterns lacks a sound theoretical basis. The potential occurrence and extent of geographic genital variation is therefore an interesting issue in a systematic rather than in an evolutionary context. As mentioned in the introduction, the lack of evidence of geographic variation in genitalia may be due to circular reasoning and because such finding will automatically lead to species splits. Intraspecific genital variation on a geographic scale (in terms of the biological species concept) may prove to be difficult to test. As far as there is no theory specifically assuming stability of genital morphology along a geographic gradient, the present taxonomic practice that makes no difference between any geographic patterns between taxa lacks a sound theoretical basis and follows a rather typological concept of species.

3.6 Correlation between genital size and shape

I found genital shape variation of *P. luedersiana* to correlate with size. Correlation with overall genital (centroid) size was found in the second principal shape axes and size, but not in the first and the third axes. Since larval conditions and food quality are known to affect overall body size in moths (Pöykkö & Hyvärinen 2003), the finding suggests that varying conditions in larval period may also affect genital morphology, although this was not experimentally shown. In any case, larger individuals tend to have a different genital shape than smaller ones, which is against the predictions of the lock-and-key hypothesis. The finding is also of systematic interest. It suggests that populations of different sizes may exhibit at least slightly different genitalia, even though there would not be reproductive isolation between them. A lack of reproductive isolation between populations was actually not detected (nor investigated), but since males of each population were attracted to exactly the same pheromone, the possibility that they do not belong to the same species is not plausible (see Phelan & Baker 1987). It is known that sister species (the closest relatives) usually have different pheromones in this group (Witzgall *et al.* 1996). Moreover, different body size alone usually does not imply

different species. This further supports the opinion that small genital differences between populations from different geographical areas should not automatically be treated as a reliable indication of species status. There are species with high variation in body size on a local scale as well. It should be noted that in such cases large individuals may tend to have different genitalia than small ones, and that species with substantial variation in body size may show larger genital variability.

Arnqvist and Thornhill 1998 investigated phenotypic and genetic correlation between genital shape and non-genital traits in a water strider. They found a genetic correlation between genital shape and measures of non-genital traits (i.e., pleiotropic effects). To my knowledge, no other such examinations have been done. This observation is against the lock-and-key hypothesis and does not support the superiority of genital characters in taxonomy either.

3.7 Morphometrics and usefulness of genitalia in taxonomy

I compared the usefulness and power of subjective visual differentiation and traditional and geometric morphometrics in species identification using the genital morphologies of five species complexes, each having two to four species with genitalia closely resembling each other (V). At the same time, the study serves as a test of species-specificity of genital morphologies. In each complex, the species were easily identifiable by structures other than genitalia (a feature not very usual among Lepidoptera because of usually rapid genital diversification). The results indicated that both traditional and geometric morphometrics is more powerful in classifying species correctly than is subjective visual differentiation. The two morphometric methods were equally good, probably due to the reason that size information was completely removed from the geometric data, and on the other hand, because there was deficient information on shape in traditional morphometric data. These two methods combined provided the most accurate identifications, indicating that both size and shape are of great value in discriminating between different morphologies. The value of size is probably due to the generally small intraspecific variation in it (I, II). Although centroid size was not used in geometric analyses, it is evident that geometric shape data, with centroid size included, would be the most powerful morphometric method in species discrimination.

Zelditch and colleagues (2004) stated that morphometrics can help to answer two kinds of taxonomic questions:

1. Are the samples different enough to warrant judging them to be different species?
2. In what do they differ?

The first question can be further divided into two parts. First, morphometrics can be used to *explore* whether a sample consists of one or several species. A case study performed in this thesis would be a good example, even though the result was negative (no evidence for the presence of several species was found) (III). In such cases, explorative analyses, such as principal component analysis, are appropriate. Principal component analysis is not very effective in indicating the existence of two or several species in cases where

morphologies between species show much overlap. It is worth noting that exploratory analyses are not capable of classifying the samples into several species. This must be done subjectively, typically using distribution of individuals in scatter plots. In that sense the method does not basically differ from the subjective visual evaluation. Nevertheless, quantitative exploratory analyses may help to evaluate the amount and nature of variation as well as evaluating whether there is morphological overlap between species.

Secondly, morphometrics can be used to *confirm* the existence of several species. In such cases, confirmatory statistical analyses, like canonical variates analysis or MANOVA, are applicable. Confirmatory analyses presuppose that the sample can be divided into several subsets *a priori*. Examples where the confirmatory approach might be useful are species having two or more (distantly related) hosts, or species with discrete variation in such morphological parts that are known to commonly express polymorphism (e.g., wing patterns). Such cues alone usually do not validate species status, but are merely indicatives that several species may actually be involved. Even if the morphological differences are very small or are only average, confirmatory analyses may reveal the presence of several genetically isolated, but morphologically similar species. Morphometric findings, however, do not alone have any biological significance. This provides that certain biological conditions are satisfied as well. Particularly important is to take biogeographic patterns into consideration (see Huber 2004). This equally concerns genital as well as all morphological characteristics. Genital characteristics are often useful in taxonomy, because they evolve rapidly and therefore show differences even between sibling species, even in situations where genetic data cannot provide help to discriminate recently diverged species (Nice & Shapiro 1999). Moreover, genital morphology is useful regardless of what the evolutionary causes behind the genital evolution are.

The conventional metric morphometric approach has long been used to justify taxonomic classifications and revisions, especially delimitation of species. The method has been used to answer both types of questions formulated by Zelditch and colleagues (2004), i.e. whether a sample consists of one or several species and how do the species differ (2004), but as noted above, the differences are usually difficult to interpret due to the lack of visualizing tools. Consequently, descriptions of differences have mostly been based on subjective comparisons. The traditional approach is also not powerful in capturing shape information. This is a clear disadvantage of the method, because it is shape rather than size that often bears good diagnostic features.

Geometric morphometrics is effective in capturing shape, and for taxonomists a particularly useful property of this approach is the possibility to depict the limits of shape variation and therefore to answer the second question by Zelditch and her co-authors (2004). Furthermore, the method enables visualization of typical (average) shape. Usually in taxonomic revisions, important structures and differences are visualized, but this does not permit other researchers to evaluate whether the depicted individual represents average or extreme type. Since an essential purpose of taxonomic revisions is to provide diagnostic features of species, there is often a risk for bias that extreme individuals will become illustrated. A finding that actual variation is larger than presented in literature, or even shows overlap, is familiar for most taxonomists. Fortunately, geometric morphometrics is nowadays increasingly used to justify species delimitations in difficult cases (e.g., Rohlf *et al.* 1996, Drotz *et al.* 2001, Fadda & Corti 2001, Pretorius & Clarce

2001, Querino *et al.* 2002, Baylac *et al.* 2003, Gumiel *et al.* 2003, Becerra & Valdecasas 2004).

There are features in moth genitalia which often restrict the use of landmark-based morphometrics, or at least make it more challenging. Moth genital evolution, and usually that of other insects too, is generally so rapidly diverging that finding corresponding (homologous) landmarks may appear to be difficult. Secondly, geometric morphometrics works in a non-Euclidean (curved) space, and to get this data in a more applicable form, the shape coordinates are usually projected onto a linear tangent space. This causes distortion of the shape coordinates, but as far as differences between the shapes are small, this distortion remains negligible (Rohlf 1999, Zelditch *et al.* 2004). In insect genitalia, the differences between species may however be large, and consequently the distortion is remarkable. In such cases, this possibility of too large a distortion should therefore be taken into consideration and be checked. Thirdly, it became very clear in this study that there is typically a lack of easily and unambiguously definable landmarks in insect genitalia. Their shape is typically curved with few tight angles, intersecting lines or other well-defined landmarks. For this reason, the method applied in this study uses a combination of "true" landmarks and sliding semilandmarks, and in each analysis the outline shapes of genital structures were applied. This approach was shown to work well. Finally, insect genitalia often consisted of several more or less separate units, and it would be beneficial to gather information about these parts into a single analysis. Such an analysis, which could be very useful in taxonomy, has been developed (Adams 1999), but apparently has never been applied for taxonomic purposes.

4 Concluding remarks

In this thesis, I investigated genital variation in moths. Insect genital evolution is generally rapid and divergent. Evolutionary causes for this peculiar feature are still open to question, and the phenomenon has long been widely utilized by taxonomists. In several studies, hypotheses of genital evolution were addressed and tested (I, II, IV). Usefulness of the morphometric approach in taxonomy was investigated by examining genital variation within and between species (III, V). The results were discussed both in evolutionary and systematic contexts.

In general, I have shown that moth genital characteristics express variation, which may be considerable. This variation is more pronounced in shape properties, whereas genital size is less variable than body size. The lock-and-key hypothesis predicts stabilizing selection and hence small variation both in shape and size of genitalia. Since I found particularly shape to vary a lot, and since it is shape rather than size that tends to diverge rapidly, the findings do not support this hypothesis. Meanwhile, the findings are mostly in good agreement with the sexual selection hypothesis, but the exact mechanism by which this selection might be driven remained largely unanswered. The finding of low size variability, possibly indicating stabilizing selection, is most compatible with the one-size-fits-all concept of the cryptic female choice hypothesis. It should be noted, however, that different sexual selection mechanisms are not mutually exclusive, but may grade into one another. Existence of some structures frequently found, such as large sclerotized hooks in the internal genitalia of moths, is hard to explain by cryptic female choice alone. Such structures are known to evolve as a result of sexual conflict (see Johnstone & Keller 2000 and references therein). The mechanisms of sexual selection should be concentrated on, but this requires methods other than their morphological examination. Many findings in this thesis, like small size variation in genitalia, do not support the pleiotropy hypothesis.

Taxonomists apply, possibly often unconsciously, the principles of the lock-and-key hypothesis. Genital characteristics are often considered superior to many other features, particularly in species delimitation. This study shows that presuming that genital characters vary little within species may lead to unsound taxonomic conclusions. It is possible that the low number of observations of large (geographic or local) genital variation within species is due to the fact that they are so often defined by genitalia. This

study shows that genital characters are not invariable and that their shape may be affected by the size of the individual (IV). Genital characters are useful in taxonomy, but as far as their morphology is affected by mechanisms other than the lock-and-key, they should not be treated superior to other characters. There is only little support for the idea that genital characters might act as an isolation mechanism, but many observations that species with different genital morphology may copulate successfully.

Morphometric methods, geometric morphometrics in particular, are powerful in differentiating species, and should be used more frequently in species level taxonomy to make species delimitation less subjective. Both size and shape of genitalia are valuable and combined use of centroid size and landmark shape data in examining taxonomy of difficult species groups is a recommended approach.

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