Comparative evidence for the evolution of genitalia by sexual selection

Göran Arnqvist

Department of Animal Ecology, University of Umeå, S-901 87 Umeå, Sweden

Rapid divergent evolution of male genitalia is one of the most general evolutionary trends in animals with internal fertilization; the shapes of genital traits often provide the only reliable characters for species identification[1]. Yet the evolutionary processes responsible for this pattern remain obscure. The long-standing lock-and-key hypothesis, still popular among taxonomists, suggests that genitalia evolve by pre-insemination hybridization avoidance; that is, hybrid inferiority drives the evolution of male genitalia with a proper mechanical fit to female genitalia. The sexual selection hypothesis[2,3], in contrast, proposes that divergent evolution of genitalia is the result of sexual selection, brought about by variation in postinsemination paternity success among males. Here, by comparing pairs of related clades of insects that differ in mating system, I assess how the opportunity for postmating sexual selection affects the rate of divergent evolution of male genitalia. Genital evolution is more than twice as divergent in groups in which females mate several times than in groups in which females mate only once. This pattern is not found for other morphological traits. These findings provide strong empirical evidence in favour of a postmating sexual selection mechanism of genital evolution.

Under the postmating sexual selection hypothesis, selection on male genitalia is caused by mechanisms that generate variation in postinsemination paternity success among males. Such mechanisms include: first, any of several female processes that affect male paternity success (that is, cryptic female choice[3–5]); second, competition between male gametes for fertilization (that is, sperm competition[6,7]); and third, evolutionary arms races between males and females over the control of fertilization (that is, sexual conflict[4,8–10]). The key prediction of this hypothesis concerns the relationship between mating system and the rate of genital evolution[1,3]. In taxa in which females typically mate with only one male (monandry), there can be little variation in male postinsemination paternity success and postmating sexual selection on genitalia will thus be weak or absent. If females mate with many males (polyandry), on the other hand, there will be ample opportunity for variation in male postinsemination paternity success and therefore for postmating sexual selection also. Under the lock-and-key hypothesis, selection for hybridization avoidance is suggested to impel the evolution of male genitalia with a proper mechanical fit. In contrast to postmating sexual selection, such selection for pre-insemination reproductive isolation would be expected to be more intense in monandrous species than in polyandrous species. A given occurrence of interspecific matings will generally be more evenly distributed among polyandrous females than among monandrous females, leading to lower variation in female fitness in polyandrous species and therefore to a weaker selection for pre-insemination reproductive isolation. Here I analyse a series of phylogenetic contrast, comparing morphological divergence in pairs of related clades of insects with differing mating systems (Fig. 1a). This is the first general quantitative assessment of the rate of genital evolution under polyandry relative to that under monandry.

Comparisons of the rate of evolutionary divergence of complex morphological traits in a set of related species have been hampered by problems with identifying homologous structures, as well as by a lack of appropriate methods for quantifying shape variation. Previous comparative studies have often resorted to various subjective ratings of morphological complexity[11–13]. Here I use one of the new tools of geometric morphometrics[14], which not only provides objective and quantitative descriptors of shape but also avoids the problem of defining homologous landmarks (that is, structural points with correspondence resulting from descent from the same point in a common ancestor) across species[14,15]. By

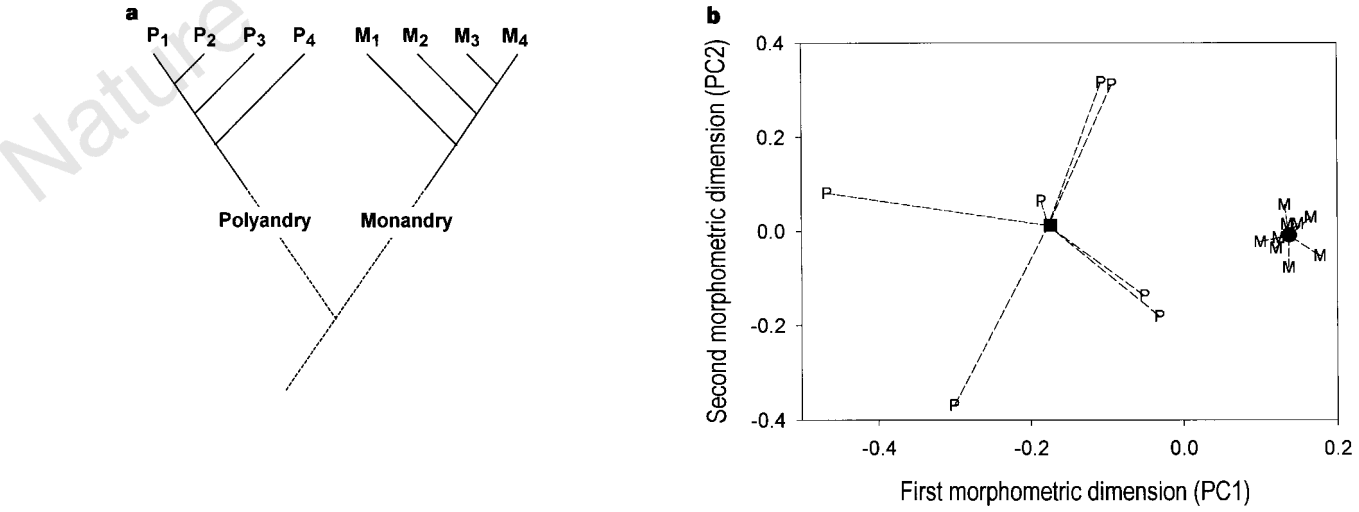

Figure 1 Comparison of the rate of genitalic evolution in polyandrous and monandrous clades. **a**, This study is based on a number of phylogenetic contrasts, where pairs of clades that share a common ancestry are compared. In each contrast, a measure of interspecific morphological dissimilarity within a clade that exhibits a polyandrous mating system (P_{1-4}) is divided by the same measure in a related monandrous clade (M_{1-4}) to form a morphometric distance ratio. Thus, dissimilarities between the two clades are ignored. A ratio higher than unity implies that morphological evolution has been more divergent in the polyandrous clade. **b**, Morphological dissimilarity within a clade was measured as the average Euclidean distance from the species to the mean of the clade in a common multidimensional shape space. The procedure is illustrated here, in two dimensions only, for male genitalia of several species in the two Dipteran genera *Dryomyza* (polyandrous, P) and *Lucilia* (monandrous, M). Dashed lines represent the distances from each species to the mean (filled symbols) of the clade. In this case, species in the polyandrous clade are about four times as different from one another as are the species in the monandrous clade (see Table 1). PC, principal component.

Table 1 Morphometric distance ratios for genital and general traits for 19 different phylogenetic contrasts

Polyandrous clade			Monandrous clade		Morphometric distance ratio	
Order	Family	Genus	Family	Genus	Genital trait	Other trait
Ephemeroptera	Siphlonuridae	*Siphlonurus* (4)	Caenidae	*Caneis* (4)	1.51	1.13
Lepidoptera	Pieridae	*Pieris* (3)	Satyridae	*Lasiommata* (3)	17.60	0.07
	Pieridae	*Colias* (4)	Satyridae	*Coenonympha* (4)	3.23	0.08
	Nymphalidae	*Eueides* (4)	Nymphalidae	*Eueides* (4)	1.47	1.42
	Nymphalidae	*Heliconius* (14)	Nymphalidae	*Heliconius* (13)	1.34	0.80
	Tortricidae	*Choristoneura* (4)	Tortricidae	*Epiphyas* (4)	1.50	–*
	Noctuidae	*Euoxa* (5)	Psychidae	*Deborrea* (5)	1.23	0.50
	Noctuidae	*Helicoverpa* (8)	Psychidae	*Cryptothelea* (7)	1.02	0.83
	Noctuidae	*Heliothis* (9)	Psychidae	*Oiketicus* (9)	1.21	0.49
Diptera	Dryomyzidae	*Dryomyza* (7)	Calliphoridae	*Lucilia* (9)	4.26	–*
	Drosophilidae	*Drosophila* (23)	Culicidae	*Anopheles* (28)	1.52	–*
	Tephritidae	*Rhagoletis* (24)	Tephritidae	*Bactrocera* (9)	1.31	1.27
	Anthomyiidae	*Coenosia* (10)	Anthomyiidae	*Delia* (11)	3.80	–*
	Cecidomyiidae	*Rhopalomyia* (13)	Cecidomyiidae	*Mayetiola* (7)	1.53	–*
	Chironomidae	*Stictochironomus* (6)	Chironomidae	*Clunio* (6)	0.93	0.49
	Chironomidae	*Chironomus* (17)	Chironomidae	*Pontomyia* (4)	2.08	–*
Coleoptera	Anobiidae	*Ernobius* (16)	Anobiidae	*Xestobium* (3)	4.34	30.29
	Elateridae	*Agriotes* (6)	Elateridae	*Ctenicera* (5)	1.39	–*
	Dermestidae	*Dermestes* (4)	Dermestidae	*Trogoderma* (9)	1.31	0.46
			Average morphometric ratio (R):		2.19	0.72
			Non-parametric tests of H_0: $R = 1$		$P < 0.001$	$P = 0.388$
			Parametric tests of H_0: $R = 1$		$P < 0.001$	$P = 0.424$

The morphometric distance ratio represents the average Euclidean distance of the species in a polyandrous clade to their mean (centroid), divided by the corresponding average distance for the monandrous clade in the contrast. Non-parametric tests of average morphometric ratios were performed with sign tests, and parametric tests with t-tests of $\log_{10}$ transformed data. Numbers within parentheses represent the numbers of species in each genus included in the analysis. A list of all species included, and a specification of the traits used, can be found as Supplementary information or obtained from the author on request.
* No comparable data available.

describing the outlines of the genitalia of each species with a nonlinear function (see Methods), and by subsequently analysing morphological shape variation among species as variance in the parameters of the fitted functions, this method allows the ordination of all the species in each contrast in a common multivariate morphological shape space (Fig. 1b).

The results of this analysis show that male genitalia evolve much more divergently in taxa in which females mate many times. The shape of male genitalia of polyandrous species were more dissimilar than were those of monandrous species in 18 out of 19 contrasts, and the average morphological distance between the genitalia of polyandrous species was more than twice that of monandrous species (see Table 1 for tests). This pattern did not differ between orders (Kruskal–Wallis analysis of variance, $P = 0.84$), and the taxonomic distance between the two clades in each contrast did not significantly affect the relative degree of genital divergence within clades (within versus between-family contrasts; Mann–Whitney U-test, $P = 0.80$). There was no association between the distance ratios of genitalia and the distance ratios of other traits across contrasts (Spearman rank correlation, $P > 0.9$). The analysis did not reveal any influence of mating system on evolutionary divergence for morphological traits other than genital traits (Table 1), and the distance ratios of genital traits were indeed significantly larger than those of other traits (paired Wilcoxon signed rank test, $P = 0.023$; Kolmogorov–Smirnov two-sample test, $P = 0.003$).

Many factors other than selection could potentially influence measures of interspecific evolutionary divergence within a given clade (such as age of clade, biogeographic characteristics, taxonomic resolution, genetic architecture and mating-system characteristics other than female mating frequency). Given the confounding role of such factors, it is remarkable that monandrous and polyandrous clades differed so consistently in the relative rates of divergent evolution of male genitalia. This study offers three important and consequential insights. First, it provides strong evidence in favour of the postmating sexual selection mechanism of genital evolution, and thus enhances our knowledge of the processes behind this general evolutionary trend[1–3,16]. Future research should attempt to determine which forms of postmating sexual selection are responsible for genital evolution[3,10,16]. Second, the results indicate that the same process (that is, sexual selection) may be responsible for the evolutionary elaboration of both primary and secondary sexual traits, suggesting that this old dichotomy, which Darwin[17] realized was problematic but nevertheless adopted, should be reconsidered[16,18]. Third, as traits evolving by sexual selection tend to be more phenotypically and genetically variable than other traits[19,20], this study calls for quantifications of the degree of intraspecific variability in genital traits. The prevailing typological view of intromittent genitalia in taxonomy, especially in species definitions, may need to be reconsidered. □

Methods

Case selection and data acquisition. I searched for clades suitable for the phylogenetic contrasts[21,22] in previous reviews[23] and comparative studies[24–26], as well as in reference databases and on the internet. Three criteria had to be met for inclusion of a clade in the analysis. First, reliable data on female mating frequencies had to be at hand, typically in the form of female spermatophore/ejaculate counts in natural populations or detailed field and/or laboratory studies of mating behaviour. Second, the phylogeny of the species included in a given contrast had to be well established. Third, taxonomic revisions containing high-quality illustrations of male genitalia had to be existent, as such illustrations were used to characterize each species.

I located 19 phylogenetic contrasts, representing four different orders, that met these criteria; all of these contrasts were independent in the sense that no clade was represented in more than one contrast (Table 1). This selection was based on a large number of published articles, as well as on personal contacts with a large number of colleagues. A complete list of these sources can be found as Supplementary information or obtained from the author on request. For each species, I captured the outlines of two trait types (male genitalia and, when available, a general trait) with a digitizing tablet (Summasketch III), using illustrations presented in published taxonomic revisions. For consistency, only one such source was used for each clade to avoid artifactual intraclade variation in morphology. The general traits were wings (nine contrasts), body parts (two contrasts) or legs (one contrast). When more than one general trait was at hand, the trait that exhibited most interspecific divergence between species in the contrast was included in the analysis.

Elliptic Fourier analysis. For each contrast and trait type, the outlines of all species were included in a common elliptic Fourier analysis[14,15,27], using the software EFA-Win (http://life.bio.sunysb.edu/morph/soft-out.html). The

Fourier analyses were made invariant of size, position and rotation, and all used 30 harmonics (yielding 120 Fourier coefficients). These functions provided a near perfect fit even to the most complex outlines.

Multivariate ordination. For each contrast and trait type, the 120 Fourier coefficients for each species were treated as variables in a principal component analysis, performed on the covariance matrix[28,29]. The first seven principal components, collectively describing >98% of the shape variation in all cases, were retained for ordination. These principal components form orthogonal dimensions in a multidimensional shape space, where all species occupy a given location (Fig. 1b). To quantify the morphological dissimilarity between the polyandrous species relative to that of the monandrous species, I calculated a morphometric distance ratio, representing the average Euclidean distance in this multidimensional space of the species in the polyandrous clade to their mean (centroid) divided by the corresponding average distance for the monandrous clade in the contrast (Fig. 1b). This ratio measures the amount of morphological variance within the polyandrous clade relative to that within the monandrous clade, ignoring variance due to differences between the clades. In contrasts in which the number of species in the two clades were skewed (difference > 1), the elliptic Fourier analysis and the subsequent multivariate ordination procedure were repeated many times (>10) using a random subsample from the more speciose clade to match the number of species in the less speciose clade, and the average morphometric distance ratio from these repeated measures was used for analysis.

Received 29 January; accepted 23 March 1998.

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Supplementary information is available on *Nature*'s World-Wide Website (http://www.nature.com) or as paper copy from Mary Sheehan at the London editorial office of *Nature*.

Acknowledgements. I thank L. Rowe and P. Watson for comments. This study was made possible by the expert advice from many entomologists (see Supplementary information), and was generously funded by a grant from the Swedish Natural Science Research Council.

Correspondence and requests for materials should be addressed to the author (e-mail: Goran.Arnqvist@animecol.umu.se).

Smad2 role in mesoderm formation, left–right patterning and craniofacial development

Masatoshi Nomura & En Li

Cardiovascular Research Center, Massachusetts General Hospital East, Department of Medicine, Harvard Medical School, Charlestown, Massachusetts 02129, USA

Signalling by the transforming growth factor-β (TGF-β) superfamily of proteins depends on the phosphorylation and activation of SMAD proteins by heteromeric complexes of ligand-specific type I and type II receptors with serine/threonine-kinase activity[1]. The vertebrate SMAD family includes at least nine members, of which Smad2 has been shown to mediate signalling by activin and TGF-β[2–5]. In *Xenopus*, Smad2 can induce dorsal mesoderm, mimicking Vg-1, activin and nodal[2,4]. Here we investigate the function of Smad2 in mammalian development by generating two independent *Smad2* mutant alleles in mice by gene targeting. We show that homozygous mutant embryos fail to form an organized egg cylinder and lack mesoderm, like mutant mice lacking *nodal*[6,7] or *ActRIB*, the gene encoding the activin type-I receptor[8]. About 20 per cent of *Smad2* heterozygous embryos have severe gastrulation defects and lack mandibles or eyes, indicating that the gene dosage of Smad2 is critical for signalling. Mice *trans*-heterozygous for both *Smad2* and *nodal* mutations display a range of phenotypes, including gastrulation defects, complex craniofacial abnormalities such as cyclopia, and defects in left–right patterning, indicating that Smad2 may mediate nodal signalling in these developmental processes. Our results show that Smad2 function is essential for early development and for several patterning processes in mice.

We generated two independent mutant alleles of *Smad2* by gene targeting in mouse embryonic stem (ES) cells. *Smad2*^{mh1} was produced by replacing part of the conserved MH1 domain with a PGK-neo cassette (Fig. 1a). To test whether this mutation was null, we generated ES cell lines homozygous for *Smad2*^{mh1} (data not shown) and analysed Smad2 expression by western blotting. We found that the amount of Smad2 protein in heterozygous ES cells was significantly reduced. In contrast, neither the normally sized protein nor a truncated form of Smad2 was detected in *Smad2*^{mh1} homozygous ES cells (Fig. 1b), indicating that *Smad2*^{mh1} is a null allele. The second allele, *Smad2*$^{mh2\text{-}lacZ}$, is a 'knock-in' insertion of a *lacZ* reporter gene into the carboxy-terminal MH2 domain created by replacing exon 8 with an *IRES*β*geo* cassette[9] (Fig. 2a). The phenotypes resulting from these two mutations were essentially identical.

The offspring from intercrossed *Smad2*^{mh1} heterozygous mice were examined at various developmental stages. Homozygous embryos were recovered at 7.5 days post-coitum (or E7.5), but not at or after E10.5 (Fig. 1c and Table 1), indicating that the

Table 1 Genotype of offspring from the *Smad2*^{mh1} heterozygote crosses

Stage	Total	Resorption	Genotype*		
			+/+	+/–	–/–
E7.5	117	1	33	53 (12)	30
E8.5	39	0	10	21 (4)	8
E10.5–E16.5	203	65	59	79 (9)	0
Newborn	112	–	48	64 (2)	0

* Defects are indicated in parentheses among heterozygous embryos: about 22% (16/74) showed gastrulation defects at E7.5 and E8.5, and about 11% (9/79) showed mandible or eye defects at E10.5–E16.5.