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Gradual evolution of male genitalia in a sibling species complex of millipedes (Diplopoda : Spirobolida : Rhinocricidae : *Anadenobolus*)

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Abstract. Jamaican millipedes in the *Anadenobolus* species complex provide an unusual case study of arthropods having undergone speciation in the absence of conspicuous divergence of male genitalia. Using landmark-based morphometrics, we examined shape deformation of the male anterior copulatory device in three genetically divergent yet morphologically cryptic species. A multivariate analysis of variance and relative warp analysis of nonuniform components show that although male genitalic shape is statistically different among species, many specimens are 'misplaced' in morphological space, perhaps consistent with a condition analogous to incomplete lineage sorting. A simulation of neutral nuclear gene coalescence suggests that such incomplete sorting is expected, given the depth of mtDNA divergences observed across species. The pronounced contrast between deep molecular *v.* incomplete genitalic divergence is at odds with the paradigm of selection-driven rapid change in male copulatory structure during arthropod speciation. Alternatively, we suggest that male genitalic divergence is evolving neutrally or in concert with other components of the genome (pleiotropy). Although we recognise the empirical validity of rapid genitalic divergence via sexual selection or sexual conflict, such models must be empirically tested using multiple lines of evidence. Accepting the rapid and divergent hypothesis without such multiple evidence scrutiny may result in a gross underestimation of evolutionary diversity and, subsequently, the misinterpretation of processes shaping genitalic change.

Introduction

Evolutionary processes underlying the divergence of animal genitalia remain one of the more puzzling phenomena in evolutionary biology (Arnqvist *et al*. 1997). The fact that little is understood about genitalic change is 'truly perplexing' (Arnqvist 1997) given their prominent role as species-specific diagnostic characteristics in many arthropod groups (Tanabe *et al*. 2001). In short, we lack a basic understanding of the processes that generate variation in the very character system often recognised as the manifestation of speciation itself. Because genitalic features are not usually 'adaptive' in the traditional sense, they are unlikely subject to the same selective forces operating on other phenotypic attributes. However, selection resulting from geographic variation in environmental conditions can indirectly drive divergence of mating signals and preferences (Schluter 2001; Kwiatkowski and Sullivan 2002) such that genitalia, or other premating and postmating barriers, evolve as a secondary consequence of ecological divergence. Moreover, speciation is considered by some to be inevitable for allopatric populations maintained over extended periods of time; thus, mating isolation is sometimes thought to evolve, facilitated by random genetic drift (Lande 1981), simply in response to the absence of forces maintaining reproductive compatibility (Turelli *et al*. 2001).

Eberhard (1985) considers the species-specific diagnosibility of male genitalia to be a reflection of both the rate and extent to which they diverge; any structure so consistently useful as a taxonomic character must have evolved rapidly, as this divergence is inextricably coupled with speciation. For mating systems in which fertilisation is internal, male genitalia likely serve a stimulatory role during copulation and thus are under *strong* sexual selection by female choice (Eberhard 1985). Sexual selection, one of the more prevalent explanations for observed 'species-specific' differences in genitalia, provides an attractive hypothesis because of its predictive and thus testable value, particularly when questions are posed in a comparative manner with multiple lines of evidence (e.g. Arnqvist *et al*. 1997; Markow 2002; Masta and Maddison 2002).

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There are at least two phylogenetic patterns of molecular/genitalic divergence that would be concordant with, or at the very least not contradictory to rapid, divergent evolution of male genitalia as a result of sexual selection by female choice or sexual conflict (hereafter referred to as 'SSFC-SC'). The first involves molecular phylogenetic patterns consistent with recent population/species divergence (e.g. relatively short branches, unresolved nodes, incomplete lineage sorting), concomitant with a pattern of divergent genitalia and putative premating isolation. Knowles (2000) and Masta and Maddison (2002) reported such patterns of divergence for grasshoppers and jumping spiders, respectively. Both examples involve taxa considered to have diverged as recently as the Pleistocene and to exhibit patterns of molecular and genitalic or mating divergence indicative of very rapid and recent genitalic evolution attributable to SSFC-SC. An alternative pattern that may not necessarily support rapid, divergent evolution of genitalia would be distinct genitalia associated with relatively long phylogenetic branches. Hedin (1997) found divergent male genital morphology in cave spider species to be concurrent with similar patterns of molecular divergence. Although such a pattern does not preclude rapid genitalic evolution early in speciation, genitalia could have likewise evolved in a neutral, or gradual, manner. Alternatively, a pattern that would appear to be in direct conflict with SSFC-SC entails a phylogeny where groups with similar genitalia are subtended by long phylogenetic branches, indicating old speciation events in the absence of significant male genitalic divergence (see Bond *et al*. 2001). We report an example of this third alternative—speciation accompanied by gradualistic change in male genitalia—in the millipede (Diplopoda) *Anadenobolus excisus* Karsch species complex.

The *Anadenobolus excisus* species complex comprises three sibling species of Spirobolida millipedes (family Rhinocricidae) that are widely distributed across the Caribbean island of Jamaica (Bond and Sierwald 2002, 2003). Although morphologically quite similar, these species form exclusive, highly divergent groups based on mitochondrial DNA sequence differences (Fig. 1 summarised from the phylogeny presented by Bond and Sierwald 2002 based on GenBank Accession nos. AF501371–AF510514). *Anadenobolus excisus* occupies the eastern end of the island, occurring throughout the John Crow Mountains. *Anadenobolus holomelanus* (Pocock) and *A. dissimulans* Bond and Sierwald occur throughout the central northern and western aspects of Jamaica with narrow sympatric ranges in the central northern areas of their distribution. In this zone of hypothesised secondary contact, populations of *A. holomelanus* and *A. dissimulans* remain genetically cohesive as shown by character displacement in overall body size (Bond and Sierwald 2002). These taxa have thus attained species status under both biological and phylogenetic criteria (Bond and Sierwald 2003). Although millipede species are diagnosed primarily on the basis of male genitalic differences, our previous qualitative assessments using scanning electron microscopy found no differences among these three species (Bond and Sierwald 2003). Using morphometric techniques, thin plate spline and relative warp analyses, we report studies of genitalic shape that demonstrate subtle, but seemingly incomplete, quantitative shape changes across taxa. This observation, in combination with neutral gene coalescence simulations, suggests that neutral evolution, rather than selection, may best explain patterns of male genital evolution in this complex.

Materials and methods

Morphometric analysis of gonopod shape

Male millipede genitalia, 'gonopods', comprise one or more modified leg articles. *Anadenobolus* gonopods consist of a pair of structures modified from the anterior and posterior walking legs on segment eight. Anterior gonopod shape, particularly the posterior aspect of the structure, is one of the more diagnostic features used in rhinocricid taxonomic studies (Bond and Sierwald 2003) and is therefore the aspect of the gonopod we used in our study of gonopod shape deformation (Fig. 2*A*). Gonopods were dissected from specimens and muscle tissue was dissolved using proteinase K (Qiagen Inc., Valencia, CA, USA). Specimens were photographed with a digital camera at the highest magnification possible while viewed under a Leica MZ 12.5 stereomicroscope.

Landmarks, loosely defined as discrete anatomical points, have been partitioned into three types by Bookstein (1991): type 1 corresponds to the meeting point of two or more tissues, type 2 marks points of maximum curvature, and type 3 includes external points. Land-

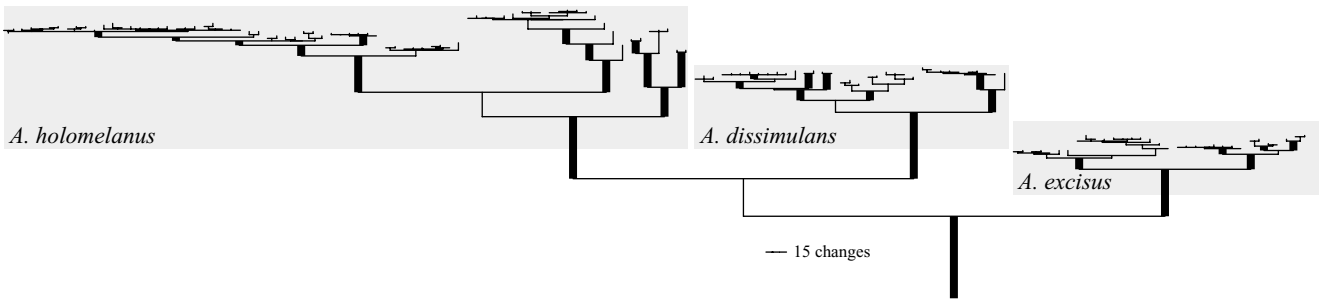

Fig. 1. *Anadenobolus excisus* phylogeny summarised from Bond and Sierwald (2002). This phylogeny is based on phylogenetic analyses of ~1030 base pairs of the *16S* rRNA mtDNA gene sequenced for 242 individuals (144 unique haplotypes). Each of the three major clades is supported by bootstrap and posterior clade probabilities greater than 98%.

marks of type 1 are homologous points among individuals, whereas landmarks of types 2 and 3 are not necessarily homologous and are referred to as pseudolandmarks. We recorded ten two-dimensional landmarks on the right aspect of the anterior gonopod for each male specimen included in the study (Fig. 2*B*). Five landmarks are considered to be type 1 (5, 6, 8–10) whereas the remaining are primarily type 2 (1–4, 7). Landmarks were plotted on digital images using the programme tpsDIG32 version 1.31(Rohlf 2001*a*).

With the aid of the computer programme tpsRelw version 1.24 (Rohlf 2001*b*), landmarks were translated to the same origin, aligned, and scaled to size using a generalised orthogonal least-squares procedure (Procrustes method; Rohlf and Slice 1990) to obtain a tangent, or consensus configuration (Rohlf *et al*. 1996). Genital shape variation was then decomposed into uniform and nonuniform components by the thin-plate spline method. Uniform or affine components correspond to overall oblique transformation of shape in addition to simple overall linear stretching or compression of specimens. These are changes in shape (elongation and/or narrowing) that do not involve bending. Real biological shape transformations are rarely if ever completely uniform, so a complete description of shape change also requires stating the nonuniform deformations. Nonuniform or nonaffine components correspond to more complex changes in shape, such as nonlinear patterns of deformation where changes can be localised to small regions. These are changes in shape that involve bending. Both uniform and nonuniform components were calculated with the programme tpsDIG32. Centroid size, which is defined as the square root of the sum of the squared distances from each landmark to their centroid, was calculated for each specimen using the programme tpsRegr version 1.24 (Rohlf 2001*c*). Centroid size was used as a size component estimate of male genitalia.

To identify and describe major trends in non-linear shape, we performed a principal components analysis on the nonuniform components. Relative warps were calculated with the scaling parameter α set to zero as recommended by Rohlf (1993), a value that weights all of the principal warps equally and thus is appropriate for exploratory systematic studies. The nonuniform components of male genitalia were then ordinated using the programme PC-ORD version 4 (McCune and Mefford 1999) following the PCA procedure with the variance/covariance centred in the cross-product matrix. To determine if genitalia shape differed significantly among lineages, we performed a one-way MANOVA (GLM) on the uniform and nonuniform components. A one-way ANOVA of centroid size was used to determine if the size component of genitalia differed among lineages. All statistical analyses were performed using the computer programme SPSS version 11.5.

Gene tree simulations

We use a coalescence approach similar to that developed by Masta and Maddison (2002) to simulate nuclear gene trees. Our hypothesis is that genitalia are nuclear-encoded phenotypes that are evolving in a neutral manner, as opposed to being shaped by selection (either sexual selection or sexual conflict). We used observed mtDNA gene trees of *Anadenobolous* as our neutral framework (complete reciprocal monophyly, interclade divergences on average four times intraclade divergences), simulated gene trees that were consistent with these observed patterns and then adjusted parameters to simulate nuclear genes. Specifically, the expected 4-fold difference in effective size between nuclear and mitochondrial genes was accounted for by decreasing branch lengths by one-fourth on simulated nuclear gene trees (Moore 1995). These simulations suggest incomplete lineage sorting of nuclear genes, given empirical patterns observed in our mtDNA (see below). We used this pattern of incomplete sorting as our neutral expectation for genitalic data. Failure to reject the null hypothesis of incomplete lineage sorting would suggest that selection is highly unlikely.

Computer simulations, using the programming system MESQUITE ver. 0.994 (Maddison and Maddison 2003), were used to generate 10000 gene trees by gene coalescence, contained within a three taxon species tree. The number of 'genes' used in the simulation reflected the total number of mtDNA haplotypes (144) identified by Bond and Sierwald (2002): *A. excisus* (Clade I) – 33, *A. dissimulans* (Clade II) – 35; *A. holomelanus* (Clade III) – 76. The effective population size (N_e) was set to 100 for all simulations. To evaluate the completeness of lineage sorting, we used Maddison's (1997) deep coalescence statistic ('the number of extra gene lineages on a species branch') assessed for two separate simulations. First, branch lengths (generations since isolation) were set to 600. At this length we conservatively estimate that average inter-clade divergence is at least

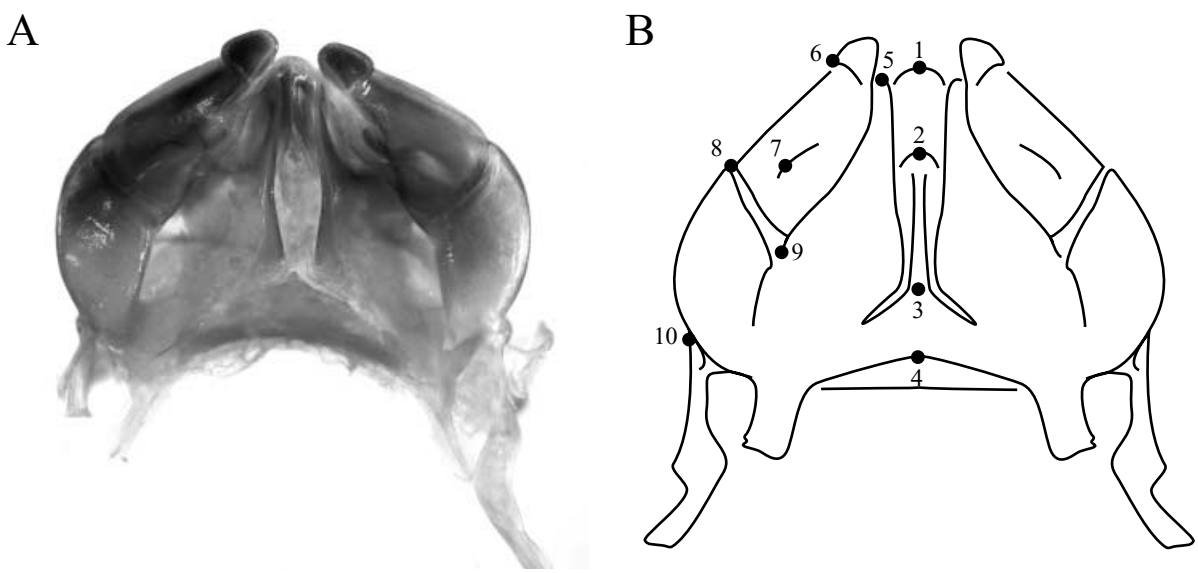

Fig. 2. Posterior aspect of the anterior gonopod (male copulatory device). *A*, Anterior gonopod dissected from *Anadenbous dissimulans* specimen. *B*, Line drawing of stylised *Anadenobolus excisus* anterior gonopod showing positions of each landmark scored for the relative warp analyses.

Table 1. Summary of between-group statistics for shape components and centroid size

Effect	Value	*F*	Hyp. d.f.	Error d.f.	*P*
MANOVA: Uniform components					
GROUP Wilk's lambda	0.853	3.433	4.000	166.000	0.010
MANOVA: Nonuniform components					
GROUP Wilk's lambda	0.207	6.084	28.000	142.000	0.000
ANOVA: Centroid size					
GROUP		0.771	2		0.466

on average four times the average intra-clade divergence (this ratio ranged from 2 to 4 times for the mtDNA data). We consider such deep divergence to be an accurate simulation of the mtDNA data. Second, deep coalescence was considered for trees simulated with branch lengths of 150, a value that is one quarter the length of the previous simulation (this value simulates nuclear genes with four times the theoretical N_e of the mtDNA genes).

Results

Anterior gonopod landmarks scored for 86 specimens representing over 50 localities (see Bond and Sierwald 2002 appendix 2 for a summary of localities included in this analysis) reveal an interesting pattern of genitalic divergence. The MANOVAs for both uniform and nonuniform components of shape show significant variation (Wilk's lambda $P = 0.01$ and $P < 0.01$ for uniform and nonuniform components, respectively) among the three cryptic species (Table 1). An ANOVA of centroid size, however, did not exhibit significant variation among species ($P = 0.47$).

The first two principal components (PC) based on the nonuniform components of male genitalia explain 59% of the variation among lineages. In the ordination scatterplot of PC1 and PC2, the individual mtDNA lineages (described as nominal species by Bond and Sierwald (2003) could not be divided into discrete, non-overlapping subgroups on the basis of nonlinear shape alone (Fig. 3). However, gonopod shape for each of the major lineages appears to be 'sorting out' according to species (approximately delineated in Fig. 3), as illustrated by the continuous gradient in the ordination plot. Specimens of *Anadenobolus excisus* (referred to as Clade 1 in Bond and Sierwald 2002) almost exclusively occupy one side of the ordination space, whereas the other side of the ordination is populated largely by members of *A. holomelanus* (Clade 3). *Anadenobolus dissimulans* specimens (Clade 2) occupy the ordination space between the other two species.

Coalescent gene-tree simulations with relative branch lengths of 600-generation times since isolation gave a very high probability of complete lineage sorting (i.e. the probability of deep coalescence of $P = 0.98$), comparable to that observed in the mtDNA dataset. However, simulations using relative branch lengths set to one quarter the completely sorted value (150) gave a very low probability of complete lineage sorting (Fig. 4; $P = 0.08$). Therefore, we would not necessarily expect the pattern of reciprocal monophyly observed in the mtDNA data to be reflected by a large percentage of nuclear genes.

Discussion

Members of the *Anadenobolus excisus* species complex do not appear to fit a model of rapid, divergent genitalic evolution expected for SSFC-SC; but instead their genitalia appear to be evolving in a neutral manner. Based on the degree of molecular divergence in *Anadenobolus* (mtDNA lineage separation times >5 million years before present, Bond and Sierwald 2002), we hypothesise that population/species and male gonopod divergence is decoupled (Hedin 1997; Bond *et al*. 2001) in this species complex. The rate of genitalic evolution, as depicted by the ordination of gonopod shape deformation, has been very conservative with respect to mtDNA divergence and does not definitively reflect the pattern of incipient speciation one would expect based on such patterns of extreme molecular divergence.

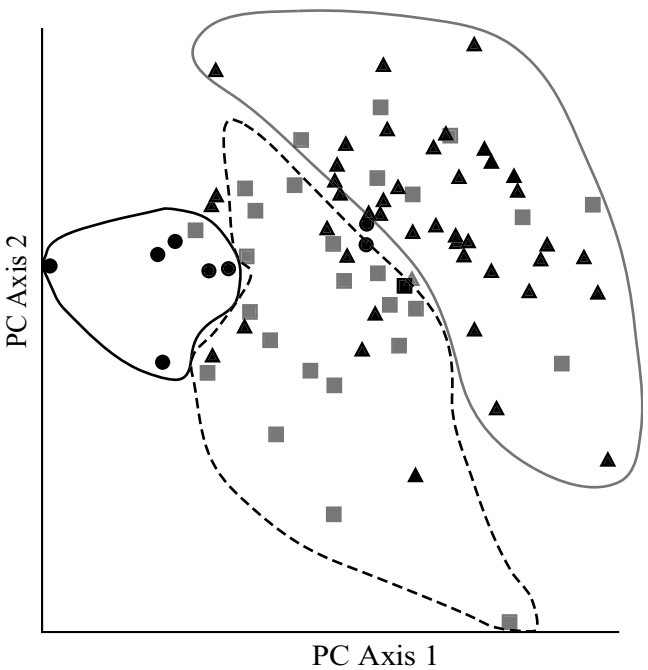

Fig. 3. Ordination plot of the nonuniform components, 59% of the variation is explained by the two axes depicted. Circles = *Anadenobolus excisus* (Clade 1); grey squares = *A. dissimulans* (Clade 2); triangles = *A. holomelanus* (Clade 3).

Based on current estimated rates of mtDNA evolution and our gene tree simulations, we would predict that considerable additional time is required for gonopod shape to sort in a fashion consistent with the mitochondrial genes (Fig. 4). The results of the ordination (Fig. 3) agree with a neutral hypothesis, with gonopod shape sorting in a neutral manner analogous to genes engaged in random lineage sorting. That is, 'polyphyly' of gonopod morphology potentially reflects the retention of ancestral morphologies. Although gonopod shape among the three species is 'statistically' different, the ordination demonstrates some overlap. Given a sufficient amount of additional time, we would anticipate gonopod shape eventually 'sorting' to reciprocal monophyly (*sensu* Avise *et al*. 1983).

An alternative explanation for the sharing of genital morphology across species boundaries is interspecific gene flow. This gene flow would have to be confined to the nuclear genome, as available data clearly indicate a lack of mitochondrial introgression (Bond and Sierwald 2002), and is expected to be more prevalent in geographic regions of species sympatry. We argue that the introgression hypothesis fails to explain our data, for two reasons. First, if introgression is more likely in sympatry, we might expect sympatric populations to share morphology, whereas geographically isolated populations might be expected to show distinctive genital morphologies. Such a pattern is not evident in the ordination scatterplot of PC1 and PC2 (Fig. 3), where none of the individual nominal species comprise discrete, non-overlapping subgroups. For example, *A. excisus*, which is completely allopatric from the other two species (Bond and Sierwald 2002), does not occupy a morphological space exclusive of the other taxa. While *A. excisus* does appear slightly more distinctive as compared to *A. dissimulans* and/or *A. holomelanus* (Fig. 3), we note that these latter species also share a more recent common ancestor. The second, more direct, argument against nuclear gene flow in sympatry follows from the observation of morphological character displacement where *A. dissimulans* and *A. holomelanus* occur in sympatry (Bond and Sierwald 2002). Populations of these species are found both in both allopatry and sympatry, but have evolved, and maintain, significant differences in body size where sympatric. Although these differences might be maintained by selection in the face of gene flow, it is more parsimonious to argue that the observed body size differences reflect a lack of gene exchange.

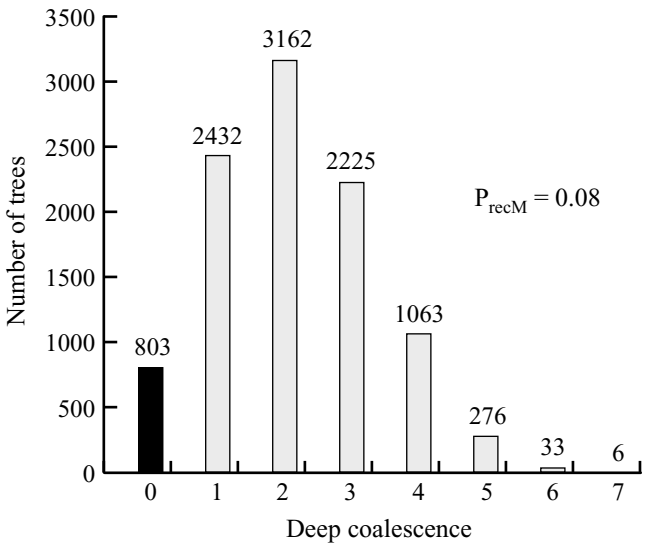

Fig. 4. Distribution of deep coalescence for gene tree simulations (branch length = 150 generations), P_{recM} = probability of reciprocal monophyly or complete lineage sorting.

These results bring into question the primary character system on which many millipede morphospecies are delineated, and to some degree, how we think about genitalic evolution (at least for many arthropod groups). First, putative millipede species based on qualitative genitalic differences may be overly inclusive, masking multiple monophyletic groups comprising multiple species (Bond and Sierwald 2002). As a result we may be overlooking a substantial amount of evolutionary diversity with such lumping. Second, the prima facie assumption that genitalia evolve rapidly and divergently in concert with speciation is inherently problematic because it influences our perception of how genitalia must evolve. If they evolve in a manner that is 'punctuated' by speciation then this evolutionary event must necessarily be rapid. It follows that if speciation and genitalic change are synchronised, then genitalia are features that should be used consistently to recognise reproductively isolated species. Together both points form a tautology that grossly affects how we characterise the evolution of diversity and how we measure it. For example, as discussed earlier, millipede species are primarily described and identified on the basis of male genital morphology. Based on this premise, the prevailing morphological species concept dictates that speciation and divergence of male millipede genital morphology be tightly coupled. Because they are tightly coupled changes in genital morphology must be rapid and divergent to be consistent with SSFC-SC.

We propose that a lack of major qualitative male genital shape change in *Anadenobolus*, concomitant with their substantive genetic divergence, is more consistent with either a pleiotropic or neutral hypothesis of genitalic evolution. The pleiotropic hypothesis postulates that genitalia evolve in a neutral manner in concert with other, more general morphological features (Eberhard 1985; Arnqvist *et al*. 1997). That is, genetic changes that occur in a species during divergence result in 'an incidental change in the structure of the genitalia' (Mayr 1970: 64). Our evidence for this hypothesis is anecdotal but nonetheless consistent with the observed pattern of gonopod shape variation and limited morphological divergence observed in this group of millipedes. Given the constraints of genitalic evolution as a

function of pleiotropic effects, we would predict concurrent stasis, or neutral evolution in parallel, of both general and genitalic morphology. Arnold (1973) suggested that genitalia are more likely to 'store' pleiotropic changes that would otherwise be eradicated in organ systems more closely checked by natural selection.

Any changes in male genitalic structure that reduce mating efficiency, however, may be subject to normalizing selection or result in compensatory changes in the female genitalic phenotype. Such compensatory changes in female genitalia are unknown. Since female vulvae of millipedes have rarely provided diagnostic characters at any taxonomic level (Kraus 1966: 24), these structures are seldom included in α-taxonomic research. Moreover, it is highly unlikely that for many millipede groups the morphological structure of female vulvae would support any compensatory changes, since the sac-like, membranous female genitalia are flexible and less precisely structured (Demange 1959; Kraus 1968; Tadler 1993) and thus more accommodating to changes in male morphology (i.e. compensation is an intrinsic property of the female system). Finally, an alternative explanation may be that gonopod morphology is relatively unimportant and thus neutrally evolving in an unlinked manner in this sibling species complex. Because we know very little about the details of millipede mating behaviour, there may be many other premating and copulatory cues that function as 'proxy' genitalia and may in fact 'fit' a model of SSFC-SC. The marginal differences observed in gonopod shape may be consistent with a neutral, 'genitalia do not matter' hypothesis, since like molecules, morphological variation can potentially be geographically associated.

Conclusions

The Jamaican *Anadenobolus* sibling species complex provides us an important and unique opportunity to understand how arthropod genitalia may evolve neutrally, since this group has undergone speciation but appears only subsequently to have begun male genital differentiation. We draw this conclusion by comparing rates of observed molecular divergence and degree of genital morphological divergence using morphometric techniques. We find that deeply diverged molecular lineages appear to be slowly sorting genitalic shape in a neutral manner inconsistent with rapid and divergent evolution of male genitalia predicted by the hypothesis of SSFC-SC.

Our example illustrates one of the three possible patterns of genitalic/molecular divergence (Pattern 3): pattern 1 = divergent genitalia/non-divergent neutral genotype—sexual selection by female choice, but also consistent with sexual conflict (SSFC-SC; e.g. Masta and Maddison 2002); pattern 2 = divergent genitalia/divergent neutral genotype—undeterminable owing to insufficient information (e.g. Hedin 1997); and pattern 3 = non-divergent genitalia/divergent neutral genotype—pleiotropy or neutrally evolving genitalia, no SSFC-SC (e.g. Bond *et al*. 2001). Although SSFC-SC represents an interesting and exciting mode of genitalic evolution, it is clearly not the only process by which genitalic shape differences evolve. As demonstrated by this study, the de facto acceptance of sexual selection by female choice (or sexual conflict) as the likely explanation for the process that drives the evolution of genitalic shape change may bias how we measure and analyse diversity. As a consequence, we may overlook species and likewise fail to appreciate other equally interesting processes influencing genitalic morphological evolution.

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