Genetic variation corroborates subspecific delimitation in the Namib fog-basking beetle, *Onymacris unguicularis* (Haag) (Tenebrionidae, Coleoptera)

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Abstract

The fog-basking beetle, *Onymacris unguicularis* (Haag, 1875), is currently listed as a polytypic form comprising two subspecies. A flightless substrate specialist, the beetle is endemic to vegetationless dunes in the Namib, where southern populations constitute the nominate subspecies, *O. u. unguicularis*, and populations some 300 km to the north compose *O. u. schulzeae* Penrith, 1984. Their taxonomic descriptions are based on minor differences in pronotal and prosternal shape, and the phylogenetic validity of these subspecies has yet to be ascertained. Here we reassess the polytypic status of *O. unguicularis* by (1) examining diagnostic phenotypic characters in conjunction with a geometric morphometric analysis, and (2) conducting phylogenetic analysis of mitochondrial DNA sequences. Our results confirm pronotal and prosternal differences, which are complemented by geometric morphometric resolution of the subspecies. Phylogenetic analysis recovered two reciprocally monophyletic lineages that exhibit perfect phylogeographic congruence with phenotypic variation. Our genetic data identify southern and northern populations as distinct lineages, corroborate morphometric data regarding subspecific delimitation, and therefore support the recognition of *O. u. unguicularis* and *O. u. schulzeae* as valid taxa under the general lineage concept.

Keywords

Subspecies, integrative taxonomy, Namib Desert, *Onymacris*, Tenebrionidae
Introduction

Darkling beetles (family Tenebrionidae) figure prominently in the arthropod fauna of Africa's Namib Desert, where they compose ~80% of all coleopterans (Louw 1983). Many exhibit unique adaptations to the Namib's substrate, thermal, and moisture conditions (Endrödy-Younga 1978; Seely et al. 2005), the most remarkable of which involves water-gathering behavior practiced by the fog-basking beetle, Onymacris unguicularis (Hamilton and Seely 1976). As its common name implies, *O. unguicularis* ‘basks’ in the advective fogs that characterize this coastal desert and provide an important water source for Namib biota in general (Henschel and Seely 2008). Fog basking typically occurs before dawn, at which time these otherwise diurnal beetles ascend the dunes (at temperatures 20–30°C below optimal activity conditions), tilt headwards into incoming fog, and drink condensate that forms on their dorsum (Hamilton and Seely 1976; Seely et al. 1983). Although fog basking has been observed in a second species from the northern Namib (*Onymacris bicolor* (Haag, 1875)), investigations have centered largely on *O. unguicularis* (Seely et al. 1983, Nøgaard and Dacke 2010; Nøgaard et al. 2012), making it one of the more widely recognized beetles worldwide.

*Onymacris unguicularis* has also been the subject of taxonomic investigation; Penrith (1984) examined morphological variation throughout the species’ range, which is apportioned south to north in a patchy network along the Namib’s coastal segment (Fig. 1). Ecologically, these flightless beetles exhibit further restriction, being habitually if not exclusively confined to vegetationless dunes within the desert’s major sand seas. Penrith (1984) identified phenotypic distinctions between northern vs. southern populations, which are separated by ~300 km of duneless plains. Based on their morphological differentiation and apparent absence of gene flow, she proposed northern and southern populations be recognized as subspecies (Figs 2–3). Thus, Penrith (1984) designated southern populations as the nominate subspecies and named the northern populations *Onymacris unguicularis schulzeae*—honoring Lieselotte Prozesky-Schulze, who first reported differences between northern/southern populations on the basis of larval characteristics (Schulze 1964). Penrith’s (1984) view of subspecies reflects the classic use of this taxonomic category in recognizing “geographic forms which cannot rank as full species” by noting that her morphological diagnosis could not “on the present evidence, separate the northern population more than subspecifically from southern populations.”

Efforts in subspecies delimitation mirror those of species delimitation conceptually if not methodologically and, similarly, engage controversy (Mayr and Ashlock 1991; Mortiz 1994; Burbrink et al. 2000; Zink 2004; Cronin 2006; Phillimore and Owens 2006; Jorgensen et al. 2013). Despite contention over subspecific rank, its taxonomic utility, and evolutionary validity, the category nonetheless remains the sole infraspecific unit recognized by the International Code of Zoological Nomenclature (ICZN 1999). Moreover, certain animal groups (e.g., birds, butterflies, beetles) still contain significant numbers of traditionally-recognized subspecies. Braby et al. (2012) recently provided a critical update of the subspecies concept, justifying viability and recommending that it correspond closely in theory and practice to the general lineage species...
concept (de Queiroz 1998, 2007). They proposed its application be restricted to extant groups of populations “representing partially isolated lineages of a species that are allopatric, phenotypically distinct, have at least one fixed diagnosable character state, and that these character differences are correlated with evolutionary independence according to population genetic structure.”

Subspecific taxa are now routinely reassessed using molecular phylogenetic analysis as a key component of integrative or coalescent approaches to recover evolutionarily independent lineages. These investigations generally yield one of two outcomes. Patterns of genetic variation may exhibit discordance with traditionally defined subspecies, either phenotypically or geographically, if not both (Burbrink et al. 2000, Zink
In effect, the subspecies fail to be recovered as historically independent lineages—their morphological distinctions being reinterpreted as local adaptation, clinal variation, etc.—and are dismissed as valid taxonomic entities. Alternatively, genetic differentiation corroborates the phenotypic variation defining subspecies, in which case researchers may justify trinomial retention (Braby et al. 2012), elevation to full species (Glor and Laport 2012), or some combination thereof (Fuchs et al. 2011).

In this study we examined morphological and genetic variation in *Onymacris unguicularis*, adopting Braby et al.’s (2012) criteria to evaluate the validity of its polytypic status. Our inquiry involved a reassessment of Penrith’s (1984) diagnostic morphological characters in conjunction with (1) morphometric analysis of additional phenotypic variation and (2) phylogenetic analysis of mitochondrial DNA sequences.

**Materials and methods**

**Morphological analysis**

Penrith’s (1984) morphological evidence for subspecific recognition involved shape differences of the pronotum and prosternal process (Figs 4–7). The pronotum is more strongly transverse in *O. u. schulzeae*, and its prosternal process is generally broader, featuring a blunt apex that is largely hidden in lateral aspect (Fig. 8). Conversely, the prosternal process in *O. u. unguicularis* is evident in lateral aspect, its apex often appearing as tooth-like projection (Fig. 9).
To reassess Penrith’s (1984) diagnostic morphological characters and explore additional aspects of phenotypic variation, we examined a series of pinned specimens from the Ditsong National Museum of Natural History (formerly Transvaal Museum), Pretoria, South Africa. Material included 93 \( O. u. unguicularis \) representing 11 populations and 30 \( O. u. schulzeae \) (all paratypes) representing four populations (Appendix). Two and five additional specimens of \( O. u. unguicularis \) and \( O. u. schulzeae \), respectively (representing a portion of our genetic sample), were also examined. Specimens were photographed to provide dorsal and ventral images of each beetle. Images were made using a Visionary Digital Imaging System (Visionary Digital™, Richmond, VA).

Penrith (1984) quantified pronotal shape differences between subspecies as a ratio of pronotal length divided by pronotal width (PL/PW). We repeated these measurements as part of our morphological analysis, and also quantified differences in the prosternal process as a length/width ratio (Figs 5 and 7), using the software program Image J. Additionally, Penrith (1984) noted possible differences in elytral shape, which appears to be “less elongate, broader, and more abruptly tapered posteriorly” in \( O. u. schulzeae \). We therefore used a geometric morphometric analysis to assess putative differences in dorsal (elytral) shape. Mindful that sexual dimorphism may contribute to elytral shape variation, we acknowledge its potential to confound signal attributable to subspecific variation. However, only one species of \( Onymacris, O. plana \) (Pérignuey, 1888), exhibits pronounced sexual dimorphism in elytral shape; in all others the female’s elytra are only “slightly broader than those of the male,” with “much overlap”
In *O. unguicularis*, frequency distributions of maximal elytral width, expressed as a percentage of elytral length, exhibit complete overlap between the sexes (Penrith 1975), and elytral shape in northern populations has been dismissed as being “scarcely dimorphic” (Penrith 1984). We should note that sexual dimorphism is evident in *O. unguicularis*: males possess longer legs and, uniquely within *Onymacris*, bear setose brushes on the anterior femora (Penrith 1975). Given the species’ limited elytral dimorphism and our relatively small sample of *O. u. schulzeae* (*n* = 35), we elected to combine the sexes in our morphometric analysis.

We identified eleven dorsal landmarks—eight type 1 and three type 3 (Brookstein 1991; Fig. 10)—and used the programs tps-Util and tps-DIG2 (Rohlf 2012) to assemble dorsal (elytral) image files for analysis and score landmarks, respectively. Landmarks were aligned and scaled to size using the generalized least squares Procrustes superim-

Figures 8–9. Lateral aspects of the prosternal process depicting a blunt apex (8) in *Onymacris unguicularis schulzeae* and toothed apex (9) in *O. u. unguicularis*.

Figure 10. Landmarks for the geometric morphometric analysis of dorsal (elytral) shape.
position method (Rohlf and Slice 1990), which removes information not relevant to shape (location, scale, and rotational effects). Relative warps were calculated with $a$ set to zero, thus weighting all principal warps equally. Superimposition, calculation of relative warps, and calculation of centroid size were preformed using the program MorphoJ (Klingenberg 2011).

**Molecular phylogenetic analysis**

Sixteen beetles were captured, preserved (100% ethanol), and processed for DNA analysis; the specimens included 12 *O. u. unguicularis*, representing three geographic localities, and four *O. u. schulzeae*, representing two relatively close localities (Fig. 1; Appendix). *Onymacris laeviceps* Gebien, 1938 and *O. plana*, identified as sister taxa to *O. unguicularis* in a generic-level phylogeny (Lamb and Bond 2013), served as outgroups. The mitochondrial genes cytochrome oxidase I (cox1) and cytochrome oxidase II (cox2) were amplified using the primers and PCR conditions listed in Table 1.

Amplification products were cleaned using exoSAP-IT (USB Corp.) and sequenced on an Applied Biosystems 3130 capillary sequencer. Sequences were edited and assembled in Sequencher 4.9 (GeneCodes, Ann Arbor, MI) and aligned using ClustalX ver. 2.0 (Larkin et al. 2007), after which sequences were translated to ensure a correct reading frame.

We used Bayesian inference (BI) and maximum likelihood (ML) methods to analyze the concatenated gene (cox1-cox2) dataset. We used Kakusan 4 (Tanabe 2007) to select nucleotide substitution models for BI, partitioning protein-coding genes by codon position and assessing each gene/codon partition using the Bayesian Information Criteria (BIC4 criterion). BI analysis was conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and involved two concurrent runs of four simultaneous Markov Chain Monte Carlo chains for 20,000,000 generations, with trees sampled every 1,000 generations. Topologies in the first 25% of the posterior distribution were discarded as burn-in. Likelihood values for all post-analysis trees and parameters were evaluated for convergence and burn-in using the “sump” command in MrBayes and the computer program Tracer ver. 1.5 (Rambaut and Drummond; http://evolve.zoo.ox.ac.uk/software.html?id=tracer). Trees remaining after burn-in were used to calculate posterior probabilities using the “sumt” command. The ML analysis, executed in RAxML ver. 7.2.8 (Stamatakis 2006), comprised 1,000 random sequence addition replicates (RAS).

**Table 1.** PCR primers and amplification conditions.

| Gene | Primer     | Annealing     | Cycles | Reference        |
|------|------------|---------------|--------|------------------|
|      | TY-J-1460  | 50°C          | 35     | Simon et al.(1994) |
|      | TL2-N-3014 | sequencing only |       |                  |
|      | C1-J-2183  |               |        |                  |
|      | TL2-J-3037 | 50°C          | 35     |                  |
|      | TK-N-3785  |               |        |                  |
using the commands “-# 1000” and “–m GTRGAMMA.” Bootstrap support values were calculated using the same search parameters with 1,000 replicates, and bootstrap results were applied to the best tree recovered in the RAS search.

**Results and discussion**

**Morphometrics**

Ratios generated for both the protonal and prosternal datasets differed significantly between subspecies (p < 0.0001), with minimal overlap for each character (Table 2). In the geometric morphometric analysis, the first two principal components based on the non-uniform components of dorsal (elytral) shape account for 78.54% of the variation between subspecies. An ordination plot of PC1 and PC2 revealed that the two subspecies are relatively well separated along the PC1 axis (Fig.11). Dorsal shape separation probably reflects proportionally longer elytra in *O. u. unguicularis*, which become apparent in side-by-side comparisons with *O. u. schulzeae* (Figs 2–3). In light of these findings, we measured elytral length and width (at the midpoint of elytral length) for all specimens to determine whether a simple ratio (EL/EW) might reflect the subspecific separation observed in our geometric morphometric analysis. We also noted the position of greatest elytral width for each specimen, scored as midpoint, anterior to midpoint, or posterior to midpoint. Despite broad overlap, elytral ratios differed significantly (p < 0.0001) between subspecies (Table 2); elytral width is widest anterior to midpoint in both subspecies but is positioned closer to the pronotal suture in *O. u. schulzeae*. Of the three ratios, we consider that for the pronotum to be the strongest diagnostic metric.

**Molecular phylogenetics**

Concatenated sequence data for the *cox1* (1574 bp) and *cox2* (680 bp) genes yielded eleven haplotypes among the 16 beetles surveyed: eight haplotypes for *O. u. unguicularis* and three for *O. u. schulzeae* (Genbank accession numbers KF835703-KF835721). No haplotypes were shared between subspecies. Mean haplotype divergence (calculated from uncorrected pair-wise distance values) was limited across geographic localities for both *O. u. unguicularis* (*cox1* = 0.046%; *cox2* = 0.027%) and *O. u. schulzeae* (*cox1* = 0.008%; *cox2* = 0.014%) but differed substantially between the two subspecies (*cox1* = 3.87%; *cox2* = 3.01%). The BI (harmonic mean –ln = 4690.15) and ML (–ln = 4172.41) analyses generated topologically identical trees in which subspecies were shown to be reciprocally monophyletic (Fig. 12). Moreover, subspecific monophyly was strongly supported (Bayesian posterior probabilities = 1.0; ML bootstraps = 100%), in contrast to the marginal to moderate support observed for haplotype relationships of geographic localities within subspecies (Fig. 12).
Table 2. Pronotal, prosternal, and elytral ratio means and ranges.

| Character | Subspecies | N  | Mean       | Range    |
|-----------|------------|----|------------|----------|
| pronotum  | unguicularis | 95 | 1.66 ± 0.08 | 1.47–1.83 |
|           | schulzeae  | 35 | 1.97 ± 0.13 | 1.73–2.35 |
| prosternum| unguicularis | 94 | 2.22 ± 0.17 | 1.86–2.71 |
|           | schulzeae  | 33 | 2.01 ± 0.14 | 1.65–2.34 |
| elytra    | unguicularis | 95 | 1.44 ± 0.08 | 1.25–1.61 |
|           | schulzeae  | 34 | 1.35 ± 0.07 | 1.24–1.47 |

Figure 11. Scatterplot of the principal component scores derived from geometric morphometric analysis of dorsal shape.

Conclusions

We employed Braby et al.’s (2012) integrative approach to evaluate the polytypic status of *O. unguicularis* and found support for each criterion in their template for subspecies delimitation. *Onymacris unguicularis* is an ultrapsammophile confined to major dune fields within the northern (Cuine, Skeleton Coast) and southern (Namib) sand seas. Separated by 300 km of unsuitable substrate, these populations are unquestionably allopatric, satisfying Braby et al.’s first criterion. Regarding criterion two, phenotypic distinctiveness, we confirmed qualitative differences in pronotal and prosternal shape and verified putative distinctions in elytral shape. Patterns in larval variation—the ninth abdominal tergum being shorter and broader in northern populations (Schulze
1964)—complement differences in adult morphology and augment the case for phenotypic distinctiveness. Support for Braby et al.’s third criterion, character difference correlations with genetic variation, involves a phylogeographic profile that is perfectly congruent with the north-south partition in phenotypic variation. More importantly, the reciprocal monophyly observed between northern and southern haplotypes (with associated levels of genetic divergence) identifies respective paths of evolutionary independence. These data demonstrate that northern and southern populations of *O. unguicularis* are phylogenetically distinct under the general lineage concept. Thus, we endorse Penrith’s (1984) taxonomic interpretation in recognizing *O. u. unguicularis* and *O. u. schulzeae* as valid taxa.

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Appendix

Localities for genetic and morphometric samples.

| Subspecies | Dataset | N  | Locality                          | Voucher numbers* |
|------------|---------|----|-----------------------------------|------------------|
| unguicularis | morphometric | 10 | Anigab                            |                  |
|            |         | 9  | Blauberg                           |                  |
|            |         | 10 | Bogenfels                          |                  |
|            |         | 1  | Chaneis                            |                  |
|            |         | 12 | Gobabeb                            |                  |
|            |         | 8  | Grillental                         |                  |
|            |         | 9  | Hottentot Bay                      |                  |
|            |         | 5  | Luderitz                           |                  |
|            |         | 10 | Spencer Bay                        |                  |
|            |         | 11 | Swakopmund                         |                  |
|            |         | 11 | Walvis Bay                         |                  |
|            | genetic | 4  | Dune 7, near Walvis Bay; -22.9691, 14.5946 | TL022 TL023 TL024 TL025 |
|            |         | 5  | Gobabeb; -23.5691, 15.0424         | TL026 TL027 TL028 TL029 TL030 |
|            |         | 3  | 20 km E Luderitz; -26.7110, 15.2828 | TL031 TL032 TL033 |
| schulzeae   | morphometric | 3  | near Foz du Cunene, Angola         |                  |
|            |         | 4  | Lacrau, 13 km N Fos du Cunene, Angola |                  |
|            |         | 11 | Kaokoveld coast, between Koichab and Unjab rivers |                  |
|            |         | 12 | Unjab River, 8 km from mouth       |                  |
|            | genetic | 5  | near Torra Bay; -20.3345, 13.2929   | TL034 TL035 TL036 |
|            |         | 3  | near Torra Bay; -20.3345, 13.2929   | TL037 |
|            |         | 1  | near Torra Bay; -20.2738; 13.2655   |                  |

*as coded in Genbank