

Integrating DNA data and traditional taxonomy to streamline biodiversity assessment: an example from edaphic beetles in the Klamath ecoregion, California, USA

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ABSTRACT

Conservation and land management decisions may be misguided by inaccurate or misinterpreted knowledge of biodiversity. Non-systematists often lack taxonomic expertise necessary for an accurate assessment of biodiversity. Additionally, there are far too few taxonomists to contribute significantly to the task of identifying species for specimens collected in biodiversity studies. While species level identification is desirable for making informed management decisions concerning biodiversity, little progress has been made to reduce this taxonomic deficiency. Involvement of non-systematists in the identification process could hasten species identification. Incorporation of DNA sequence data has been recognized as one way to enhance biodiversity assessment and species identification. DNA data are now technologically and economically feasible for most scientists to apply in biodiversity studies. However, its use is not widespread and means of its application has not been extensively addressed. This paper illustrates how such data can be used to hasten biodiversity assessment of species using a little-known group of edaphic beetles. Partial mitochondrial cytochrome oxidase I was sequenced for 171 individuals of feather-wing beetles (Coleoptera: Ptiliidae) from the Klamath ecoregion, which is part of a biodiversity hotspot, the California Floristic Province. A phylogram of these data was reconstructed via parsimony and the strict consensus of 28,000 equally parsimonious trees was well resolved except for peripheral nodes. Forty-two voucher specimens were selected for further identification from clades that were associated with many synonymous and non-synonymous nucleotide changes. A ptiliid taxonomic expert identified nine species that corresponded to monophyletic groups. These results allowed for a more accurate assessment of ptiliid species diversity in the Klamath ecoregion. In addition, we found that the number of amino acid changes or percentage nucleotide difference did not associate with species limits. This study demonstrates that the complementary use of taxonomic expertise and molecular data can improve both the speed and the accuracy of species-level biodiversity assessment. We believe this represents a means for non-systematists to collaborate directly with taxonomists in species identification and represents an improvement over methods that rely solely on parataxonomy or sequence data.

Keywords

Barcoding, DNA taxonomy, molecular systematics, species identification.

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INTRODUCTION

Conservation and land management decisions can be misguided by inaccurate or misinterpreted knowledge of biodiversity. Vertebrates and plants have been the focus of biodiversity assessment

because of relative ease of species identification or perceived ecological significance (i.e. indicators or umbrellas species) (e.g. Rubinoff, 2001; Ferrier *et al.*, 2004). To a lesser extent, arthropods have been considered, but their identification is often limited to higher taxonomic levels such as family or 'morphospecies'. The

use of indicator taxa, as well as consideration of families as equivalent units of comparison, may over- or underestimate biodiversity values (Krell, 2004). Because these practices usually neglect species-level identification of the megadiverse arthropods, their interpretation and application remains questionable. Conservation legislation focuses on species so biodiversity inventories should strive to include species data. A more accurate assessment of arthropod species diversity and ecological associations could help improve conservation decisions and hasten efforts to document and describe biodiversity.

Species identification of arthropods based on morphology can be problematic and time intensive. While some taxa are relatively well known and easily identified (e.g. dragonflies, butterflies, etc.), most are difficult to identify without specialized training. This often limits taxon identification to family and precludes accurate assessment of biodiversity, given the arbitrary nature of family ranks. Non-systematists (e.g. parataxonomists, etc.) often lack experience needed to evaluate species variability and navigate technical nuances of identification keys (Krell, 2004). Taxonomists provide the most accurate identifications for those taxa on which they specialize; however, their services and numbers are severely limited (Hammond, 1995). Also, a poor understanding of species limits for particular taxa may confound even the experts (Knowlton, 1993).

The use of DNA sequences for species taxonomy (i.e. DNA taxonomy) may circumvent some of the aforementioned problems (Stoeckle, 2003). Information regarding the distribution of genetic variation and population histories within species can be used to enhance conservation efforts and develop more effective land management policies (Hibbet & Donoghue, 1996; Moritz, 2002). It can be employed on a larger scale to address biodiversity assessment as well. Insect species have been identified with a phylogenetic analysis of DNA characters via parsimony optimization (e.g. Wells *et al.*, 2001; Foster *et al.*, 2004). The effectiveness of DNA taxonomy requires an initially correct species identification of some subset of specimens, as well as assessment of DNA variation within the species (Sperling, 2003; Mallet & Willmott, 2003; Foster *et al.*, 2004). While the use of DNA characters to assess biodiversity is enticing, similar issues that plague morphology-based species identifications preclude application of DNA data to an unknown biota. Particularly, species limits in terms of DNA differences are unknown for all but a few well-studied taxa. Thus, potential paraphyly and polyphyly of recognized species will confound the distinction between gene history and the discovery of new species (e.g. Funk & Omland, 2003). Consequently, overestimation of diversity is likely if all monophyletic groups of individuals are considered species (Will & Rubinoff, 2004).

An alternative to DNA taxonomy, 'DNA barcoding', uses overall nucleotide similarity, which does not distinguish between homologous and homoplasitic DNA characters, to identify insect species and higher taxonomic levels (Hebert *et al.*, 2003). In part, this method proposes the use of 10× mean intraspecific mtDNA sequence variation to identify the species boundaries among animals (Hebert *et al.*, 2004a,b). Thus, a group of individuals would be identified as a new species if the group exhibits greater

than 10× mean intraspecific variation observed in the taxon understudy. This scheme would likely compound the problem with identifying species boundaries because the DNA data are not evaluated in an evolutionary context and 10× mean intraspecific variation is not likely universally applicable.

Morphology and DNA-based species identification methods have different deficiencies. Morphology-based identification suffers from a lack of sufficient taxonomic expertise to identify species and, if needed, to revise species limits. DNA-based identification suffers from its limited application and from a lack of understanding of molecular variability among organisms. There is little precedence to help evaluate expected genotypic/haplotypic variation within and among species. The synergistic effect of morphological and molecular data in phylogenetic analyses is well documented (e.g. Gatesy *et al.*, 1999; Cognato & Vogler, 2001), and these data are no longer considered incompatible. Similarly, consideration of traditional taxonomy and molecular variation may counter the deficiencies of each, and improve species identification and subsequent biodiversity assessment.

This study presents the use of DNA data for an initial assessment of species identification and diversity of feather-wing beetle (Coleoptera: Ptiliidae) specimens sampled within a biodiversity 'hotspot'. The Klamath-Siskiyou ecoregion is one of the most diverse temperate coniferous forests in the world, with high levels of endemism and rare habitat types (DellaSala *et al.*, 1999; Noss *et al.*, 1999). The terrestrial biodiversity of the region is imperilled due to fragmentation of critical habitat and introduction of invasive species (DellaSala *et al.*, 1999). Comparatively, little research has been conducted to characterize the organisms of this region. This is especially true for the soil and litter inhabiting macro-arthropods that are the most diverse organisms in the Klamath-Siskiyou ecoregion (Moldenke, 1999). These organisms play a crucial role in decomposition and nutrient cycling and are essential for the long-term sustainability of forests. Thus, leaf-litter arthropods can be useful indicators of forest health (Van Straalen, 1997). In this regard, biodiversity and conservation research in this region should aim to consider these arthropods at the species level.

Patterns of genetic variation among these organisms will likely provide insight into the evolutionary processes that have shaped the history of species within the Klamath ecoregion (Avise, 2000). Ptiliid beetles provide a good starting point to address the history of this region because they are one of the most common beetles in the ecoregion and provide sufficient sample size for population genetic analysis (Caesar *et al.*, 2005). An obvious but important requirement for the measurement of genetic variation and population history is that the individuals compared are the same species. However, taxonomic knowledge of these tiny (< 1 mm) feather-winged beetles is limited. Thus, we combined the use of molecular phylogenetics and traditional taxonomy to improve species identification of ptiliids and subsequent biodiversity assessment. Because of the taxonomic dearth, we initially included all ptiliid individuals collected in our study. DNA from the mitochondrial cytochrome oxidase I (COI) gene was sequenced and analysed in a phylogenetic context. Individuals from lineages that demonstrated amino acid and synonymous

nucleotide changes were determined to species by the second author (M.S.), a ptiliid taxonomist. This scheme to assess biodiversity produced more accurate and faster assessment of ptiliid diversity. Also, we examined whether 10× intraspecific sequence divergence reflects species boundaries among ptiliid.

METHODS

Soil arthropods were extracted from leaf litter sampled in May and June of 2002 and 2003 via Berlese funnels (for details see Caesar *et al.*, 2005). Specimens were sorted to ptiliid morpho-species and approximately 20 individuals representing three morphospecies were sent to M.S. for initial species determination. A 740 base-pair region of mtDNA COI was amplified, purified, and sequenced for 167 individuals. DNA extraction protocols were modified to preserve diagnosable voucher specimens for subsequent identification based on morphology. Details concerning sampling and molecular techniques are described in Caesar *et al.* (2005). DNA sequences are available from GenBank at <http://www.ncbi.nlm.nih.gov/GenBank/index.html> (for *Acrotrichis xanthocera*, AY550852–AY550882; for the other taxa, DQ313332–DQ313376).

Phylogenetic relationships of the 167 ptiliid COI sequences were estimated using *PAUP** version 4.0b2a (Swofford, 1998)

under the maximum parsimony (MP) optimality criterion. A heuristic search was performed using the tree bisection reconnection algorithm and 100 random addition sequence replicates, with all characters equally weighted and unordered. For each replicate, no more than 2000 trees with a score greater than one were saved (to reduce the computational time of analyses). Support for individual nodes was assessed with 10,000 pseudo-replicates with the bootstrap procedure via 'fast' stepwise-addition.

We used amino acid residue substitution, as inferred from the resulting phylogenetic analysis, as one criterion to identify lineages that were evolutionary divergent (Rubinoff & Powell, 2004). Non-synonymous nucleotide substitutions occur less frequently than synonymous substitutions, thus lineages with non-synonymous nucleotide substitutions have likely diverged over a longer period of time than those without. These lineages potentially represent species with diagnostic morphological characters. However, lineages exhibiting synonymous substitutions were also considered for species identification. Codon positions were inferred based on the *Drosophila yakuba* mitochondrial genetic code (Clary & Wolstenholme, 1985) and mapped on the phylogram (Fig. 1) using *MACCLADE*, version 4 (Maddison & Maddison, 2000). Unambiguous amino acid changes were recorded (Table 1). Percentage DNA difference for clades of

Table 1 Unambiguous amino acids state changes determined with the presented phylogeny. Character numbers are sequential amino acid positions which start at the 5' end of mtDNA COI

DNA sequence Character no.	Clade	Amino acid change	Character no.	Clade	Amino acid change
4	D	Gln-His	197	E	Ileu-Leu
			200	D	Met-Ileu
22	D	Leu-Met	203	E	Leu-Met
	60	Met-Ileu	207	85	Thr-Ileu
23	F	Ala-Thr	211	E	Phe-Ileu
26	D	Ileu-Leu	212	E	Phe-Leu
30	F	Val-Ileu	213	E	Ileu-Leu
44	110	Asp-Asn	216	H	Met-Ileu
75	E	Lys-Gln		E	Met-Ileu
78	D	Try-Phe	221	E	Ileu-Val
79	D	Thr-Ser		H	Ileu-Ser
81	H	Ser-Ala	223	3	Asn-Ser
	68	Ala-Thr	226	F	Ser-Met
85	D	Ala-Ser	227	D	Ser-Ileu
100	E	Val-Ileu	228	D	Phe-Ser
109	H	Ileu-Val	229	D	Ileu- Ser
110	E	Ileu-Val	231	F	Asn-His
137	E	Phe-Leu	232	E	Phe-Met
	L	Phe-Leu	233	E	Pro-Ala
138	I	Val-Ileu	234	D	Thr-Ser
139	E	His-Gln	239	F	Leu-Ileu
141	D	Phe-Tyr	242	E	Tyr-Met
149	E	Met-Leu	243	68	Pro-Gln
151	F	Asn-Gln	244	68	Pro-Gln
156	E	Ileu-Thr		73	Pro-Leu
167	F	Leu-Ileu		78	Pro-Leu
190	E	Ala-Ileu		85	Pro-Leu
192	F	Thr-Met	245	E	Ala- Ser

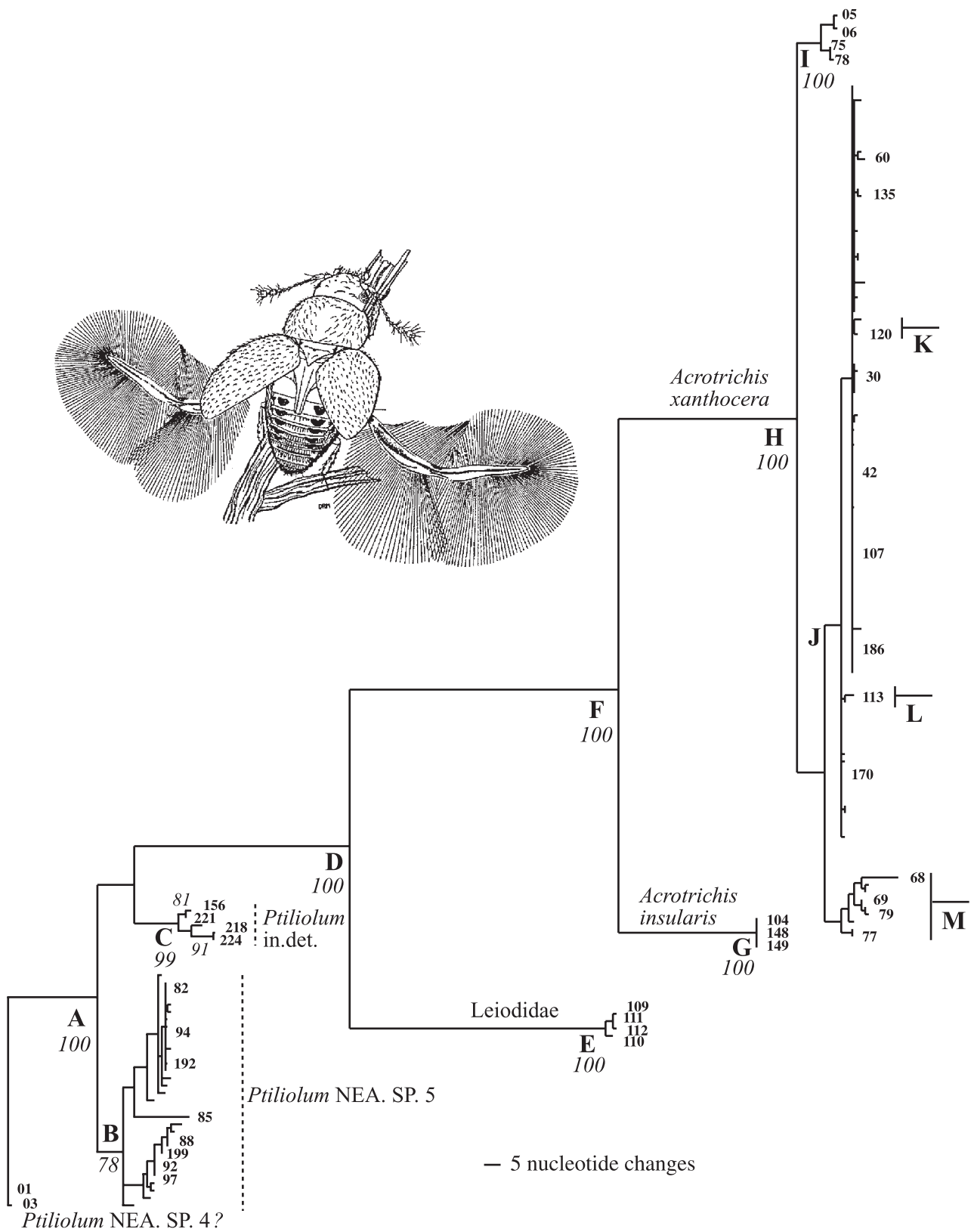


Figure 1 Unrooted phylogram of 167 ptiliid individuals sequenced for 740 bp of mtDNA COI. The tree is a strict consensus of 28,000 equally parsimonious trees. Letters label clades and branches. Numbers below branches are bootstrap values. Numbers at terminals refer to individuals that were examined by the taxonomic expert (Sörensson) who identified the species. The inset illustrates a *Ptenidium* species and diagnostic ptiliid feather-wings. '?' indicates authors unsure of species.

individuals was measured as the branch length or mean of branch lengths stemming from the branch supporting the clade to the terminal branches of individuals.

Based on the tree topology, an additional subset of specimens (a total of 42 individuals, or 25% of all specimens) were selected from clades that were subtended by branch lengths equal or greater than five nucleotide changes or those branches that exhibited unambiguous amino acid changes (Fig. 1). For each clade, three or more individuals were selected in order to increase the potential of sampling both males and females. These specimens were identified by M.S. using morphological characters, published taxonomic keys, and comparison to museum specimens.

RESULTS

Phylogenetic analysis of the ptiliid specimens yielded 28,000 equally parsimonious trees (EPTs) based on 268 parsimony informative characters. The strict consensus of these EPTs was mostly resolved except for peripheral clades (Fig. 1). The consistency index (CI = 0.708, excluding uninformative data) indicated a low level of homoplasy and many of the resolved clades had high bootstrap values (Fig. 1). At least one amino acid substitution was observed for these clades, except for clades A and B. Amino acid substitutions were observed for clade L, however, bootstrap values were low. Clades D and E exhibited the most amino acid substitutions, 16 and 19, respectively. Also, a variable amount of unambiguous amino acid change was observed for a few terminal lineages. Of these, a few had relatively long branch lengths and little amino acid change, which indicated substantial synonymous nucleotide change. Most amino acid substitutions were characterized as transitions within chemically similar groups, i.e. within hydrophobic or hydrophilic residues (Table 1). Changes between hydrophobic and hydrophilic residues occurred less frequently and among these, changes between threonine and either methionine, isoleucine, or alanine were most common (Table 1).

Forty-two voucher specimens selected from the labelled lineages were identified to the lowest taxonomic level (Fig. 1). Six taxa were identified and each associated with a single lineage (Fig. 1). *Acrotrichis xanthocera* was the most common species, although some of the specimens represented apparently undescribed or indeterminate species. One additional *Acrotrichis* species was identified. Three *Ptiliolum* species were found, but species identification was not possible because either the species were undescribed or the diagnostic morphology was bleached beyond recognition by the process of DNA extraction (for one specimen). One species of Leiodidae, which is a soil beetle that resembles ptiliids, was also recovered (Fig. 1).

Neither the amount nor the type of nucleotide or amino acid change is indicative of taxonomic level. For example, 19 amino acid changes were observed for the clade of Leiodidae, while nearly the same was observed for the *Ptiliolum* clade that currently represents a subfamily separate from the Acrotrichinae. Amino acid changes also varied widely among species lineages. No amino acid change was observed among *Ptiliolum* species,

while all *Acrotrichis* species exhibited at least one amino acid change. Hydrophilic to hydrophobic changes and vice versa occurred among terminal lineages and internal branches (Table 1, Fig. 1).

Percentage DNA difference also varied among species. The branches that subtend *A. xanthocera* and *Acrotrichis insularis* clades exhibited 9.1 and 5.6% DNA difference, respectively. Intraspecific variation measured at 2.0% for *A. xanthocera*. There was no sequence difference among *A. insularis* individuals, although the sample size was smaller than *A. xanthocera*. The leiodid exhibited similar amounts of interspecific sequence difference as compared to *A. xanthocera*, but the intraspecific difference was less. Interspecific and intraspecific percentage DNA difference for *Ptiliolum* species was less and ranged between 2.7 and 3.3% and 0.9–1.3%, respectively. These nucleotide differences among taxonomic levels pertain to individuals found in the Klamath-Siskiyou ecoregion. A broader study that includes individuals from across the species ranges would be needed to adequately estimate the 'total' intraspecific and interspecific nucleotide differences.

DISCUSSION

Mistakes of taxon identification for poorly studied organisms are expected, even by informed laypersons or trained parataxonomists (Krell, 2004). Phylogenetic analysis using DNA characters provides a repeatable scheme to test initial assessment of taxa and morphospecies. Monophyly should be recovered for individuals assigned to the same taxon if the initial assessment of diagnostic morphological characters reflects taxon limits. If taxa are not recovered as monophyletic, then a taxonomic expert should re-evaluate individuals for different or additional diagnostic morphological features. In our case, the initial assessment of morphospecies did associate with monophyletic groups of individuals, although we underestimated the overall species diversity in our samples by half. A specialist should also evaluate specimens representing lineages that exhibit unambiguous amino acid changes for morphological features that would support species status (Table 1). In our case, two described species were identified on this basis, while other clades represented undescribed species or unidentifiable individuals. We also exposed the misidentification of a few individuals belonging to the closely related beetle family Leiodidae. The absence of amino acid change for a clade or lineage does not preclude species status, as observed for the *Ptiliolum* species (Fig. 1). Clades of these species (A, B, and C, Fig. 1) are supported by synapomorphies that comprise synonymous nucleotide changes and exhibit large to medium bootstrap values. Thus, assessment of species diversity as inferred from DNA data should not ignore clades of individuals that exhibit relatively high branch support in the absence of non-synonymous substitutions.

As lamented in the introduction, taxonomic expertise for leaf-litter arthropods is inadequate to meet the needs of ecological and conservation studies. It has been suggested that comparison of DNA sequence difference measured as branch lengths on a phylogenetic tree (e.g. Morgan *et al.*, 2000; Foster *et al.*, 2004) or

overall pairwise taxon difference (Hebert *et al.*, 2003) could identify lineages or groups of species. For some taxa, such as birds, the occurrence of groups of individuals with a DNA sequence difference that is 10× the mean pairwise intraspecific DNA sequence difference may predict an undescribed species (Hebert *et al.*, 2004b). This method requires knowledge of species limits that may or may not be known for a particular taxon and is, therefore, not universally applicable. Such a generalization underestimates the diversity of ptiliid species based on the intraspecific sequence divergence (2.0%) of the unambiguously identified *A. xanthocera*. For example, individuals exhibiting 20% or less sequence divergence would be grouped as species. This action would group both of the *Acrotrichis* species included in this study as one species. While 10× the mean pairwise intraspecific DNA sequence difference may be an unrealistic amount of expected divergence, considering only the mean interspecific sequence divergence within ptiliid genera does not improve recognition of species. The three *Ptiliolium* species would be recognized as one species given the mean interspecific sequence divergence within *Acrotrichis* (7.6%). Conversely, species diversity for *Acrotrichis* would be overestimated given the mean interspecific sequence divergence within *Ptiliolium* (2.9%). These estimates of sequence divergence may change with the inclusion of specimens from a broader geographical range. For ptiliids, and likely for other poorly known organisms, it appears premature to recognize species based only on an approximate amount of sequence difference. DNA phylogenies reveal shared common ancestry of individuals, yet these lineages may or may not be recognized as species. Determination and verification of species status will depend on expert taxonomic knowledge and/or further investigation of morphological, biological, and ecological evidence.

This study demonstrates that the complementary use of taxonomic expertise and molecular data can improve the assessment of biodiversity. Instead of recognizing only the family level taxon for ptiliid individuals, or three ptiliid morphospecies, we discovered that at least five ptiliid and one leiodid species reside in the sampled portion of the Klamath-Siskiyou ecoregion. *Acrotrichis xanthocera* is a widespread species that occurs throughout all sites, although haplotype variation is segregated between eastern and western populations (Caesar *et al.*, 2005). The added species data confirm observations that soil arthropods tend to have patchy distributions. The conclusions of our earlier study on intraspecific genetic variation in *A. xanthocera* might have been severely skewed had we not ensured species identification (Caesar *et al.*, 2005). Considering the shortage of taxonomic expertise and reliance on parataxonomists for identification, it is likely that such mistakes have gone unnoticed in other biodiversity surveys.

Perhaps the greatest benefit of this study is the use of phylogenetic analysis to guide selection of representative voucher specimens for examination by a specialist. Those specimens that represent long branches in a phylogram or exhibit amino acid substitutions likely represent a subset that would interest a specialist. This is best done in the absence of a specific protocol that invokes a generalized amount of nucleotide substitution to represent species or higher taxa, as such a metric is not likely to be universal. Often taxonomic specialists are requested to identify

bulk samples, which is extremely time consuming and peripheral to a specialist's research. In our case, the DNA data allowed for an informed sort of specimens and only 25% of specimens were examined by M.S. This saved approximately 20.8 h of detailed dissections and microscopic study. Based on a conservative estimate, a trained taxonomic specialist with access to an extensive reference collection might spend an average of 10 min identifying each specimen. In this study, species identifications would have taken 27.8 h (167 specimens × 10 min). However, approximately 7 h were needed to confirm species identifications because only specimens prescreened by phylogenetic analysis were sent to M.S. We can only speculate as to the impact this approach would have on much larger or long-term studies. Identification requests that are made based on phylogenetic analysis and guided specimen selection are much more likely to be received with enthusiasm and completed in a timely fashion. This allows the user of the taxonomic information to play a larger role in the specimen identification, and provides a repeatable and testable justification for delimiting species. This is a great improvement over previous parataxonomic approaches (Krell, 2004) or any method in which accurate species identification is not possible. For these reasons, we feel that implementation of these methods will help synergise biodiversity studies through closer collaboration of conservation biologists and taxonomists.

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