

Phylogenomic reconstruction of transcriptome data confirms the basal position of Prodoxidae moths within the order Lepidoptera

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Abstract. Yucca moths (Prodoxidae) have long been considered by taxonomists to be basally positioned within the Lepidoptera in the superfamily Adeloidea. Recently, phylogenomic reconstructions of ordinal lepidopteran relationships using transcriptome data confirmed the basal position of the Adeloidea and the positioning of the *Tegeticula* pollinating yucca moths within the superfamily. However, to date, no phylogenetic studies have been conducted attempting to position the *Prodoxus* bogus yucca moths using whole genome data. We incorporated our own transcriptome libraries into publicly available lepidopteran data in order to phylogenetically confirm the evolutionary position of the Prodoxidae within the Lepidoptera and to assess the position of *Prodoxus* relative to *Tegeticula*. Our phylotranscriptomic reconstruction verified the Prodoxidae as the sister taxa to the basal Adelidae (Adeloidea), and *Prodoxus* as sister to *Tegeticula*. However, topological relationships among our four focal *Tegeticula* species contradicted recent findings from RAD-seq analyses. We show that this apparent paradox is in fact an artefact of the phylogenetic methods employed in building the ordinal level phylogeny (i.e. sequence alignment based on the first two nucleotide positions only) and that the true *Tegeticula* relationships are recovered by using all three nucleotide base positions to correctly infer more recent evolutionary events. Our work shows the utility of next-generation sequencing (NGS) technologies whilst highlighting some technical considerations that may confound phylogenetic interpretation according to taxonomic scale. We add to the growing consensus that NGS techniques offer a prime opportunity to elucidate previously challenging questions in evolutionary biology.

Key words. Prodoxidae, yucca moths, *Tegeticula*, *Prodoxus*, Lepidoptera, phylogenomics, transcriptomics.

1. Introduction

The obligate pollination mutualism between yuccas (Agavaceae) and the yucca-moths (*Tegeticula* Zeller, 1873; Prodoxidae; Lepidoptera) is a model system for tackling numerous issues in evolutionary biology such as the origins of mutualism (BRONSTEIN 1994; PELLMYR & HUTH 1994), the evolution of cheating (SEGRAVES et al. 2008), host specialisation (ALTHOFF et al. 2012), and adaptive radiation (PELLMYR & LEEBENS-MACK 1999; DARWELL et al. 2016). The mutualism originated at least 30 Ma ago

(PELLMYR & LEEBENS-MACK 1999) and within it resides a recent adaptive speciation burst that yielded alternative pollinating strategies and the evolution of two cheating species that have completely eschewed their ancestral pollination obligations (SEGRAVES et al. 2008; DARWELL et al. 2016). However, the Prodoxidae themselves are considered to be much older and also contain the bogus yucca moths (*Prodoxus* Riley, 1892) (whose relationship with yuccas is antagonistic and considered basal

to *Tegeticula*) and members of the genus *Greya* Busck, 1903, which pollinate some species of Saxifragaceae (RICH et al. 2008).

Taxonomic work has placed the Prodoxidae within the superfamily Adeloidea based on shared morphological traits such as a piercing ovipositor with the eighth abdominal segment withdrawn into the seventh (DAVIS 1986). The Adeloidea is currently defined as comprising four other lepidopteran families: the cosmopolitan Heliozelidae, Adelidae and Incurvariidae along with the much less diverse Cecidosidae (NIELSEN & DAVIS 1985; DAVIS 1999; VAN NIEUKERKEN et al. 2011; BAZINET et al. 2017). The Adeloidea have long been considered a relatively basal clade within the Lepidoptera (although positioned within the derived sub-order Glossata featuring a coiled proboscis they possess the primitive monotrysian – cf. Ditrysia – single female reproductive opening; see WIEGMANN et al. 2000) and recent transcriptomic phylogenies positioned them near the base of the phylogeny (REGIER et al. 2013) and confirmed the position of *Tegeticula* within them (BAZINET et al. 2017). However, no attempt has been made to demonstrate membership of *Prodoxus* within the Prodoxidae using data generated from genome-wide marker scans.

The next-generation sequencing (NGS) revolution is rapidly expanding the evidence-base and resolving power to address evolutionary and ecological questions in non-model organisms (e.g. WAGNER et al. 2013; BURGAR et al. 2014; ALVAREZ et al. 2015). Within a phylogenetics framework, a number of different sequencing methodologies provide markers that have successfully recovered the evolutionary relationships of species radiations that had previously proven problematic with standard sequencing technologies (DARWELL et al. 2016). Commonly used methods applicable to phylogenomics include restriction site associated DNA sequencing (RAD-seq; e.g. JONES et al. 2013; WAGNER et al. 2013; DARWELL et al. 2016), ultra-conserved elements (UCEs; e.g. BLAIMER et al. 2015) and transcriptomics (e.g. KAWAHARA & BREINHOLT 2014; MISOF et al. 2014; EGGER et al. 2015), although choice of marker is typically contingent on taxonomic considerations such as the phylogenetic extent of the focal study taxa.

However, NGS technologies are evolving and care should be taken to assess possible biases and errors for specific library making protocols and sequencing instruments. It is conceivable that upstream sequencing conditions may prove sufficiently idiosyncratic that data generated on different sequencing machines at different times (MASTRETTA-YANES et al. 2015) may contain subtle artefactual structure that influence analyses, for example, by yielding disparate loci sets between different ortholog identification assays (e.g. BLAST; Stacks).

To address these biological questions and the methodological issues of NGS marker choice, we incorporated our own transcriptome data from representative species of the Prodoxidae (four *Tegeticula* and one *Prodoxus*) with data from a recently published lepidopteran phylotranscriptomic study by KAWAHARA & BREINHOLT

(2014), which used four model lepidopteran species with complete published reference genomes to identify orthologous loci. For the four *Tegeticula* species a recent RAD-seq (DARWELL et al. 2016) phylogeny, which used the Stacks program (CATCHEN et al. 2013) to identify orthologous loci amongst the *Tegeticula* species radiation, placed *T. yuccasella* Pellmyr, 1999 and *T. baccatella* Pellmyr, 1999 most closely together within a clade of recently radiated species that oviposit into yucca plant locules. However, the cheating moth, *T. intermedia* Pellmyr, 1999, was positioned in the sister-clade of superficially ovipositing moths that has also undergone recent rapid radiation and which also contains the second cheater species found in the genus. These results suggest that the evolution of cheating in these species was contingent on the evolutionary shift into superficial oviposition by these moths. Both these clades of locular and superficially ovipositing species are thought to have radiated around 3–7 Ma ago (PELLMYR & LEEBENS-MACK 1999). Finally, the fourth species, *T. synthetica* Davis, 1967, was placed within the sister lineage to all other *Tegeticula* species, implying a long evolutionary history as a distinct lineage relative to most of its congeners.

We constructed phylogenies in order to: (i) identify the evolutionary position of Prodoxidae within the Lepidoptera and their status as Adeloidea using a different transcriptome dataset than that of BAZINET et al. (2017); (ii) identify the position of *Prodoxus* relative to the pollinating yucca moths, *Tegeticula*; and, (iii) examine consistency in the phylogenetic relationships among the four investigated *Tegeticula* species according to employed NGS marker by comparing phylogenies generated by this current transcriptomic analysis and the RAD-seq findings of DARWELL et al. (2016).

2. Materials and methods

2.1. Transcriptome assembly

We constructed RNA Seq libraries for the pollinating yucca moth species *Tegeticula baccatella*, *T. yuccasella* and *T. synthetica*, the cheating yucca moth *T. intermedia*, and the bogus yucca moth *Prodoxus quinquepunctellus* Chambers, 1875 (all superfamily Adeloidea; family Prodoxidae). RNA was extracted from combined thorax and abdomen tissues for each species. Illumina barcoded RNA Seq libraries were constructed according to the manufacturer's protocol using the Illumina TruSeq kit. After checking each library quality and concentration on an Agilent 2100 Bioanalyzer, they were sent to BGI for sequencing on an Illumina HiSeq. A minimum of 5 Gb of paired-end 25 bp reads were generated from each sample. Raw data were filtered for adaptors and low quality base calls and then assembled using Trinity (GRABHERR et al. 2011). The reads were mapped back to the resulting assemblies using RSEM v1.2.0 (LI & DEWEY 2011) and

isoforms with less than 1% of the reads mapping to a component were removed.

2.2. Orthologue identification and phylogenomic dataset construction

We downloaded the LEP1-COS nucleotide custom orthologue (EBERSBERGER et al. 2009; WATERHOUSE et al. 2013) set used by KAWAHARA & BREINHOLT (2014) for their phylogenomic investigation into the relationships of 46 species from 19 major lepidopteran superfamilies. We used the BLAST suite of executables (tblastn; CAMACHO et al. 2009) to identify candidate orthologous loci for each of our five prodoxid species referenced against the 6,568 LEP1-COS single-copy orthologous genes derived from the four model lepidopteran species with complete published reference genomes (*Bombyx mori* Linnaeus, 1758, *Danaus plexippus* Linnaeus, 1758, *Heliconius melpomene* Linnaeus, 1758 and *Manduca sexta* Linnaeus, 1763) used by KAWAHARA & BREINHOLT (2014) as their focal library construction taxa. We then used custom-made Python scripts to parse out the longest identified candidate orthologue with an e-value less than e^{-20} and a minimum percentage identity threshold greater than 60% from each of the 6,568 LEP1-COS single-copy orthologue genes for each prodoxid species. Python scripts were then used to trim our selected candidate orthologues to the identified correct start/end positions indicated by BLAST and, if necessary, translate them to their reverse complement nucleotide sequences. Our set of 6,568 candidate genes were then stripped down to the equivalent 2,696 genes used by KAWAHARA & BREINHOLT (2014) to build their supermatrix providing our stipulated threshold e-value and percentage threshold criteria were met. Consequently, our set of identified gene orthologues was less than 2,696 for each prodoxid species. Our identified orthologues were aligned with the final supermatrix NEXUS data file of KAWAHARA & BREINHOLT (2014). To do this, the KAWAHARA & BREINHOLT (2014) alignment was split into separate FASTA files representing individual genes according to partitioning information retrieved from the downloaded NEXUS file. Alignments of each individual gene were made using MAFFT sequence alignment software version 7 (KATO & STANDLEY 2013) using the 'linsi' command. Gene alignments consisting of the 46 species from KAWAHARA & BREINHOLT (2014) and our five prodoxid species were re-concatenated to form a single supermatrix FASTA file with all indel regions caused by the five prodoxid species removed. Finally, following KAWAHARA & BREINHOLT (2014), and in order to both remove noise likely inherent in the degenerate DNA code and reduce computational demands, the third base positions were removed from the supermatrix. Additionally, from this initial alignment we constructed a further alignment featuring only the Prodoxidae species and with the third base codon positions reinstated for the analysis.

2.3. Phylogenomic analysis

We estimated our Lepidoptera phylogeny using Maximum-Likelihood inference methods in RAxML v.8.1.3 (STAMATAKIS 2014). Following KAWAHARA & BREINHOLT (2014), the supermatrix was partitioned by nucleotide positions demarcating each aligned gene. We used the GTRGAMMA model of sequence evolution. Best ML tree searches from a random topology were conducted using the '-f d' option for 100ML searches. Identical methods were employed for the phylogenetic reconstruction of the prodoxid-only alignment.

3. Results

With respect to the 2,696 orthologous gene regions used by KAWAHARA & BREINHOLT (2014) we identified 2,361, 2,328, 2,315, 2,362 and 2,354 gene orthologues for *Tegeticula baccatella*, *T. yuccasella*, *T. synthetica*, *T. intermedia*, and *Prodoxus quinquepunctellus*, respectively. The resulting supermatrix, consisting of the first and second base positions only, was 2,550,030 base pairs in length and featured 46.5% missing data.

Phylogenomic analysis from the combined KAWAHARA & BREINHOLT (2014) and Prodoxidae datasets using only the first and second nucleotide positions produced a fully resolved tree with topological relationships of all lepidopteran superfamilies identical to those inferred by KAWAHARA & BREINHOLT (2014) (Fig. 1). Support values were high at virtually all nodes. Prodoxid yucca moths comprise a well-supported ($p = 100\%$) monophyletic group and form a sister clade to the basal *Nemophora* (Adeloidea: Adelidae). Moreover, our *Prodoxus* representative, *P. quinquepunctellus*, is positioned within the Adelidae adjacent to the *Tegeticula* pollinating yucca moths. Notably, the relationships among the four focal *Tegeticula* are not consistent with those found by DARWELL et al. (2016), where *T. yuccasella* and *T. baccatella* were proximately positioned and the cheating yucca moth, *T. intermedia*, in a distinct clade. Although, the alternative arrangement of *T. yuccasella* + *T. intermedia* exhibits lowered support in our full analysis featuring the first two nucleotides ($p = 0.66$). However, the Prodoxidae-only alignment featuring all three nucleotide positions (3,173,031 bp), rendered the phylogenetic relationships among these *Tegeticula* species as identical to those of DARWELL et al. (2016; Fig. 2).

4. Discussion

The next-generation sequencing (NGS) revolution promises to unveil a welter of ecological and evolutionary patterns that have remained hitherto obscured despite at-

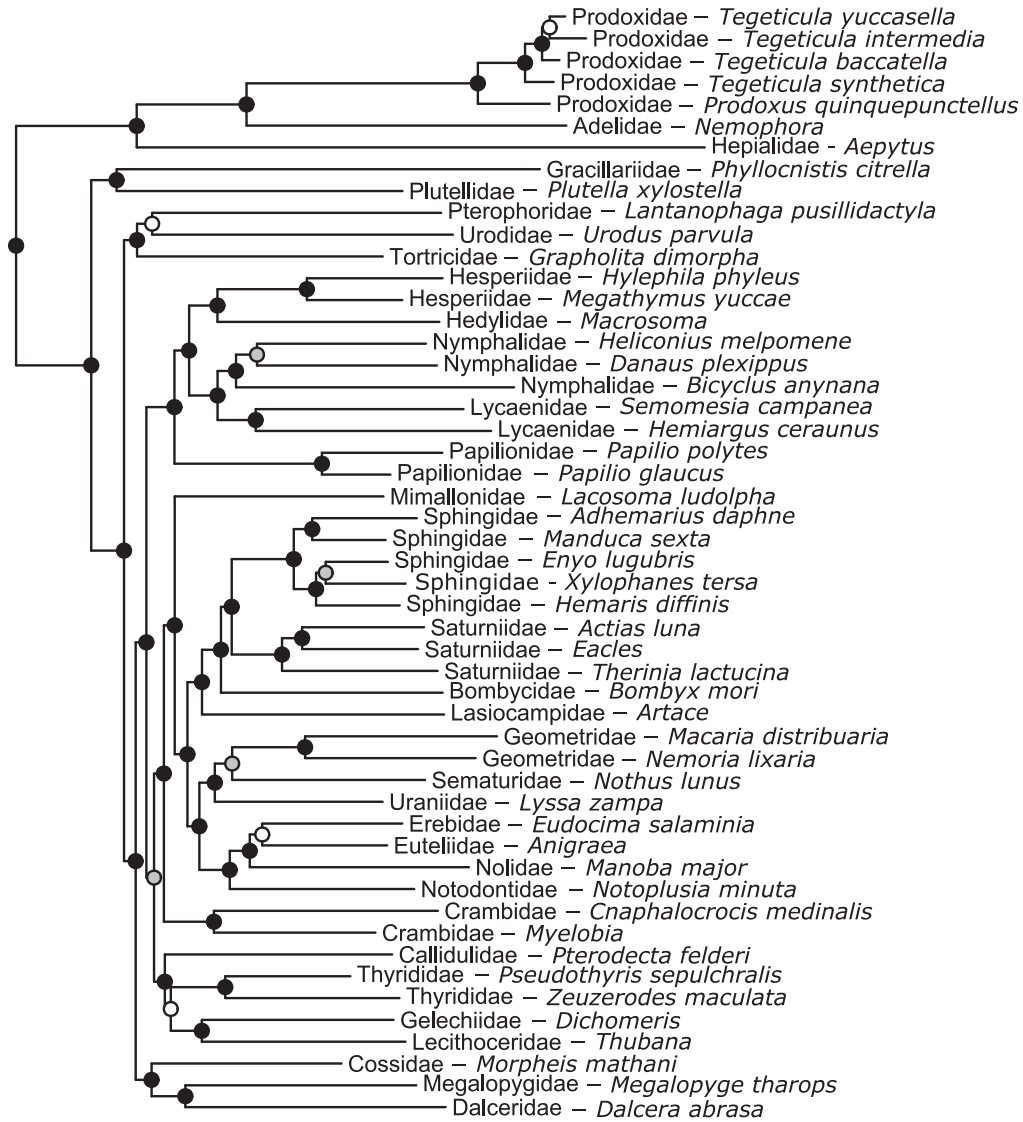


Fig. 1. Full RAxML transcriptome phylogeny incorporating the Prodoxidae, constructed using the first two nucleotide positions only. Circles at internal nodes indicate support values (black $p > 95$; grey $0.95 > p > 0.75$; white $p < 0.75$). The Prodoxidae form a monophyletic clade allied with the basal lepidopteran genus *Nemophora* (Adelidae).

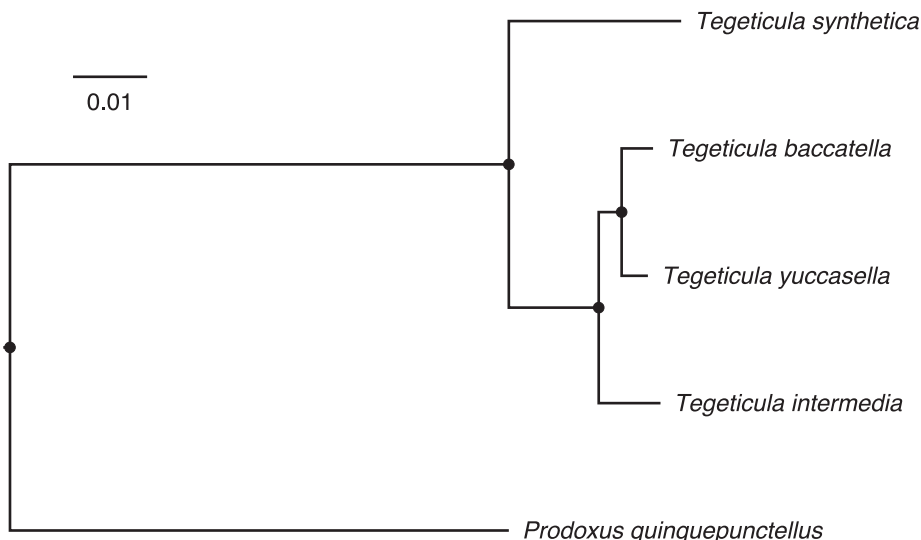


Fig. 2. RAxML transcriptome phylogeny constructed using all three nucleotide positions for Prodoxidae species only. All node support values are 100%. *T. yuccasella* + *T. baccatella* is rendered monophyletic.

tempts by workers to elucidate them. Since the advent of widespread molecular genetic analyses there have been numerous instances of hypothesised evolutionary relationships based on traditional taxonomic appraisal being rejected in light of molecular findings (e.g. ANDERSEN et al. 2014), and among the prodoxid yucca moths there has been only one attempt with genomic data to verify their inclusion in the Adeloidea (BAZINET et al. 2017) and no attempts to recover the position of *Prodoxus* using such markers. Furthermore, it is unknown whether the findings generated from NGS analyses are likely to be consistent according to choices made regarding type of marker employed and various other methodological issues.

In agreement with the findings of BAZINET et al. (2017), incorporation of our own transcriptomic data into the LEP-COS1 dataset featuring all 19 major lepidopteran superfamilies does indeed support the positioning of the Prodoxidae within the Adeloidea, as their nearest phylogenetic neighbour is the genus *Nemorpha* (Adeloidea: Adelidae). Additionally, our inclusion of prodoxid transcriptome data had no influence on the relationships found among the lepidopteran superfamilies by KAWAHARA & BREINHOLT (2014). Furthermore, the numbers of orthologous loci in our analysis was similar to the 2,696 identified by KAWAHARA & BREINHOLT (2014) suggesting that the distinct sequencing analysis events employed across the two studies provided readily comparable datasets. In addition, our results support the positioning of *Prodoxus* as sister to the *Tegeticula* pollinating yucca moths.

However, for our initial full dataset analysis, following the methods of KAWAHARA & BREINHOLT (2014) to deduce lepidopteran superfamilial relationships using only the first two nucleotide positions, topological arrangements among the three most derived *Tegeticula* from our study species is not consistent with the RAD-seq phylogeny presented by DARWELL et al. (2016). Whilst *T. synthetica* remained the most basally positioned *Tegeticula*, the two locular ovipositing species were split by the cheating species, *T. intermedia*. Not only does this constitute a topological rearrangement of these species in comparison to those derived from RAD-seq markers, it would also imply that the evolution of cheating among these mutualistic moths was not dependent on the stepping-stone evolution of the alternative superficial ovipositing strategies as previously posited. Thus, the evolutionary implications of these alternate views of the relationships among *Tegeticula* are quite profound.

However, phylogenetic reconstruction featuring only the Prodoxidae species derived from the orthologue alignment featuring all three nucleotide positions shows a rearrangement of *Tegeticula* relationships consistent with those obtained from RAD-seq marker analyses (DARWELL et al. 2016). Here, the two locular ovipositing species are rendered monophyletic and support the notion that the cheating species, *T. intermedia*, is likely to have arisen within the superficially ovipositing species clade whose life-history behavioural transitions most intuitively cor-

respond to the evolution of cheating. Thus, whilst using only the first two nucleotide positions to assess the phylogenetic relationships of distantly related taxa is entirely appropriate, our analyses show there is a minimal phylogenetic scale where this is applicable. Among our study taxa, relationships between genera and at the deeper nodes within a genus (*Tegeticula*) appear robust. However, at more recent timescales (~3–7 Ma ago), the utility of this approach appears to break down among the *Tegeticula* pollinators. Nevertheless, the consistency of *Tegeticula* relationships between the alignment featuring all three base nucleotide positions and the RAD-seq findings of DARWELL et al. (2016), despite the employment of different NGS markers, is reassuring and suggests that different NGS marker technologies are likely to yield similar evolutionary inference.

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6. References

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