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Widespread and persistent invasions of terrestrial habitats coincident with larval feeding behavior transitions during snail-killing fly evolution (Diptera: Sciomyzidae)

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Widespread and persistent invasions of terrestrial habitats coincident with larval feeding behavior transitions during snail-killing fly evolution (Diptera: Sciomyzidae)

Eric G Chapman1, Andrey A Przhiboro2, James D Harwood1, Benjamin A Foote3 and Walter R Hoeh3*

Abstract

**Background:** Transitions in habitats and feeding behaviors were fundamental to the diversification of life on Earth. There is ongoing debate regarding the typical directionality of transitions between aquatic and terrestrial habitats and the mechanisms responsible for the preponderance of terrestrial to aquatic transitions. Snail-killing flies (Diptera: Sciomyzidae) represent an excellent model system to study such transitions because their larvae display a range of feeding behaviors, being predators, parasitoids or saprophages of a variety of mollusks in freshwater, shoreline and dry terrestrial habitats. The remarkable genus *Tetanocera* (Tetanocerini) occupies five larval feeding groups and all of the habitat types mentioned above. This study has four principal objectives: (i) construct a robust estimate of phylogeny for *Tetanocera* and Tetanocerini, (ii) estimate the evolutionary transitions in larval feeding behaviors and habitats, (iii) test the monophyly of feeding groups and (iv) identify mechanisms underlying sciomyzid habitat and feeding behavior evolution.

**Results:** Bayesian inference and maximum likelihood analyses of molecular data provided strong support that the Sciomyzini, Tetanocerini and *Tetanocera* are monophyletic. However, the monophyly of many behavioral groupings was rejected via phylogenetic constraint analyses. We determined that (i) the ancestral sciomyzid lineage was terrestrial, (ii) there was a single terrestrial to aquatic habitat transition early in the evolution of the Tetanocerini and (iii) there were at least 10 independent aquatic to terrestrial habitat transitions and at least 15 feeding behavior transitions during tetanocerine phylogenesis. The ancestor of *Tetanocera* was aquatic with five lineages making independent transitions to terrestrial habitats and seven making independent transitions in feeding behaviors.

**Conclusions:** The preponderance of aquatic to terrestrial transitions in sciomyzids goes against the trend generally observed across eukaryotes. Damp shoreline habitats are likely transitional where larvae can change habitat but still have similar prey available. Transitioning from aquatic to terrestrial habitats is likely easier than the reverse for sciomyzids because morphological characters associated with air-breathing while under the water's surface are lost rather than gained, and sciomyzids originated and diversified during a general drying period in Earth's history. Our results imply that any animal lineage having aquatic and terrestrial members, respiring the same way in both habitats and having the same type of food available in both habitats could show a similar pattern of multiple independent habitat transitions coincident with changes in behavioral and morphological traits.

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Background

Some of the most important evolutionary innovations in the history of life on Earth resulted from transitions between aquatic (freshwater) and terrestrial habitats. The colonization of land by unicellular aquatic plants [1,2] and their eventual transformation into vascular plants helped shape terrestrial environments and paved the way for the evolution of the majority of the eukaryotic species alive today. Other key lineage diversifications that occurred following transitions from aquatic to terrestrial habitats include those of tetrapod vertebrates [3], millipedes [4], scorpions [5], other arachnids [2,6,7], earthworms [8] and nematodes [9]. Whereas the ancestral insect originated in a terrestrial environment [10-12], insects are one of the most successful colonizers of freshwater habitats, as at least 12 of the 31 insect orders have representatives occupying these environments during at least one life history stage [13]. Transitions between aquatic and terrestrial habitats are generally rarer than other habitat changes (e.g., between epigeal and arboreal) because of the substantial physical differences between them [14]. In addition to differences in the physical requirements of living in water versus on land (e.g., differences in oxygen concentration), one presumed barrier is that the suite of available food items are typically distinct, as there are major differences between aquatic and terrestrial food webs [15,16]. Therefore, in order to transition between these habitats, a lineage typically must adapt to new physical conditions while concomitantly modifying its feeding behaviors.

The family Sciomyzidae, or “snail-killing flies” (Diptera: Acalyptratae: Sciomyzoidea), is an ideal taxon with which to study the evolution of feeding behaviors and associated habitat transitions. Their life histories are well-studied, as 240 of the 539 species have known larval feeding habits [17]. Sciomyzid larvae display a wide range of feeding behaviors, including predation, parasitism, or saprophagy of terrestrial, semi-aquatic and aquatic non-operculate snails, operculate aquatic snails, semi-terrestrial suckcineid snails, slugs, snail eggs, finger-nail clams and freshwater oligochaete worms [17,18]. This represents, by far, the most extensive radiation of primarily mala-cophagous (= mollusk-feeding) species when compared to all other dipteran lineages [17,18]. A total of 109 species from six other dipteran families attack mollusks [19], whereas ~99% of the 240 sciomyzid species with known life cycles attack mollusks [17,18,20]. Sciomyzids have three larval stages and most species exhibiting parasitoid behavior have very specific host requirements in the 1st and 2nd larval stage but become more generalized predators in the 3rd stage. These species have been referred to as parasitoids or parasitoids/predators in sciomyzid literature, so, for simplicity, we refer to these species as parasitoids herein. There have been two different approaches to organizing sciomyzid species into behavioral/ecological groups: (i) based on commonalities in larval microhabitat, mode of feeding and prey type ([21]: 8 groups; [22]: 10 groups; [20]: 9 groups; [17,18]: 15 groups), and (ii) based on an ordination analysis of 36 egg and larval morphological characters, larval behaviors, and habitat that identified nine “Eco-Groups,” each possessing a unique combination of states from these 36 characters [23].

The Sciomyzidae includes three subfamilies: the Huttonininae with two genera [24], the Salticellinae with one genus (Salticella) and the Sciomyzinae with the remaining 58 genera. The Sciomyzinae is comprised of two tribes, the Sciomyzini with 12 genera and the Tetanocerini with the remaining 46 genera [17]. All of the Sciomyzini and Salticellinae have terrestrial larvae, whereas 14 tetanocerine genera have at least one species with aquatic larvae [17]. The larvae of the Huttonininae remain unknown [17]. Recent phylogenetic analyses of morphological data suggest that the Sciomyzinae and its two tribes are monophyletic [23,25]. The family Phaeomyiidae, with five described Palaearctic species in two genera (Akebono and Pelidnoptera), was at one time considered to be a subfamily of the Sciomyzidae, but was subsequently elevated to family by Griffiths [26], who proposed its sister status to Sciomyzidae.

The evolution of feeding behaviors in Sciomyzidae has been discussed in numerous papers (e.g., [17,18,20-22]). Because larval feeding on decaying animal matter occurs in other dipteran lineages, including families in the Sciomyzoidea (e.g., Dryomyzidae; [17]), it has been suggested that the ancestral sciomyzid was probably similar to the extant Atrichomelina pubera (Sciomyzini), a generalist that feeds on dead, dying or living aquatic and semi-aquatic, non-operculate snails on damp terrestrial substrates [27,28]. Steyskal’s [29] classification of the Sciomyzidae lead to sciomyzine larvae being characterized as terrestrial (including those inhabiting moist surfaces) saprophages/predators/parasitoids, while tetanocerine larvae are typically characterized as aquatic predators. Knutson & Vala [18] mapped their feeding groups onto the morphological phylogeny presented in Marinoni & Mathis [25] to infer the ancestral feeding behavior for the family and to discuss the evolution of such behaviors based on the position of each genus in the phylogeny. They concluded that while Steyskal’s [29] generalizations have exceptions, the distribution of feeding behaviors known today support these general characterizations. They further concluded that the terrestrial habits of many of the species in the Tetanocerini represent a derived condition within the tribe. Unfortunately, the utility of Knutson and Vala’s [18] study was somewhat limited due to the incomplete resolution of intergeneric relationships and the absence of replicate
| Family     | Genus                | Species                   | Feeding Group                                      | Specimen | GenBank Accession Numbers |
|------------|----------------------|---------------------------|----------------------------------------------------|----------|--------------------------|
|           |                      |                           | Feeding Group                                      | Citation | Number | COI | COII | 16S | 28S | Ef-1a |
| Drosophilidae | Drosophila melanogaster | Meigen 1830              | Yeast, mold                                       | [80]     | AJ400907 | AJ400907 | AJ400907 | M21017 | NM_170570 |
| Phaeomyiidae | Peltidoptera nigripennis | (Fabricius 1794)          | Millipede parasitoid                               | [81]     | F272    | JN860439 |         |        |        |
| Sciomyzidae | Sciomyzini            | Atrichomelina             | Facultative predator/saprophage of snails and clams on damp shorelines | [28]     | F160    | JN860438 | JN837567 | JN816281 | JN837498 |
| Sciomyzidae | Sciomyzina            | Atrichomelina pubera      |                                                    |          | F161    | AY875151 | AY875182 | AY875089 | AY875120 | JN816247 |
| Sciomyzidae | Sciomyza              | Sciomyza simplex          | Predator of shoreline-stranded aquatics            | [82]     | F175    | AY875152 | AY875183 | AY875090 | AY875121 | JN816248 |
| Sciomyzidae | Tetanocerini          | Anticheta melanosoma      | Predator of exposed snail eggs                     | [83]     | F254    | JN860440 | JN837568 | JN816327 | JN837499 | JN816250 |
| Sciomyzidae | Tetanocerini          | Anticheta                 |                                                    |          | F248    | JN860441 | JN837569 | JN816328 | JN837500 | JN816251 |
| Sciomyzidae | Tetanocerini          | Dichetophora              |                                                    |          | F260    | JN860449 | JN837570 | JN816329 | JN837501 | JN816252 |
| Sciomyzidae | Tetanocerini          | Dichetophora finlandica   |                                                    |          | F260    | JN860449 | JN837570 | JN816329 | JN837501 | JN816252 |
| Sciomyzidae | Tetanocerini          | Dictya borealis           | Predator of aquatic snails in the water            | [84]     | F257    | JN860442 | JN837570 | JN816329 | JN837501 | JN816252 |
| Sciomyzidae | Tetanocerini          | Dictya expansa            | Predator of aquatic snails in the water            | [84]     | F263    | JN860443 | JN837571 | JN816330 | JN837502 |
| Sciomyzidae | Tetanocerini          | Dictya floridensis        | Predator of aquatic snails in the water            | [85]     | F258    | JN860444 | JN837572 | JN816331 | JN837503 |
| Sciomyzidae | Tetanocerini          | Dictya gaigei             | Predator of aquatic snails in the water            | [84]     | F267    | JN860445 | JN837573 | JN816336 | JN837504 |
| Sciomyzidae | Tetanocerini          | Dictya pictipes           | Predator of aquatic snails in the water            | [84]     | F261    | JN860446 | JN837574 | JN816332 | JN837505 | JN816253 |
| Sciomyzidae | Tetanocerini          | Dictya steyskali          | Predator of aquatic snails in the water            | [84]     | F270    | JN860447 | JN837575 | JN816333 | JN837506 |
| Sciomyzidae | Tetanocerini          | Dictya stricta            | Predator of aquatic snails in the water            | [84]     | F271    | JN860448 | JN837576 | JN816334 | JN837507 | JN816254 |
| Sciomyzidae | Tetanocerini          | Dictya texensis           | Predator of aquatic snails in the water            | [84]     | F268    | JN860450 | JN837578 | JN816335 | JN837509 |
| Species                | Feeding Behavioral Group | GenBank Numbers                          |
|-----------------------|--------------------------|------------------------------------------|
| **Dictyacium**        |                          |                                          |
| D. firmum             | Unknown                  | F187 JN860451 JN837579 JN816337 JN837510 |
|                       |                          | F188 JN860452 JN837580 JN816338 JN837511 |
| **Elgiva**            |                          |                                          |
| E. connexa            | Predator of aquatic      | F150 AY875153 AY875184 AY875091 AY875122 |
|                       | snails in the water      | F151 JN860453 JN837581 JN816282 JN837512 |
|                       |                          | F152 JN860454 JN837582 JN816283 JN837513 JN816255 |
| E. solicta            | Predator of aquatic      | F5 AY875154 AY875185 AY875092 AY875123 |
|                       | snails in the water      | F6 JN860455 JN837583 JN816284 JN837514 |
| **Ethiolimnia**       |                          |                                          |
| E. geniculata         | Unknown                  | F255 JN860456 JN837584 JN816339 JN837515 JN816256 |
| **Euthycera**         |                          |                                          |
| E. arcuata            | Parasitoid of slugs      | F222 JN860457 JN837585 JN816340 JN816257 |
|                       |                          | F223 JN860458 JN837586 JN816341 JN837516 |
|                       |                          | F224 JN860459 JN837587 JN816342 JN837517 |
| **Hedria**            |                          |                                          |
| H. mixta              | Predator of submerged    | F168 JN860460 JN837588 JN816285 JN837518 |
|                       | aquatic snails           | F169 AY875155 AY875186 AY875093 AY875124 |
| **Hoplodictya**       |                          |                                          |
| H. acuticornis        | Parasitoid of succineid  | F277 JN860461 JN837589 JN816343 JN837519 JN816258 |
|                       | snails                   | F278 JN860462 JN837590 JN816344 JN837520 |
| **Hydromya**          |                          |                                          |
| H. dorsalis           | Predator of shoreline-   | F249 JN860463 JN837591 JN816345 JN837521 JN816259 |
|                       | stranded aquatics        |                                          |
| **Ilione**            |                          |                                          |
| I. albiseta           | Predator of submerged    | F122 JN860464 JN837592 JN816286 |
|                       | aquatic snails           |                                          |
| **Limnia**            |                          |                                          |
| L. boscii             | Parasitoid of succineid  | F120 AY875156 AY875187 AY875094 AY875125 JN816260 |
|                       | snails                   | F121 JN860465 JN837593 JN816287 JN837522 |

Table 1 Species analyzed in this study, the feeding behavioral group [17] to which each taxon belongs, and GenBank numbers for the sequences used in this study (Continued)
| Species                        | Feeding Behavioral Group | GenBank Numbers          |
|-------------------------------|--------------------------|--------------------------|
| *Limnia ottawensis* Melander 1920 | Unknown                  | F154 AY875157 AY875188 AY875095 AY875126 JN816261 |
| *Limnia sandovalensis* Fisher & Orth 1978 | Unknown                  | F155 AY875158 AY875189 AY875096 AY875127 |
| *Pherbecta*                  |                          | F156 JN860466 JN837594 JN816288 JN837523 |
| *Pherbecta limenitis* Steyskal 1956 | Predatory of shoreline-stranded aquatics | F237 JN860467 JN837595 JN816346 JN837524 JN816262 |
| *Pherbina*                  |                          | F250 JN860468 JN837596 JN816347 |
| *Pherbina conyleti* (Scopoli 1763) |                      | F250 JN860468 JN837596 JN816347 |
| *Poecilographa*              |                          | F250 JN860468 JN837596 JN816347 |
| *Poecilographa decora* (Loew 1864) |                      | F212 JN860469 JN837597 JN816348 JN837525 JN816263 |
| *Psacadina*                  |                          | F230 JN860470 JN837598 JN816349 JN837526 |
| *Psacadina zemyi* (Mayer 1953) | Predatory of shoreline-stranded aquatics | F251 JN860471 JN816350 JN837527 JN816264 |
| *Renocera*                   |                          | F88 AY875159 AY875190 AY875097 AY875128 |
| *Renocera amanda* (Cresson 1920) | Parasitoid of fingernail clams below the water’s surface | F90 AY875160 AY875191 AY875098 AY875129 |
| *Renocera johnsoni* (Cresson 1920) | Parasitoid of aquatic snails in the water | BA Foote (unpublished) |
| *Renocera pallida* (Fallen 1820) | Parasitoid of fingernail clams above the water’s surface | [94] F193 JN860473 JN837600 JN816351 JN837529 |
| *Sepedon*                    |                          | F194 JN860474 JN837601 JN816352 JN837530 JN816266 |
| *Sepedon armipes* Loew 1859   | Predator of aquatic snails in the water | [95] F28 AY875161 AY875192 AY875099 AY875130 |
| *Sepedon fuscipennis* Loew 1859 |                      | F116 JN860475 JN837602 JN816360 |
| *Sepedon praemiosa* Giglio-Tos 1893 | Predator of aquatic snails in the water | F117 AY875162 AY875193 AY875100 AY875131 |
| *Tetanocera*                 |                          | F118 AY875163 AY875194 AY875101 AY875132 JN816267 |
| *Tetanocera amurensis* Hendel 1809 |                      | F198 JN860478 JN837605 JN816290 JN837533 |
Table 1 Species analyzed in this study, the feeding behavioral group [17] to which each taxon belongs, and GenBank numbers for the sequences used in this study (Continued)

| Species                               | Feeding Behavioral Group                        | GenBank Numbers               |
|---------------------------------------|------------------------------------------------|------------------------------|
| **Tetanocera annae**                  | Predator of aquatic snails in the water        | JN860479, JN837606, JN816291, JN837534 |
| Steyskal 1938                         |                                                 | F200 JN860480, JN837607, JN816292 |
| **Tetanocera annaudi**                | Unknown                                         | JN860484, JN837611, JN816295, JN837536 |
| Orth & Fisher 1982                    |                                                 | F24 JN860485, JN837612, JN816296, JN837537 |
| **Tetanocera arrogans**               | Parasitoid of succineid snails                 | F159 JN860486, JN816297, JN837538, JN816272 |
| Meigen 1830                           |                                                 | [39]                          |
| **Tetanocera bergi**                  | Predator of aquatic snails in the water        | F57 JN860487, JN837613, JN816298, JN837539 |
| Steyskal 1954                         |                                                 | F247 JN860488, JN837614, JN816299, JN837540 |
| **Tetanocera clara**                  | Parasitoid of slugs                            | JN860489, JN837615, JN816300, JN816274 |
| Loew 1862                             |                                                 |                              |
| **Tetanocera elata** (Fabricius 1781)| Parasitoid of slugs                            | JN860490, JN837616, JN816302, JN837541 |
| **Tetanocera ferruginea**             | Predator of aquatic snails in the water        | JN860491, JN837617, JN816301, JN837542 |
| Fallén 1820                           |                                                 |                              |
| **Tetanocera freyi**                  | Unknown                                         | JN860492, JN837618, JN816303 |
| Stackelberg 1963                      |                                                 | F127 JN860493, JN837619, JN816304, JN837543 |
| **Tetanocera fuscinervis**            | Predator of shoreline-stranded aquatics        | JN860494, JN837620, JN816305, JN837544 |
| (Zetterstedt 1839)                    |                                                 |                              |
| **Tetanocera hyalipennis**            | Predator of shoreline-stranded aquatics        | JN860495, JN837621, JN816306, JN837545 |
| Roser 1840                            |                                                 |                              |
| **Tetanocera kertesi**                | Predator of terrestrial snails                 | JN860496, JN837622, JN816307, JN837546 |
| Hendel 1901                           | LV Knutson (pers. comm.)                       | F191 JN860497, JN837623, JN816358, JN837547 |
| **Tetanocera latifibula**             | Predator of aquatic snails in the water        | JN860498, JN837624, JN816359, JN837548 |
| Frey 1924                             |                                                 | F146 JN860499, JN837625, JN816360, JN837549 |
| **Tetanocera marshi**                |                                                  | F147 JN860500, JN837626, JN816361, JN837550 |
| **Tetanocera nigra**                  |                                                  | F148 JN860501, JN837627, JN816362, JN837551 |
| **Tetanocera rossii**                 |                                                  | F149 JN860502, JN837628, JN816363, JN837552 |
| **Tetanocera schultzei**              |                                                  | F150 JN860503, JN837629, JN816364, JN837553 |
| **Tetanocera solandri**               |                                                  | F151 JN860504, JN837630, JN816365, JN837554 |
| **Tetanocera xiphiodes**              |                                                  | F152 JN860505, JN837631, JN816366, JN837555 |
| Species                        | Feeding Behavioral Group | GenBank Numbers                |
|-------------------------------|--------------------------|--------------------------------|
| Tetanocera loewi            | Predator of aquatic      | F189 JN860499 JN837625 JN816307 JN837549 |
| Steyskal 1959                | snails in the water      | F226 JN860500 JN837626 JN816308 JN837550 |
| Tetanocera melanostigma      | Parasitoid of succineid  | F2 AY875172 AY875203 AY875110 AY875141 |
| Steyskal 1959                | snails                   | [101] |
| Tetanocera mesapora          | Predator of aquatic      | F40 AY875173 AY875204 AY875111 AY875142 |
| Steyskal 1959                | snails in the water      | [96] |
| Tetanocera montana           | Predator of aquatic      | F142 AY875174 AY875205 AY875112 AY875143 |
| Day 1881                     | snails in the water      | F143 JN860501 JN837627 JN816309 JN837551 |
| Tetanocera obtusifibula      | Predator of aquatic      | F275 JN860504 JN837630 JN816353 JN837554 |
| Melander 1920                | snails in the water      | F276 JN860505 JN837631 JN816354 JN837555 |
| Tetanocera oxia              | Parasitoid of succineid  | F204 JN860506 JN837632 JN816312 |
| Steyskal 1959                | snails                   | [101] |
| Tetanocera phyllophora       | Predator of terrestrial  | F39 AY875175 AY875206 AY875113 AY875144 |
| Melander 1920                | snails                   | [98,102] |
| Tetanocera pietjeja          | Parasitoid of slugs      | F1 JN860507 JN837633 JN816314 JN837556 |
| Loew 1862                    |                          | F13 AY875176 AY875207 AY875114 AY875145 |
| Tetanocera plumosa           | Predator of aquatic      | F205 JN860508 JN837634 JN816313 JN816276 |
| Loew 1847                    | snails in the water or   | [73] |
|                             | on damp shorelines       | F11 AY875177 AY875208 AY875115 AY875146 |
| Tetanocera robusta           | Predator of aquatic      | F43 JN860509 JN837635 JN816317 JN837557 |
| Loew 1847                    | snails in the water      | [96] |
| Tetanocera rotundicornis     | Parasitoid of succineid  | F10 AY875178 AY875209 AY875116 AY875147 |
| Loew 1861                    | snails                   | [101] |
| Tetanocera silivatica        | Predator of shoreline-   | F16 JN860510 JN837636 JN816317 JN837558 |
| Meigen 1830                  | stranded aquatics        | F134 JN860511 JN837637 JN816315 JN837559 JN816277 |
| Tetanocera silvatica         |                          | F157 JN860512 JN837638 JN816316 JN837560 |
| Meigen 1830                  |                          | [101] |
|                             |                          | F206 JN860513 JN837639 JN816318 JN837561 |
|                             |                          | [100] |
|                             |                          | F35 JN860515 JN816321 JN837562 JN816279 |
|                             |                          | F172 AY875179 AY875210 AY875117 AY875148 JN816278 |
|                             |                          | F173 JN860514 JN837640 JN816320 JN837563 |
|                             |                          | [103] |
|                             |                          | F209 JN860516 JN837641 JN816322 |
Table 1 Species analyzed in this study, the feeding behavioral group [17] to which each taxon belongs, and GenBank numbers for the sequences used in this study (Continued)

| Species                     | Predation Type                          | GenBank Numbers                  |
|-----------------------------|-----------------------------------------|----------------------------------|
| Tetanocera soror            | Predator of aquatic snails in the water | F210 JN860517 JN837642 JN816323 |
| Melander 1920               |                                         |                                  |
| Tetanocera valida           | Parasitoid of slugs                     | F84 AY875180 AY875211 AY875118 AY875149 JN816280 |
| Loew 1862                   |                                         |                                  |
| Tetanocera vicina           | Predator of aquatic snails in the water | F94 AY875181 AY875212 AY875119 AY875150 |
| Macquart 1843               |                                         |                                  |
| Trypetoptera canadensis     | Predator of terrestrial snails          | F164 AY875164 AY875195 AY875102 AY875133 JN816268 |
| (Macquart 1843)             | BA Foote (unpublished)                  |                                  |
| Trypetoptera punctulata     | Predator of terrestrial snails          | F217 JN860476 JN837603 JN816355 JN837531 JN816269 |
| (Scopoli 1763)              |                                         |                                  |

Sequence coverage:

| Gene | Coverage |
|------|----------|
| COI  | 114: 100% |
| COII | 110: 96.5% |
| 16S  | 111: 97.4% |
| 28S  | 101: 88.6% |
| EF1α | 34: 29.8% |
intrageneric taxon sampling within the Marinoni & Mathis [25] phylogeny. A more recent study on intergeneric scioomyzid relationships [23], which included more morphological characters than did Marinoni & Mathis [25], was similarly limited, as the taxon sampling was nearly identical and the relationships among many of the genera not well-supported. Therefore, a well-resolved species-level phylogeny focusing on a lineage that exhibits a variety of feeding behaviors and occupies multiple habitats would enable a better understanding of the evolutionary processes involved in transitions among habitat, mode of feeding and host/prey selection.

Within the Tetanocerini, the genus Tetanocera is of particular interest because it is one of the most diverse scioomyzid genera with respect to feeding behaviors. Twenty-eight of its 39 species have known life cycles (see Table 1 for a partial list) and its species occupy five of the 15 feeding groups of Knutson & Vala [17,18]: (i) general predators of non-operculate aquatic snails in the water (14 species), (ii) general predators of non-operculate aquatic snails occurring on damp shorelines (3 species), (iii) general predators of terrestrial snails (2 species), (iv) parasitoids of slugs (4 species) or (v) parasitoids of succineid (semi-terrestrial) snails (5 species). The life cycles of 11 species remain unknown. Members of the largest feeding group within Tetanocera (i above) spend their larval stages just under the surface of the water, whereas the remaining groups have terrestrial larvae. Only one other scioomyzid genus occupies five feeding groups (Sepedon), whereas most only occupy one or two [17].

In a previous paper, a DNA sequence-based phylogeny of scioomyzids was used to examine the evolution of larval characters that appeared correlated with larval habitat [30]. Character states in four larval characters were found to be significantly correlated with aquatic to terrestrial transitions in Tetanocera where each larval character changed in the same way as multiple lineages made independent habitat transitions. In the present study, we build on these findings by examining feeding behavior evolution, as feeding behaviors are dependent on both larval morphological adaptations to different environments and specific requirements related to finding and subduing different prey species. Given the diversity of feeding behaviors within Sciomyzidae and Tetanocera, it is important to determine whether there were single or multiple origins of feeding behaviors. Such an analysis would simultaneously show whether there was convergent evolution of larval habitat and the relative frequencies of habitat transitions. Multiple evolutionary hypotheses regarding feeding behaviors and habitat transitions are presented in the literature (e.g., [17,18,20,21,23,27-29]) and all should be considered plausible until rigorously evaluated using modern phylogenetic comparative methods. Therefore, the present study has four specific objectives: (i) construct a robust estimate of phylogeny for Tetanocera and Tetanocerini based on multiple mitochondrial and nuclear genes, (ii) estimate the evolutionary transitions in larval feeding behaviors, habitats and host/prey that have occurred during the evolution of Tetanocerini and Tetanocera, (iii) test prior hypotheses regarding the monophyly of feeding and ecological groupings and (iv) identify the mechanisms underlying habitat and feeding behavior evolution in Tetanocera.

**Results**

**Phylogenetic analyses**

We used Bayesian inference (BI) and maximum likelihood (ML) to analyze a concatenated 5-gene data set. The BI MAP tree with BI posterior probabilities (x100) and ML bootstrap nodal support values is shown in Figure 1. The BI MAP and best ML tree (Additional file 1: Figure S1) were largely congruent (also see Additional file 1: Figure S2, Additional file 1: Figure S3 for BI consens and ML bootstrap trees, respectively). Both recovered a monophyletic Tetanocerini (BI PP = 1.0; ML bootstrap = 100), a monophyletic Sciomyzini (PP = 1.0; BS = 100), and placed Pherbina, now in Phaeomyiidae but once considered a subfamily of Sciomyzidae (e.g., [31]) as the sister lineage to the Tetanocerini, suggesting its potential status as a tribe within the Sciomyzinae (PP = 0.97; BS = 80; Figure 1). All genera with multiple species are monophyletic except for 1) Limnia, which is rendered polyphyletic by Trypetoptera and Pherbina in the BI MAP tree and by Trypetoptera in the best ML tree, (2) Trypetoptera, rendered polyphyletic by Limnia ottawensis in both trees and (3) Renocera, rendered polyphyletic by Ethiolimnia and Dichetophora in both trees. The polyphyly of these genera are each supported by at least one node with high BI PP and ML BS values (Figure 1).

Both BI and ML recovered a monophyletic Tetanocera (PP = 1.0; BS = 96; Figure 1). Within Tetanocera, both trees have T. robusta + T. annae (Figures 1,2,3: clade ➀) as sister to the remaining species. Both analyses recovered Tetanocera clade ➀ (Figures 1,2,3) with identical relationships. This clade includes all five behavioral groups known for the genus (Table 1; Figure 2). The other major Tetanocera clade common to both trees (Figures 1,2,3: clade ➁) contains eight aquatic predators, one shoreline predator, one terrestrial predator and two species with unknown life cycles, with relatively minor differences in species relationships between the BI and ML trees. Finally, both analyses recovered T. silvatica + T. freyi (Figures 1,2,3: clade ➂) as sister species: in the BI MAP tree, clade ➂ is sister to clade ➀ + clade ➁, however in the best ML tree, clade ➂ is sister to clade ➀.
The BI MAP and best ML trees were not significantly different from one another as judged by ML methods via GARLI and CONSEL (Table 2).

We optimized Knutson and Vala’s [17] larval behavioral groups on the BI MAP tree using ML methods in Mesquite (Figure 2; see Additional file 1: Figure S1).

Figure 1 Majority rule consensus of 20,000 post burn-in trees from a 160 million generation Bayesian analysis of COI, COI and 16S mtDNA and 28S nuclear DNA from 64 sciomyzid and one phaeomyiid species under a partitioned substitution model. Bayesian posterior probabilities (x100) appear above the nodes and maximum likelihood bootstrap values (200 bootstrap replicates) appear below the nodes. Nodal support values for individuals of the same species were generally high, but were left off due to spatial constraints (as were those for species of Dictya), but appear in the supplemental figures. Drosophila melanogaster sequences were used to root the analysis. Numbers after species names are specimen numbers (Table 1).

Behavioral group optimizations

We optimized Knutson and Vala’s [17] larval behavioral groups on the BI MAP tree using ML methods in Mesquite (Figure 2; see Additional file 1: Figure S4 for
optimization on the best ML tree). From these optimizations, we infer that (i) the evolution of aquatic larvae occurred relatively early in tetanocerine phylogenesis, (ii) from this aquatic ancestor, at least 10 lineages made independent, evolutionary reversals to terrestrial existence and, during the process, made at least 15 feeding behavioral transitions, (iii) the ancestor of Tetanocera was a general predator of non-operculate snails just below the water surface and (iv) a minimum of five Tetanocera lineages made independent, evolutionary reversals to terrestrial existence during which at least seven transitions in feeding behaviors occurred. All of these transitions were judged significant by ML criteria. The optimization of larval habitat (Figure 3) demonstrates an identical aquatic to terrestrial transition pattern (as compared to Figure 2) within the Tetanocerini subsequent to the

Figure 2 Maximum likelihood optimization of Knutson and Vala’s [17] larval feeding groups on the topology from Figure 1 (pruned to include only one terminal per species) analyzed with Mesquite using the MK1 model of character evolution. Only character states that are statistically significantly better than the others (ancestral character state estimates with a log likelihood two or more units higher than all others) are shown in the pie charts at the nodes. A solid (one color) node indicates that state is significantly better than all other possible states. Grey indicates unknown character states. Numbers after species names are specimen numbers (Table 1).
The divergence of *Sepedon*. Removal of species with unknown life cycles had no significant effects on either optimization.

We also estimated the evolution of habitat changes using the dispersal-extinction-cladogenesis (DEC) model implemented in the program Lagrange [32]. We found the optimal ratio of aquatic-to-terrestrial vs. terrestrial-to-aquatic transitions was between 11:1 and 13:1 which was significantly better than the null model with no bias in habitat transition rates (i.e., the global ML estimate was more than two log-likelihood units higher; Additional file 1: Table S1) and congruent with the Mesquite optimization. Therefore, the DEC model-estimated ancestral states plotted on Figure 3 are those with Lagrange set to a 12:1 ratio of aquatic-to-terrestrial vs. terrestrial-to-aquatic transitions. This procedure significantly estimated a terrestrial habitat for the sciomyzid ancestral

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**Figure 3** Maximum likelihood optimization of larval habitat on the topology from Figure 1 (pruned to include only one terminal per species) analyzed with Mesquite using the AsymmMk model of character evolution. Only character states that are statistically significantly better than the others are shown along the branches. A solid (one color) node indicates that state is significantly better than all other possible states. Numbers after species names are specimen numbers (Table 1). Lagrange-estimated ancestral character states are denoted by blue (aquatic) and black (terrestrial) boxes. Only those nodes with a single state estimated to be significantly better than all other states are plotted. The full Lagrange output is shown in Additional file 1: Figure S5.
Table 2 Results of the likelihood-based approximately unbiased (AU), Shimodiara-Hasegawa (SH), weighted Kishino-Hasegawa (WKH), and weighted Shimodiara-Hasegawa (WSH) tests calculated using CONSEL

| Constraint                                              | -ln L  | Difference | Test          |
|---------------------------------------------------------|--------|------------|---------------|
| Tetanocera feeding group analysis (59-taxon data set)    |        |            |               |
| Unconstrained                                          | -38937 2.126 | (Best)     |               |
| Aquatic snail predators1*                               | -39212682 | 280.556    |               |
| Aquatic snail predators2*                               | -39252447 | 320.321    |               |
| Shoreline snail predators1                              | -38971338 | 394.12     |               |
| Shoreline snail predators2*                             | -39004527 | 724.01     |               |
| Slug parasitoids                                        | -38940105 | 88.89      |               |
| Terrestrial snail predators*                            | -39051229 | 119.103    |               |
| Renocerinae monophyly analysis (entire 115-taxon data set) |        |            |               |
| Unconstrained                                          | -73022049 | (Best)     |               |
| Renocerinae                                             | -73056937 | 34.89      |               |
| Comparison of Bayes MAP (Figure 1) and best ML (Additional file 1: Figure S1) trees (entire data set) |        |            |               |
| ML tree                                                 | -72999441 | (Best)     |               |
| Bayes MAP tree                                          | -73005315 | 5.875      |               |

Knutson and Vala [17] feeding group constraints were done with an abbreviated data set containing 59 terminal taxa (all Tetanocera plus 4 outgroups). Trees compared were the best topology from unconstrained analysis versus an analysis where the feeding groups (see Table 1) were constrained to be monophyletic. Tetanocera plumosa, which can either live in the water or on the shoreline, was coded both ways (Aquatic2 & Shoreline2 = T. plumosa) considered a shoreline snail predator. The monophyly of Anticheta + Renocera, proposed as subfamily Renocerinae by Verbeke [74], was tested by constraining them to be outside of the Tetanocerini and Sciomyzini. The Bayesian MAP tree and ML tree were tested to see if they were significantly different from one another. P-values in bold are significant. Constraints with an asterisk (∗) were constrained trees that were significantly worse than the unconstrained tree in all statistical tests.

Discussion

Evolutionary transitions in sciomyzid larvae

Based on life history and larval morphological studies, Knutson & Vala [18] concluded that “terrestrial behavior and morphology in the Tetanocerini are apomorphic features of that tribe.” The present study and others support this conclusion. Wiegmann et al. [33] performed phylogenetic analyses of a comprehensive dipteran data set that yielded a monophyletic Sciomyoidea that included eight families currently classified in the Sciomyzoidae, the Huttoninidiae (elevated to family) and Conopidae, a family not previously included in the Sciomyoidea (also see [34]). The Sciomyzidae occupies a relatively derived position within the Sciomyoidea in the Wiegmann et al. [33] phylogeny. The only sciomyzoid taxon known to contain aquatic larvae is the Tetanocerini. Therefore, higher-level studies of Sciomyoidea [33] support a terrestrial ancestor for Sciomyzidae and their closest relatives. The derived position of Tetanocerini within Sciomyzidae (Figures 1, 2) suggests the freshwater aquatic habit is a unique derived feature of this clade, and terrestrial behavior and morphology in the Tetanocerini are largely derived from aquatic ancestry. Our habitat optimizations (Figure 3) strongly support a terrestrial ancestor for Sciomyzidae and both Mesquite optimizations...
Figures 2, 3) strongly support an aquatic ancestor for Tetanocerini minus Anticheta and Psacadina (i.e., the third basal-most Tetanocerini node). These analyses clearly demonstrate a (i) sciomyzid ancestor with terrestrial larva, (ii) derived aquatic habitat for the ancestor of most Tetanocerini and (iii) secondarily derived terrestrial habitat for multiple tetanocerine lineages.

The genus Tetanocera is one of the most remarkable sciomyzid genera with respect to feeding behaviors, microhabitat and host/prey preference as its species are members of five of Knutson and Vala’s [17] behavioral groups. The monophyly of Tetanocera is well-supported (BI PP = 1.0; ML bootstrap = 96; Figure 1, Additional file 1: Figure S1), and the ancestor to Tetanocera was strongly supported as being a predator of aquatic snails (Figure 2). From this ancestral condition, there were three independent transitions to shoreline predation on aquatic snails, two independent transitions to terrestrial snail predation, and one transition to slug parasitoidism with one lineage subsequently transitioning to succineid snail parasitoidism (Figures 2, 4). Furthermore, the monophyly of these three behavioral groups has been rejected (Table 2). In a more general sense, this implies that Tetanocera lineages made between three (Lagrange) and five (Mesquite) independent transitions to terrestrial habitats (Figure 3). These transitions were estimated to be statistically significant by ML (Figures 2, 3, Additional file 1: Figure S4, Additional file 1: Figure S5).

Sciomyzids violate trends in habitat transitions
In their study of evolutionary aquatic-terrestrial habitat transitions, Vermeij and Dudley [14] concluded that with the exception of tetrapod vertebrates, aquatic-to-terrestrial habitat transitions are rare as compared to the reverse. However, within Diptera, more than 20 lineages have made such transitions (inferred from Figure 1 of [33]). From the present study, it can be concluded that sciomyzid lineages have made an exceptional number of
independent transitions between aquatic and terrestrial habitats. Specifically, they have made at least one transition from terrestrial to aquatic habitats, with Mesquite estimating at least 10 lineages subsequently experiencing evolutionary reversals to terrestrial habitats (Figures 2, 3, Additional file 1: Figure S4). Additionally, the Lagrange analysis demonstrated a significant bias in the transition rate towards aquatic-to-terrestrial transitions with the overall log-likelihood maximizing near a ratio of 12:1 (Additional file 1: Table S2). Furthermore, some species in each of nine tetanocerine genera not included in our analyses (Dichetophora, Dictya, Eulimnia, Neolimnia, Perilimnia, Protodictya, Sepedon, Sepedonella, Shannonia) have terrestrial larvae [17], so it is quite likely that there have been additional aquatic-to-terrestrial transitions during the phylogeny of the Tetanocerini. If we assume that Dichetophora, Dictya, Neolimnia, Protodictya and Sepedon are monophyletic and arose after the early tetanocerine ancestor entered the water (obviously true for three of these genera in Figure 1, Additional file 1: Figure S1), then as many as five additional aquatic-to-terrestrial transitions have occurred, because each of these genera have both aquatic and terrestrial members [17,18]. Given that life cycle, habitat and host/prey information is available for only 240 of 540 sciomyzid species and 41 of the 61 genera [17], the actual number of independent aquatic to terrestrial habitat transitions could easily number in the 20s. Clearly, aquatic-to-terrestrial habitat transitions are strikingly common in the Sciomyzidae.

Possible mechanisms for aquatic to terrestrial transitions

Vermeij and Dudley [14] also concluded that predation intensities are generally lower in freshwater habitats than they are on land, therefore offering less biotic resistance to transitions from terrestrial to freshwater habitats than the reverse. However, our estimated evolutionary transitions within the Tetanocerini show the opposite pattern, with a 10:1 ratio of aquatic-to-terrestrial vs. terrestrial-to-aquatic transitions (Figures 2, 3, 4; Additional file 1: Table S2). This raises the question of why sciomyzids are going against the trend observed by Vermeij and Dudley [14]. A portion of the answer likely lies in the larval morphological adaptations necessary for survival in each habitat. Chapman et al. [30] examined changes in four larval characters that were found to be significantly correlated with aquatic-to-terrestrial transitions in Tetanocera. They found that in each independent transition, the larvae of terrestrial lineages experienced reductions or losses in three characters associated with breathing while under water and lost pigmentation (also see [37]). This trend was observed across the Tetanocerini by Vala and Gasc [38], who found a series of reductions in the same breathing-related characters as lineages moved from aquatic to shoreline to drier terrestrial habitats. In order for a terrestrial Tetanocerini lineage to enter the water, it would have to gain those adaptations necessary to respire while mostly submerged. Therefore, the relative ease of losing aquatic adaptations versus the relative difficulty of gaining such adaptations de novo is likely one of the primary reasons that there is a much higher rate of aquatic to terrestrial habitat transitions than the reverse in sciomyzids. This significant reduction in aquatic-to-terrestrial adaptive morphological constraints indicates that tetanocerine phylogeny likely tracked some ecological pressures (e.g., increased aquatic predation and/or increased terrestrial food availability) more accurately than did more constrained lineages.

In order for a lineage to make a successful transition to a new habitat, the members must be able to compete for and acquire resources in the new habitat [14]. Generally, it seems rather unlikely that a lineage could make multiple parallel transitions into a new habitat as the success of these transitions would typically depend upon a simultaneous adaptation to new physical conditions as well as the utilization of new food resources. However, in the case of the Tetanocerini, the intermediate nature of the damp, shoreline habitat likely played a significant role in facilitating parallel aquatic-to-terrestrial habitat transitions. Our analyses demonstrate that five of the 10 independent transitions to terrestrial habitats were to the shoreline habitat where the prey taxa are the same as their aquatic ancestors (Hydromya, Renocera, Tetanocera; Figures 2, 4). Both aquatic snails and fingernail clams occur on damp shorelines where they are either periodically stranded by receding or fluctuating waters, or in the case of snails, temporarily foraging out of the water or migrating between aquatic habitats [17]. The availability of the same food resources on the shoreline as in the water likely facilitated stepwise transitions to terrestrial existence in multiple sciomyzid lineages. Like aquatic snails, aquatic sciomyzid larvae can move onto the damp shoreline in search of their prey. However, shoreline-adapted species have lost their adaptations to breathing while under the surface of the water, and will actively swim to shore if placed in water [39]. Once a lineage has adapted to living on damp shorelines (possessing only vestiges of the adaptations to breathing while under water), they may find it more difficult to go back into the water (having to re-express the aquatic-adapted traits) than to move to even drier habitats or switch prey type. Once accustomed to feeding on aquatic snails on terrestrial shorelines, tetanocerine lineages then are pre-adapted to preying on non-aquatic gastropods. Relative to the Tetanocerini, derived terrestrial food items include slugs, succineid (semi-terrestrial) snails and land snails. Given this evolutionary scenario, it is easy to imagine how the ancestors of dry-land terrestrial
snail-feeding lineages like Trypetoptera, Tetanocera phyllophora and T. kerteszi used the shoreline as a stepping-stone environment facilitating a gradual movement to dry-land habitats where they became generalist predators of land snails.

A central question remains as to what selection pressures led multiple lineages of Tetanocerini to transition to terrestrial habitats with some lineages switching to prey other than aquatic pulmonate snails. Chapman et al. [30] speculated that it was a combination of (1) eliminating competition with other aquatic snail predators, (2) compensating for prolonged declines in aquatic snail populations, (3) escaping aquatic insect predators/parasitoids and (4) the reduction and/or loss of suitable aquatic habitats due to the general drying climatic trend that took place between 65 and 5 mya (beginning of the Cenozoic era to the end of the Pliocene epoch) that drove these terrestrial transitions. Wiegmann et al. [33], using the penalized likelihood method in r8s [40], estimated that the Sciomyzidae originated ~30 mya (see Fig. S3 in [33]). The oldest known fossil Tetanocerini are preserved in Baltic amber (55–24 mya [41]), and the oldest known fossil Tetanocera (although this generic assignment is questionable [41]) is from the Oligocene epoch (34–24 mya [42,43]). These data place Tetanocerini lineages within the general drying period mentioned above. Therefore, (i) the relative ease of reducing or losing morphological characters (compared to gaining them de novo), (ii) the occurrence of the same prey on damp shorelines as occur in the water and (iii) the general drying trend all likely played key roles resulting in multiple tetanocerine lineages making independent aquatic-to-terrestrial transitions during their phylogeny.

General implications for evolutionary transitions

The results of this study may have implications for how changes between aquatic and terrestrial habitats have occurred in other animals. Any lineage that (1) occurs in aquatic and terrestrial habitats, (2) respires the same way in aquatic and moist shoreline habitats (e.g., cuticular respiration or open tracheal system) and (3) has the same type of food available in both habitats (e.g., pulmonate snails) could show a similar pattern of multiple independent habitat transitions coincident with changes in behavioral and morphological traits. Borda & Sidall [44] found multiple aquatic-to-terrestrial transitions in arynchobdellid leeches, and Rubinoff [45] found either multiple independent terrestrial-to-aquatic transitions or an evolutionary reversal to terrestrial habitats in one lineage of cosmopterigid moths in Hawaii. Both of these taxa fit the above criteria. Like Sciomyzidae, at least 34 other dipteran families have both aquatic and terrestrial lineages [33] and many of the larger such families have larvae that are, in general, restricted to air-breathing (e.g., Culicidae, Dixidae, Dolichopodidae, Stratiomyidae, Syrphidae, Tipulidae and Tabanidae [13]). Air-breathing insects have open tracheal systems and must establish contact between their spiracles and the atmosphere to respire and must therefore either remain at or come to the surface periodically. Of these families, the Tipulidae (crane flies), unlike many of the families traditionally classified in the suborder Nematocera (primitive flies with long, filamentous antennae) that probably share an aquatic ancestor, may have originated in damp terrestrial, tropical habitats [11]. Wiegmann et al.’s [33] plot of aquatic habitat on their comprehensive dipteran phylogeny indicated that most of the families of suborder Brachycera (derived flies with short antennae) with aquatic lineages were likely of terrestrial origin. Therefore, the findings presented herein should broadly interest anyone studying the evolution of aquatic and terrestrial habitat transitions and associated behavioral and morphological changes in Diptera, a group that includes over 152,000 currently named species [33]. Other lineages that fit the above criteria include oligochaete worms, pulmonate gastropods, decapods, isopods, amphipods, oribatid mites, true bugs in the infraorder Nepomorpha and beetles in the suborder Adephaga, superfamilies Byrrhoidea and family Lampyridae. The results of the present study are suggestive that some lineages within these groups will also show multiple convergences on aquatic or terrestrial habits when examined with modern phylogenetic comparative methods.

Conclusions

Phylogenetic analyses of sciomyzid DNA sequences provided strong support that the Sciomyzini, Tetanocerini and Tetanocera are monophyletic (Figure 1). We significantly estimated that (i) the ancestor of the Sciomyzidae was terrestrial (Figures 2, 3), (ii) there was a single terrestrial-to-aquatic transition early in the evolution of the Tetanocerini and, subsequently, (iii) there were at least 10 independent aquatic-to-terrestrial transitions and at least 15 transitions in feeding behaviors (Figures 2, 3, 4, Additional file 1: Figure S2). The 10:1 ratio of aquatic-to-terrestrial vs. terrestrial-to-aquatic transitions goes against the general trend observed in animals. We found that the ancestor to Tetanocera was aquatic and five Tetanocera lineages made independent aquatic-to-terrestrial transitions and seven independent transitions in feeding behaviors (Figures 2, 3, Additional file 1: Figure S2). Classifications of sciomyzids into ecological assemblages of species resulted in many non-monophyletic groupings (Figures 2, 3, 4, Additional file 1: Figure S2, Additional file 1: Figure S3) whose monophyly were rejected via phylogenetic constraint analyses (Table 2). Therefore, these findings strongly support our inferences of multiple independent transitions in feeding behaviors, habitats
and prey/host usage. The damp shoreline habitat is likely a crucial transitional habitat where tetanocerine lineages that move out of the water to forage can find the same prey taxa as in the water. Once tetanocerine lineages are established on the shoreline, terrestrial molluscan taxa are available as potential food resources. From a morphological standpoint, transitioning from aquatic to terrestrial habitats is easier than the reverse, as adaptations to air-breathing just below the surface of the water are more difficult to gain than to lose. Furthermore, tetanocerine phylogeny occurred as the Earth was going through a general drying period. These factors likely explain why so many tetanocerine lineages made secondary transitions to terrestrial environments. Finally, the results herein imply that any animal lineage that has aquatic and terrestrial members, respire the same way in both habitats and have the same type of food available in both habitats could show a similar pattern of multiple independent habitat transitions coincident with changes in behavioral and morphological traits.

Materials and methods

Taxon sampling

Phylogenetic analyses were performed on DNA sequences from five genes obtained from 60 Tetanocera specimens (representing 28 species) and 53 individuals representing 21 additional genera within the Sciomyzidae (19 from the Tetanocerini (34 species)), two from the Sciomyzini (2 species) and Pelidnoptera (Phaeomyiidae) which is not currently considered to be a member of the Sciomyzidae but is thought to be its sister taxon ([23,25,26] but see [33]). Therefore, our analyses include 72% of Tetanocera species, 42% of the genera of Tetanocerini, and 15% of the genera of Sciomyzini. Drosophila melanogaster (Drosophilidae) was used as the outgroup in all unconstrained phylogenetic analyses. Table 1 contains a complete listing of the taxa analyzed in this study including GenBank accession numbers and the percentage of OTUs, species and genera sequenced for each gene. For 18 of the 28 Tetanocera species, multiple individuals were available and sequenced for replicate sampling purposes. Of the 29 Tetanocera species with known life cycles, 25 are examined. Of the 41 sciomyzid genera that have behavioral information known for the larvae of at least one species, at least one representative of 17 genera is included. Ten of Knutson and Vala’s [17] 15 feeding groups are represented.

Laboratory protocols

Field collections of adult specimens were preserved immediately in 95% ethanol. In the laboratory, specimens were transferred to vials containing 100% hexamethyldisilazane (Polysciences, Inc., Warrington, Pennsylvania, USA) for at least 24 hours, after which the liquid was decanted and the specimens allowed to dry under a fume hood. Prior to preparation for total DNA isolation, the head, legs, wings and abdomen of each specimen were removed from the thorax. Total DNA was isolated from each thorax, and the remaining body parts (which contain the morphological characters necessary for species determination) are stored as vouchers in 95% ethanol at the University of Kentucky. Each specimen and associated DNA extraction was given a unique number. Species identification, collecting locality information and habitat notes were recorded in a database.

Total DNA was isolated from single individuals using QiaGen DNaseasy Tissue Kits (QIAGEN Inc., Chatsworth, California, USA) following the manufacturer’s animal tissue protocol. We PCR-amplified fragments of the mitochondrial cytochrome c oxidase subunits I (COI) and II (COII) and 16S rDNA genes, and the nuclear 28S rDNA and elongation factor-1 alpha (EF-1α) genes using the primer pairs listed in Table 3. Each amplicon was purified in NuSieve® GTG® low melting temperature agarose (Lonza, Rockland, Maine, USA) and separated from the agarose with Wizard® PCR preps DNA purification system (Promega Corp., Madison, Wisconsin, USA).

Table 3 Genes / primer information used in this study

| Gene    | Primer pair          | References | Analyzed fragment size | Notes                                      |
|---------|----------------------|------------|------------------------|--------------------------------------------|
| Mitochondrial loci: |                        |            |                        |                                            |
| 16S     | LR-N-13398 / LR-J-12887 | [105]      | 426 bp                 | Primer sequences identical to those of “Locust” |
| COI     | LCO1490              | [46]       | 658 bp                 | Together, both COI primer pairs encompass nearly the entire gene |
| HCO-700ME |                       |            | 813 bp                 |                                            |
| COII    | TL2-J-3034 / TK-N-3785 | [105]      | 681 bp                 | Amplyfy all of COII                        |
| Nuclear loci: |                        |            |                        |                                            |
| 28S     | D1F / D6R            | [107]      | 1095 bp                |                                            |
| EF-1α   | ScioEF1a-F           | Designed herein | 876 bp                 | CAYMGDGATTTCATYAAARAACATGA               |
|         | ScioEF1a-R           |            |                        | GCRATGTTAGCCGTTGTGCAATCC                |

Analyzed fragment size is the number of base pairs remaining after primer sequences and regions of ambiguous alignment were removed.
PCR reactions (total volume = 50 µL) consisted of 1X Qiagen PCR buffer, 0.2 mM of each dNTP, 0.5 mM of each primer, 1.25 U of Qiagen Taq and 1–5 µL of template DNA. Cycle sequencing protocols followed Folmer et al. [46]; both strands were cycle sequenced using either end-labeled primers (Perkin Elmer AmpliCycle Sequencing Kits; Li-COR sequencer) or labeled dideoxynucleotides (ABI Big-Dye Terminator mix v. 3.0; Applied Biosystems, Foster City, California, USA; ABI sequencer). The separation of cycle sequencing reaction products was done in 3.7% and 5.5% polyacrylamide gels in Li-COR 4200 L-2 and 4200S-2 automated DNA sequencers, respectively, or Applied Biosystems 3730XL or 3730 DNA Analyzers.

**Phylogenetic analyses**

Bi-directional sequences were aligned using AlignIR (v. 2.0, Li-COR Biosciences, Inc., Lincoln, Nebraska, USA). Multiple sequence alignments of each gene region were produced with MAFFT [47]. The alignments of the COI, COII and EF-1a sequences contained no indels, however, indels that presented alignment ambiguities were found in the sciomyzid 16S and 28S sequences. The GUIDANCE server [48] was used to assess confidence scores for each column in the MAFFT alignments. Columns with confidence scores < 95% were removed prior to all phylogenetic analyses. The data sets analyzed herein (including program-specific commands) have been deposited on Dryad (http://dx.doi.org/10.5061/dryad.cb098).

Bayesian inference (BI) phylogenetic analyses were conducted on a concatenated (using MacClade v. 4.08 [49]) 4,549-character data set (COI = 1471 nt, COII = 681 nt, 16S = 426 nt, 28S = 1095 nt, EF-1a = 876 nt) with MrBayes (v. 3.1.2 [50,51]). The data set contained 114 terminal taxa for which we generated sequences (including 31 terminals from Chapman et al. [30]), plus one additional terminal (D. melanogaster) whose sequences were obtained from GenBank (Table 1). The data were partitioned by gene region and codon position when appropriate (11 total partitions: three gene regions × three codon positions for the COI, COII and EF-1a partitions plus a single partition each for 28S and 16S) and jModeltest (v. 12.9.0 [52]) was used to determine the best-fit model for each partition (Additional file 1: Table S2). To allow each partition to have its own set of parameter estimates, revmat, tratto, statefreq, shape, and pinvar were all unlinked during the analyses. To obtain the most accurate branch length estimates possible, the option prset ratepr = variable (assigns a separate branch length parameter for each partition) was employed as per the recommendations of Marshall et al. [53], who found that BI analyses of partitioned data with a global branch length parameter resulted in significantly longer overall tree length. Four 5-million generation pilot analyses (temp = 0.2, 0.1, 0.02, 0.01) were run to determine the optimal temperature setting to assure an appropriate acceptance rate of swaps between chains [54]. Subsequently, two independent, simultaneous BI searches were run for 160 million generations, saving a tree every 5000 generations, with four search chains each (temp = 0.01). The analysis was terminated ~100 million generations after the average standard deviation of the split frequencies fell below 0.02. The 20,000 post-burn-in trees from each run, determined by examination of the log probability of observing the data by generation plot with Tracer (v. 1.5 [55]), were used to calculate the majority rule consensus tree whose nodal support values were plotted on the BI MAP tree (= maximum a posteriori probability tree).

A maximum likelihood (ML) tree was generated using GARLI (v. 2.0 [56]) using the same partitioning scheme and model assignments as the BI analysis (above) and using the default settings except for the following: searchreps = 5, numberofprecreductions = 20, treerejectionthreshold = 100. The parameter estimates from the search replicate that obtained the tree with the highest log-likelihood value were fixed in a 200-replicate ML bootstrap analysis [57] using default settings.

**Character optimizations**

The estimation of ancestral feeding groups, based on the BI MAP tree and best ML tree, were carried out using ML methods in Mesquite (v. 2.74 [58]). We followed the behavioral groups of Knutson and Vala [17], which are based on the most recent analysis of sciomyzid life cycles. Our data set included taxa from ten sciomyzid behavioral groups plus Peliandoptera (Pheamymiidae) and the outgroup (Drosophilida) as 11th and 12th states. The Markov k-state one parameter model (MK1 [59]) was used to infer ancestral character states in the ML optimizations. We also optimized larval habitat (coded as aquatic or terrestrial) for which we utilized the Asymmetrical Markov k-state 2 parameter model (AsymmMK: available only for binary characters [60-62]) which allows forward and backward rates to be different. This model was used because the behavioral group optimization estimated a 10:1 ratio of aquatic-to-terrestrial versus terrestrial-to-aquatic transitions. To make decisions regarding the significance of ancestral character state reconstructions, we followed Pagel [63] (following Edwards [64]) who recommended that ancestral character state estimates with a log-likelihood two or more units lower than the best state estimate (decision threshold [T] set to T = 2) be rejected. Generally viewed as a conservative cutoff, this threshold has been used by numerous recent authors (e.g., [65-69]). The DEC model implemented in the program Lagrange [32] was also used to estimate ancestral habitats using the BI MAP tree.
Unlike the Mesquite ML optimization which assumes instantaneous habitat transitions, Lagrange models habitat evolution along branches (i.e., over time), therefore allowing ancestors to occur in two habitats simultaneously. While apparently rare in sciomyzids, we did have one taxon (Tetanocera plumosa; see Hypothesis testing section below) that is known to occur both in aquatic and shoreline habitats. Because the Mesquite optimization suggested a 10:1 ratio of aquatic-terrestrial vs. terrestrial-aquatic transitions, we optimized this parameter in Lagrange and used a 12:1 ratio to infer ancestral habitats.

Hypothesis testing
Because many of Knutson and Vala’s [17] behavioral groups within Tetanocera were not monophyletic in the unconstrained analyses, we conducted separate analyses constraining each of these groups to be monophyletic. Each resulting constrained tree was statistically compared (see below) to the unconstrained tree to test whether the monophyly of Knutson and Vala’s [17] behavioral groups in Tetanocera could be rejected, thereby adding statistical support to inferences of multiple independent feeding behavior and habitat transitions. One unconstrained and six constrained analyses were done with RAxML (iMAC Pthreads-version [70,71]) using the same partitioning scheme as above under the GTR + G + I model [72]. Twenty replicate searches were done for each analysis (constrained and unconstrained) and the tree with the highest log-likelihood from each was used for topology testing (below). To assure that only topology changes within Tetanocera were the major differences between constrained and unconstrained trees, all but five outgroups were removed, leaving one individual each of D. melanogaster, Atrichomelina pubera, Anticheta melanosoma, Hoplodictya acuticornis and Limnia bosci and 54 Tetanocera terminals for which behavioral group is known (see Table 1). The non-Tetanocera taxa remaining in the analyses were chosen because each represents a major lineage in the BI MAP (maximum a-posteriori probability) tree and best ML trees and they had the lowest percentage of missing data. Behavioral groups that were not monophyletic on either the BI MAP or best ML trees were constrained in separate analyses as follows (see Table 1): Aquatic1: all Tetanocera with aquatic larvae including the facultative T. plumosa which can also occur on damp shorelines [73]; Aquatic2: same as Aquatic1 excluding T. plumosa; Shoreline1: all Tetanocera with larvae occurring on damp shorelines and preying on aquatic snails excluding T. plumosa; Shoreline2: same as Shoreline1 plus T. plumosa; Slug: all slug parasites; Terrestrials: both species predatory on terrestrial snails.

The full 115-taxon data set was used to test the monophyly of the Renocerinae, proposed by Verbeke [74] to include Renocera + Anticheta, two genera quite distantly separated in the BI and ML analyses presented herein. To constrain Renocera + Anticheta as a lineage outside of the other tribes, the Sciomyzini, Tetanocerini and Renocera + Anticheta were each constrained to be monophyletic with three separate constraint statements. Finally, this data set was also used to evaluate whether the BI MAP tree and the best ML tree were significantly different from one another.

To test for significant differences in topologies between unconstrained and constrained analyses, GARLI (v. 2.0), under the same partitioning scheme and models as the BI analysis, was used to create the site-likelihoods file used as input for the topology-testing program CONSEL (v. 0.1 k [75]). CONSEL was used to do the likelihood-based approximately unbiased test (AU [76]), Shimodaira-Hasegawa test (SH [77]), weighted Kishino-Hasegawa test and weighted Shimodaira-Hasegawa test (WKH and WSH [76]). Results of the KH test [78] were omitted due to its inappropriateness for testing a posteriori significant differences among tree topologies [79].

Additional file

**Additional file 1: Figure S1.** Maximum likelihood tree produced by using the partitioning scheme and model assignments in Additional file 1: Table S2 using the default settings in Garli (v. 2.0) except for the following: searchreps = 5, numberofprecreductions = 20, treerejectionthreshold = 100. Drosophila melanogaster sequences were used to root the analysis. Numbers after species names are specimen numbers (Table 1). Figure S2. Bayesian consensus tree produced by using the partitioning scheme and model assignments in Additional file 1: Table S2. Drosophila melanogaster sequences were used to root the analysis. Numbers after species names are specimen numbers (Table 1). Figure S3. Maximum likelihood bootstrap tree (200 replicates) produced by using the partitioning scheme and model assignments in Additional file 1: Table S2 using the default settings in Garli (v. 2.0). Parameter estimates from the non-bootstrap search replicate that obtained the tree with the highest log-likelihood value were fixed. Drosophila melanogaster sequences were used to root the analysis. Numbers after species names are specimen numbers (Table 1). Figure S4. Maximum likelihood optimization of Knutson and Vala’s [17] larval feeding groups on the maximum likelihood topology. Additional file 1: Figure S1, pruned to include only one terminal per species) analyzed with Mesquite using the MK1 model of character evolution. Only character states that are statistically significantly better than the others (ancestral character state estimates with a log likelihood two or more units higher than all others) are shown in the pie charts at the nodes. A solid (one color) node indicates that state is significantly better than all other possible states. Grey indicates unknown character states. Numbers after species names are specimen numbers (Table 1). Figure S5. Model log-likelihood scores for variations of the ratio of aquatic-terrestrial/aquatic transitions using the DEC model in Lagrange with dispersal and extinction rates for each. Bold font indicates significantly better log-likelihoods (i.e., greater than 2 lnL units) than the null (1:1) model. Red font indicates the parameter setting used in the plot of ancestral character states on Figure 3. **Figure S5.** Lagrange output with ratio of aquatic-to-terrestrial vs. terrestrial-to-aquatic transitions set to 12:1. Only states within 2 log-likelihood units of the best were considered for plotting on Figure 3, and
only unambiguous states were plotted. Table S2. Gene information and evolutionary models selected by jmodelTest for BI and ML phylogenetic analyses.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Research was conducted by EGC, under the overall guidance of major professors WRH and BAF at Kent State University and postdoctoral advisor JDH at the University of Kentucky. All advisers were integral to the development, funding and execution of all aspects of research. AAP was involved in data collection of Palaearctic specimens and contributed to the writing of the manuscript.

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