Molecular Evidence for an Old World Origin of Galapagos and Caribbean Band-Winged Grasshoppers (Acrididae: Oedipodinae: *Sphingonotus*)

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Abstract

Patterns of colonization and diversification on islands provide valuable insights into evolutionary processes. Due to their unique geographic position and well known history, the Galapagos Islands are an important model system for evolutionary studies. Here we investigate the evolutionary history of a winged grasshopper genus to infer its origin and pattern of colonization in the Galapagos archipelago. The grasshopper genus *Sphingonotus* has radiated extensively in the Palaearctic and many species are endemic to islands. In the New World, the genus is largely replaced by the genus *Trimerotropis*. Oddly, in the Caribbean and on the Galapagos archipelago, two species of *Sphingonotus* are found, which has led to the suggestion that these might be the result of anthropogenic translocations from Europe. Here, we test this hypothesis using mitochondrial and nuclear DNA sequences from a broad sample of Sphingonotini and Trimerotropini species from the Old World and New World. The genetic data show two distinct genetic clusters representing the New World Trimerotropini and the Old World Sphingonotini. However, the *Sphingonotus* species from Galapagos and the Caribbean split basally within the Old World Sphingonotini lineage. The Galapagos and Caribbean species appear to be related to Old World taxa, but are not the result of recent anthropogenic translocations as revealed by divergence time estimates. Distinct genetic lineages occur on the four investigated Galapagos Islands, with deep splits among them compared to their relatives from the Palaearctic. A scenario of a past wider distribution of *Sphingonotus* in the New World with subsequent extinction on the mainland and replacement by *Trimerotropis* might explain the disjunct distribution.
Introduction

Oceanic archipelagos are natural laboratories for studying evolutionary processes [1]. The Galapagos archipelago, in particular, has provided significant insight into our current understanding of speciation [2–4]. Its remote location far off the coast of Ecuador and its well-known geologic history [5] provide a unique opportunity to study colonization and subsequent radiation processes. The islands in their current state developed less than 5 million years ago [5, 6, 7]. The ages of the central and western islands, however, are much younger and range between 0.5 and 2.5 my [8]. This variation in island age might influence patterns of divergence within the archipelago.

The location of the archipelago has determined the general colonization source: phylogeographic studies have shown that most animal and plant species endemic to the Galapagos Islands originated in South America and radiated after one or multiple colonization events [9–13]. Subsequent ‘island hopping’ led to further differentiation among island lineages [7, 13]. Hence, most organisms found in the Galapagos archipelago belong to Neotropical groups and very few studies have shown a direct relationship of Galapagos endemics to any Old World taxon (e.g. the Hemipteran Nezara viridula [14, 15]). However, for many Galapagos endemics no closely related taxa occur in the Old World. In the rare instance that Galapagos species have both New and Old World relatives, phylogeographic studies often neglect the Old World as possible colonization source.

Representatives of the grasshopper genus Sphingonotus Fieber, 1852 provide a rare case of Galapagos endemics for which representatives can be found in both the New and the Old World [16, 17]. This genus is among the most species-rich grasshopper genera worldwide [18, 19]. Its main centres of species richness and endemism are the Mediterranean, central and eastern Asia, but a limited number of species have been described from Australia, South Africa, the Caribbean and Galapagos [17, 20–22]. The genus contains many endemics with very limited geographic distributions [18], often endemic to islands [20, 22]. In North and South America the genus is replaced by the ecologically similar genus Trimerotropis Stål, 1873 [19, 23]. Both genera were until recently grouped in the same tribe (Sphingonotini) [23]. However, it has been demonstrated that they split some 35 million years ago [23]. The presence of Sphingonotus species in the Caribbean (Sphingontous haitensis (Saussure, 1861)) and Galapagos (Sphingonotus fuscoirroratus (Stål, 1861)) is puzzling as these archipelagos are far off the main distribution [24]. Only two other Sphingonotus species have been recorded from the New World, Sphingonotus brasiliensis Saussure, 1888 and Sphingonotus punensis Dirsh, 1969. The types of S. brasiliensis are lost (NHMW pers. com.) [25] and the description of the species is insufficient to judge the status of the species. Hence, we consider it as nomen dubium. Sphingonotus punensis from Puna Island close to the Ecuadorian coast is morphologically very similar to S. fuscoirroratus [26, 27] and thought to belong to the same species group. However, only a single female of the species is known [26]. Sphingonotus fuscoirroratus itself has a complex history. Originally two species (S. trinesiotis Snodgrass, 1902, S. tetranesiotis Snodgrass, 1902) with several subspecies were described from the Galapagos Islands [28], which later were synonymised [29]. This synonymy was subsequently confirmed by morphological analyses, including inner genitalia, as the island populations could not be separated [26]. Similarly, S. haitensis was originally split in three species (S. haitensis, S. jamaicensis Saussure, 1884, S. cubensis Saussure, 1884). However, currently, only a single species with two subspecies is considered valid [16]. Interestingly, both taxa have been connected to the European species Sphingonotus caerulans in the past due to extremely similar phallic structures [26] and on the basis of the outer morphology [16].

To study the reasons for this disjunct distribution pattern across both continents, we test three hypotheses using a wide geographic sampling and DNA sequences of two mitochondrial genes and a nuclear gene fragment. (i) The taxonomic assignment of the Caribbean and Galapagos species might be wrong and these species may be related to the New World genus...
Trimerotropis. (ii) It has been suggested that the occurrence of *Sphingonotus* in the Caribbean is the result of recent anthropogenic translocation of a European species [16]. (iii) Alternatively, their presence may be the result of ancient long-distance colonization from the Old World and may be the relict of a formerly wider distribution.

**Results**

We sequenced a 651 bp long fragment of the Cytochrome Oxidase I (COI) gene for a total of 104 specimens. The alignment for the NADH Dehydrogenase subunit 5 (ND5) fragment consisted of 955 bp and 104 sequences. For the nuclear Histone 3 (H3) gene fragment 293 bp were sequenced for the same set of taxa (Table 1). The ND5 alignment had 401 variable sites (42.0%), 317 of which were parsimony informative. The COI alignment had 225 variable sites (34.6%), 200 of which were parsimony informative. H3 had 26 variable sites (8.9%), 18 of which were parsimony informative.

We used two different phylogenetic reconstruction methods, MrBayes and BEAST, which both yielded similar groupings: a major split with high posterior probabilities (pp = 1 for both methods) was identified separating the New World Trimerotropini and the Old World Sphingonotini (Fig. 1). Within the Trimerotropini two groups were detected with high confidence (pp = 1 for both methods) corresponding to the chromosomal groups defined by White [30–32] and previously confirmed by Husemann and colleagues [23]. Further, within the Trimerotropini most species for which multiple individuals were sequenced were monophyletic, besides *Trimerotropis pistrinaria* Saussure, 1884 and some species of the genus *Circotettix* Scudder, 1876. *Sphingonotus haitensis* from the Dominican Republic and *S. fuscoirroratus* from four Galapagos Islands grouped within the Sphingonotini. Within the Sphingonotini *S. octofasciatus* (Serville, 1838), the genus *Thalpomena* Saussure, 1884 and the *Sphingonotus* species from China split basally from the other species in the group. The next split separates *Sphingoderus carinatus* (Saussure, 1888) from a group consisting of all other *Sphingonotus* species including *S. haitensis* and *S. fuscoirroratus*. The first taxon splitting off in this group is *S. scabriculus* Stål, 1876 from South Africa followed by the New World *Sphingonotus* species; *Sphingonotus fuscoirroratus* from San Cristobal groups together with *S. haitensis* in both analyses with high support (pp ≥ 0.99). The *S. fuscoirroratus* lineages from the other three islands form a second monophyletic group with the lineages from Santa Fe and Santa Cruz being sister clades. However, *S. fuscoirroratus* is not monophyletic in either analysis. The remaining *Sphingonotus* species from Eurasia and Africa branch off subsequently.

Both RASP analyses (S-DIVA and Bayes-Lagrange) yielded similar results suggesting an African origin for the Sphingonotini as a whole and a wider distribution (Africa and Galapagos) for the ancestral taxa of the New World *Sphingonotus* species (S1 Fig.). The molecular clock analyses dated the divergence between the two major clades (Trimerotropini from the New World and Sphingonotini from the Old World) at approximately 23.4 million years ago. The onset of the Trimerotropini radiation was dated at 9.1 million years ago. The Sphingonotini radiation was dated to be older with an age of 15.1 my. The clade including *S. fuscoirroratus* from San Cristobal and *S. haitensis* was dated to approximately 9.6 mya whereas the split between the San Cristobal lineage and *S. haitensis* was dated at 7.2 mya; the radiation of the second *S. fuscoirroratus* clade started about 7.9 mya. However, the confidence intervals around the estimates were large (S2 Fig.) and hence the results should be only taken as rough guidelines rather than hard evidence.

**Discussion**

Most oceanic islands are colonized from the closest mainland [7, 33]. For the Galapagos Islands, this means that the common source for most colonizers is the South American mainland, which
Table 1. Overview of all samples used for molecular analyses; given are sampling location, GPS-coordinates, date of sampling and respective Genbank accession numbers.

| ID | Tribe            | Genus            | Species          | Country          | County/Island/City | Collector | Genbank accessions |
|----|------------------|------------------|------------------|------------------|--------------------|-----------|--------------------|
|    |                   |                  |                  |                  |                    |           | COI                |
| T76| Chortophagini     | Chortophaga      | viridifasciata   | USA, Texas       | McLennan Co.       | MH        | JQ513034          |
| T112| Cibolacrimi      | Cibolacris       | parviceps        | USA, Texas       | Brewster Co.       | MH        | JQ513033          |
| T25| Trimerotropini   | Circotettix      | maculatus        | USA, California  | Mono Co.           | D. Ferguson| JQ513041          |
| T26| Trimerotropini   | Circotettix      | maculatus        | USA, California  | Mono Co.           | D. Ferguson| JQ513045          |
| T10| Trimerotropini   | Circotettix      | rabula           | USA, New Mexico  | Sandoval Co.       | D. Ferguson| JQ286519          |
| T108| Trimerotropini   | Circotettix      | rabula           | USA, Montana     | Yellowstone Co.    | R.D. Scott| JQ513044          |
| T9 | Trimerotropini   | Circotettix      | stenometopus     | USA, California  | Glenn Co.          | D. Ferguson| JQ513039          |
| T23| Trimerotropini   | Circotettix      | undulatus        | USA, California  | Mono Co.           | D. Ferguson| JQ513043          |
| T24| Trimerotropini   | Circotettix      | undulatus        | USA, California  | Mono Co.           | D. Ferguson| JQ513042          |
| T15| Trimerotropini   | Conozoa          | texana           | USA, New Mexico  | Valencia Co.       | D. Ferguson| JQ286500          |
| K379| Sphingonotini    | Leptopternis     | maculatus        | Tunisia           | Ouesslatia         | AH        | JQ513074          |
| K473| Sphingonotini    | Sphingoderus     | carinatus        | Tunisia           | Bou Hedma          | AH        | KJ923334          |
| K315| Sphingonotini    | Sphingonotus     | caerulans        | France            | Vergières / Crau   | AH        | KJ923368          |
| K608| Sphingonotini    | Sphingonotus     | caerulans        | Finland           | Hanko Taktom       | AH        | JQ513067          |
| K613| Sphingonotini    | Sphingonotus     | caerulans        | Italy             | Affi               | S. Lötters| KJ923335          |
| K512| Sphingonotini    | Sphingonotus     | canariensis      | Cape Verde        | Maio               | M. Lecoq  | JQ513077          |
| K403| Sphingonotini    | Sphingonotus     | candidus         | Italy             | Sardinia           | Y. Görzig | JQ513066          |
| K262| Sphingonotini    | Sphingonotus     | corsicus         | France            | Corse              | F. Pahlmann| KJ923336          |
| K90 | Sphingonotini    | Sphingonotus     | femoralis        | Niger              | Tabourax           | T. McNary | JQ513065          |
| K383| Sphingonotini    | Sphingonotus     | finotianus       | Tunisia            | Enfida             | AH        | JQ513073          |
| K456| Sphingonotini    | Sphingonotus     | fuerteventrae    | Spain              | Canary Islands,    | AH, MH    | JQ513071          |
| K424| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923337          |
| K631| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923338          |
| K632| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923339          |
| T166| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923340          |
| T167| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923341          |
| T169| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923343          |
| T170| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923344          |
| T171| Sphingonotini    | Sphingonotus     | fuscoirroratus   | Ecuador           | Galapagos Islands, | F. Otte   | KJ923345          |

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| ID   | Tribe            | Genus          | Species                        | Country                          | County/Island/City                      | Collector                      | Genbank accessions |
|------|------------------|----------------|--------------------------------|----------------------------------|----------------------------------------|-------------------------------|--------------------|
| T172 | Sphingonotini    | Sphingonotus   | fuscoirroratus                 | Ecuador                          | Galapagos Islands, Santa Fe            | D. Otte                       | KJ923346 KJ923400 KJ923388 |
| T54  | Sphingonotini    | Sphingonotus   | fuscoirroratus                 | Ecuador                          | Galapagos Islands, Santa Fe            | D. Otte                       | KJ923349 KJ923401 KP201154 |
| T56  | Sphingonotini    | Sphingonotus   | fuscoirroratus                 | Ecuador                          | Galapagos Islands, Floreana            | D. Otte                       | KJ923350 KJ923403 KP201155 |
| T66  | Sphingonotini    | Sphingonotus   | fuscoirroratus                 | Ecuador                          | Galapagos Islands, Floreana            | D. Otte                       | KJ923351 KJ923404 KP201156 |
| K14  | Sphingonotini    | Sphingonotus   | guanchus                       | Spain                            | Canary Islands, Gran Canary            | AH                            | JQ513064 EU266743 JQ513192 |
| K638 | Sphingonotini    | Sphingonotus   | guanchus                       | Spain                            | Canary Islands, Gran Canary            | R. Bland                      | JQ513063 JQ513149 JQ513193 |
| T178 | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. Independencia                    | A. Hilario                    | KP201141 KJ923405 KP201157 |
| T179 | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. San Cristobal                    | A. Hilario                    | KJ923354 KJ923406 KJ923390 |
| T180 | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. San Cristobal                    | A. Hilario                    | KJ923355 KJ923407 KJ923391 |
| T184 | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. San Juan                         | H. Takizawa                   | KP201142 KJ923408 KP201158 |
| T39  | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. Peravia                          | D. Perez, B. Hierro           | KJ923356 KJ923409 KP201159 |
| T40  | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. Pedernales                       | D. Perez, B. Hierro, R. Bastardo | KJ923357 KJ923410 KP201160 |
| T41  | Sphingonotini    | Sphingonotus   | haitensis                      | Dominican Republic               | Prov. Pedernales                       | D. Perez, B. Hierro, R. Bastardo | KJ923358 KJ923411 KP201161 |
| K651 | Sphingonotini    | Sphingonotus   | maroccanus                     | Morocco                          | Ameskroult                              | MH                            | JQ513075 JQ513150 JQ513194 |
| K616 | Sphingonotini    | Sphingonotus   | ningsianus                     | China                            | unknown                                 | unknown                       | JQ513060 JQ513151 JQ513195 |
| K470 | Sphingonotini    | Sphingonotus   | octofasciatus                  | Tunisia                          | Gafsa                                    | AH                            | JQ513058 JQ513152 JQ513196 |
| K351 | Sphingonotini    | Sphingonotus   | rubescens                      | Spain                            | Canary Islands, Fuerteventura          | AH, MH                        | JQ513069 JQ513153 JQ513197 |
| K510 | Sphingonotini    | Sphingonotus   | rubescens                      | Cape Verde                       | Fopo                                    | M. Lecoq                      | JQ513070 JQ513154 JQ513198 |
| K5   | Sphingonotini    | Sphingonotus   | rugosus                        | Spain                            | Canary Islands, Lanzarote               | AH                            | KJ923359 EU266739 KP201162 |
| K150 | Sphingonotini    | Sphingonotus   | savignyi                       | Spain                            | Canary Islands, Gran Canary             | AH                            | JQ513076 JQ513155 JQ513199 |
| K214 | Sphingonotini    | Sphingonotus   | scabriculum                    | Namibia                          | Otjiu                                   | W. Schuett                    | JQ513061 JQ513156 JQ513200 |
| K615 | Sphingonotini    | Sphingonotus   | tsinlingensis                  | China                            | unknown                                 | unknown                       | JQ513059 JQ513157 JQ513201 |
| K227 | Sphingonotini    | Thalpomena     | caerulescens                   | Morocco                          | Irhil-n'-Isemsiden                     | AH                            | JQ513057 JQ513158 JQ513203 |
| K641 | Sphingonotini    | Thalpomena     | viridipennis                   | Morocco                          | Imouzzer                                | MH, JCH                       | JQ513056 JQ513159 JQ513204 |
| T27  | Trimerotropini   | Trimerotropis  | californica                    | USA, New Mexico                  | Socorro Co.                             | D. Ferguson                   | KJ923360 KJ923412 KP201163 |
| T28  | Trimerotropini   | Trimerotropis  | californica                    | USA, New Mexico                  | Socorro Co.                             | D. Ferguson                   | JQ513048 JQ513160 JQ513205 |
| T21  | Trimerotropini   | Trimerotropis  | cincta                         | USA, New Mexico                  | Sandoval Co.                            | D. Ferguson                   | KJ923361 KJ923413 KP201164 |
| T22  | Trimerotropini   | Trimerotropis  | cincta                         | USA, New Mexico                  | Sandoval Co.                            | D. Ferguson                   | KJ923362 KJ923414 KP201165 |

(Continued)
| ID | Tribe          | Genus      | Species                  | Country       | County/Island/City | Collector       | Genbank accessions |
|----|----------------|------------|--------------------------|---------------|-------------------|----------------|-------------------|
| T1 | Trimerotropini | Trimerotropis | cyanepennis             | USA, Texas    | McLennan Co.      | MH, PDD        | JQ286498 JQ286630 JQ286565 |
| T2 | Trimerotropini | Trimerotropis | maritima                 | USA, Texas    | McLennan Co.      | MH, PDD        | KJ923368 KJ923420 KP201172 |
| T52| Trimerotropini | Trimerotropis | maritima                 | USA, Texas    | Bosque Co.        | MH, PDD        | KJ9284977 JQ286629 JQ286564 |
| T86| Trimerotropini | Trimerotropis | maritima                 | USA, Texas    | Brewster Co.      | MH             | KJ923369 KJ923421 KP201173 |
| T29| Trimerotropini | Trimerotropis | melanoptera              | USA, New Mexico | Valencia Co.  | D. Ferguson     | KJ923370 KJ923422 KP201174 |
| T30| Trimerotropini | Trimerotropis | melanoptera              | USA, New Mexico | Valencia Co.  | D. Ferguson     | KJ923371 KJ923423 KP201175 |
| T14| Trimerotropini | Trimerotropis | modesta                  | USA, Arizona  | Coconino Co.      | D. Ferguson     | KJ923372 KJ923425 KP201176 |
| T57| Trimerotropini | Trimerotropis | modesta                  | USA, Arizona  | Cochise Co.       | D.R. Swanson    | KJ923373 KP201201 KP201177 |
| T58| Trimerotropini | Trimerotropis | modesta                  | USA, Arizona  | Cochise Co.       | D.R. Swanson    | KJ923374 KP201202 KP201178 |
| T152| Trimerotropini | Trimerotropis | occidentalis             | USA, California | Glenn Co.  | D. Ferguson     | KJ923375 KJ923426 KP201179 |
| T153| Trimerotropini | Trimerotropis | occidentalis             | USA, California | Glenn Co.  | D. Ferguson     | KJ923376 KP201203 KP201180 |
| T116| Trimerotropini | Trimerotropis | ochraceipennis           | Chile         | Coquimbe          | J. Pizarro      | JQ286549 JQ286681 JQ286607 |
| T117| Trimerotropini | Trimerotropis | ochraceipennis           | Chile         | Coquimbe          | J. Pizarro      | JQ286547 JQ286679 KP201181 |
| T118| Trimerotropini | Trimerotropis | ochraceipennis           | Chile         | Coquimbe          | J. Pizarro      | JQ286546 JQ286678 KP201182 |
| T119| Trimerotropini | Trimerotropis | ochraceipennis           | Chile         | Coquimbe          | J. Pizarro      | JQ286548 JQ286680 JQ286606 |
| T128| Trimerotropini | Trimerotropis | ochraceipennis           | Chile         | Coquimbe          | J. Pizarro      | KJ923377 JQ286688 JQ286622 |
| T130| Trimerotropini | Trimerotropis | pallidipennis            | USA, Texas    | Brewer Co.       | MH              | KP201143 JQ286690 KP201183 |
| T140| Trimerotropini | Trimerotropis | pallidipennis            | Mexico        | El Coptal        | D. Salas        | JQ286533 JQ286665 KP201184 |
| T141| Trimerotropini | Trimerotropis | pallidipennis            | Mexico        | Marquez          | D. Salas        | JQ286527 JQ286659 KP201185 |
| T144| Trimerotropini | Trimerotropis | pallidipennis            | Mexico        | El Coptal        | D. Salas        | JQ286562 KP201204 KP201186 |
| T156| Trimerotropini | Trimerotropis | pallidipennis            | Mexico        | Salamanca        | D. Salas        | JQ286522 JQ286654 JQ286581 |
| T162| Trimerotropini | Trimerotropis | pallidipennis            | Mexico        | Salamanca        | D. Salas        | JQ286537 JQ286669 JQ286596 |
| T163| Trimerotropini | Trimerotropis | pallidipennis            | Mexico        | Salamanca        | D. Salas        | JQ286535 JQ286667 JQ286594 |
| T124| Trimerotropini | Trimerotropis | pistrinaria              | USA, Texas    | Whitney Co.      | MH              | KJ923379 KJ923427 KP201187 |

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is ~1000 km away from the archipelago. Our analyses, however, clearly support an Old World origin of the Neotropic \textit{Sphingonotus} species. The species from Galapagos and the Caribbean Islands group within the Sphingonotini with high support. In addition, the branch lengths of each island population are rather long, which supports the original designation of each island population as a distinct species or subspecies [28] despite limited phenotypic divergence [26].

### Phylogeography of the New World \textit{Sphingonotus} species

The inferred phylogeny interpreted against the background of contemporary species distributions lets us argue that (i) grasshoppers of the tribe Sphingonotini are mainly distributed in the Old World. However, (ii) the focal species found in the Neotropics, i.e. on the Galapagos Islands and in the Caribbean, belong to the Sphingonotini rather than to the Trimerotropini, which is the predominant tribe in the New World. Hence, our analyses reject our first hypothesis that the taxonomic assignment of the Caribbean and Galapagos species to the tribe Sphingonotini is wrong. Rather our data support the hypothesis that the Caribbean (i.e. Atlantic) and the Galapagos Archipelago (i.e. Pacific) species are members of the Sphingonotini.

It has been suggested that the occurrence of \textit{Sphingonotus} on Galapagos might be the result of a recent introduction from Europe [16]. This hypothesis can be rejected as well, since the species represent rather old lineages within the genus and are much older than most Old World species and diverged prior to any potential introduction date. While the dating is very crude the resulting age estimates are more likely an underestimate than an overestimate; the divergence between the two major clades (Trimerotropini from the New World and Sphingonotini from the Old World) was here estimated at approximately 24.4 million years ago. This dating estimate is more recent (yet both estimates have overlapping 95% HPD) than the estimate derived from a more comprehensive study which dated the split between the clades at about 35 mya [23]. The same split was dated even further back (~55 mya) by a study by Chapco & Contreras [34]. The estimate derived here is therefore a minimum estimate of the age with

| ID | Tribe          | Genus     | Species | Country      | County/Island/City | Collector  | Genbank accessions |
|----|----------------|-----------|---------|--------------|--------------------|------------|--------------------|
|    |                |           |         |              |                    |            | COI    | ND5  | H3           |
| T31 | Trimerotropini | Trimerotropis | pistriaria | USA, New Mexico | Valencia Co. | D. Ferguson | JQ513046 | JQ513165 | JQ513210 |
| T19 | Trimerotropini | Trimerotropis | pseudofasciata | USA, Utah | Tooele Co. | D. Ferguson | KJ923381 | KJ923428 | KJ921188 |
| T20 | Trimerotropini | Trimerotropis | pseudofasciata | USA, Utah | Tooele Co. | D. Ferguson | KJ923382 | KJ923429 | KJ921189 |
| T132 | Trimerotropini | Trimerotropis | saxatilis | USA, Texas | Hill Co. | M. Hanitzsch | JQ286503 | JQ286635 | JQ286570 |
| T133 | Trimerotropini | Trimerotropis | saxatilis | USA, Texas | Hill Co. | M. Hanitzsch | JQ286502 | JQ286634 | KJ921190 |
| T154 | Trimerotropini | Trimerotropis | saxatilis | USA, Missouri | unknown | A. Templeton | KJ923383 | KJ923430 | KJ921191 |
| T155 | Trimerotropini | Trimerotropis | saxatilis | USA, Missouri | unknown | A. Templeton | KJ923384 | KJ923431 | KJ921192 |
| T68 | Trimerotropini | Trimerotropis | sp | Argentina | Mendoza Prov. | V. Confalonieri | JQ286552 | JQ286684 | KJ921193 |
| T69 | Trimerotropini | Trimerotropis | sp | Argentina | Mendoza Prov. | V. Confalonieri | JQ286555 | JQ286687 | KJ921194 |
| T70 | Trimerotropini | Trimerotropis | sp | Argentina | San Luis Prov. | V. Confalonieri | JQ286554 | JQ286686 | KJ921195 |
| T71 | Trimerotropini | Trimerotropis | sp | Argentina | San Luis Prov. | V. Confalonieri | JQ286553 | JQ286685 | JQ286611 |
| T11 | Trimerotropini | Trimerotropis | verruculata suffusa | USA, New Mexico | Sandoval Co. | D. Ferguson | KP201144 | KJ923432 | KJ921196 |
| T12 | Trimerotropini | Trimerotropis | verruculata suffusa | USA, New Mexico | Sandoval Co. | D. Ferguson | KJ923385 | KP201205 | KJ921197 |

Table 1. (Continued)

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Fig 1. Phylogenetic tree resulting from Bayesian analysis of the combined data set of three genes. Red color indicates the New World Trimerotropini, blue are the Old World Sphingonotini. Black circles represent posterior probabilities \( \geq 0.95 \) in both analyses. Numbers are posterior probabilities below 0.95 for at least one of the analyses (upper value from BEAST analysis, lower value from MrBayes analysis). The numbers in parentheses represent the divergence time estimates derived from the BEAST analysis. Only the
the lineages likely being much older. The ages of the Galapagos endemics with more than 7 mya at the basis of the lineages predate the origin of the islands.

The observed relationships may be explained by long-distance dispersal via the mainland leading to the colonization of the islands with subsequent extinction on the mainland. One might even speculate that the Sphingonotini might have colonized the American continent (e.g. [23]) and later been displaced by Trimerotropini, except for the oceanic island populations. This is supported by the high age of the islands endemics predating the ages of the islands. Alternatively, the New World Sphingonotus species might have reached the islands via rare long-distance, trans-Atlantic dispersal events. The first colonization step was then likely to the Caribbean, which is supported by the phylogeny. A reasonable number of studies have shown trans-Atlantic dispersal of a variety of animal and plant taxa [35–38]. For example, a study by Carranza and colleagues [39] showed a case of long-distance dispersal, where Tarentola Geckos invaded the Caribbean from Africa [39]; South America has been colonized by Hemidactylus Geckos from Africa [40], and the Americas were colonized from Africa by the grasshopper genus Schistocerca [36].

The Galapagos lineages of Sphingonotus appear to be older than many of the islands and hence a previous mainland distribution with subsequent extinction appears more likely. A continental extinction of the genus would also explain the lack of monophyly of the New World Sphingonotini. However, with our data we are not able to support with confidence either of the following hypotheses: (1) the Sphingonotini had a wider New World distribution which has been largely replaced by the Trimerotropini except for relict occurrences of Sphingonotus on the archipelagos or (ii) the Sphingonotini of the Galapagos archipelago and Hispaniola are the result of trans-Atlantic colonization.

Island colonization and differentiation
In the past, Sphingonotus fuscoirroratus from Galapagos had been divided into two species with several subspecies [28]. Subsequently, these taxa were synonymised as only limited morphological variation between island lineages was found [26]. Our analyses suggest that each island indeed has its own distinct genetic lineage which supports the original species or subspecies status. The extent of genetic divergence of the island populations suggests that no or very little gene flow between islands exists.

Generally, inter-island radiations are typical for the Galapagos as a result of the large distance to the mainland and the relatively high distances between most islands. This can partly be confirmed here (at least for four islands). Similar radiations on the Galapagos are known for mockingbirds (Nesomimus) [10], tenebrionid beetles [41], iguanas (Conolophus) [42], and the Galapagos lava lizards [43]. The lack of monophyly of S. fuscoirroratus due to the position of the San Cristobal lineage might be caused by insufficient resolution of the data or by extinction of true sister species on the American continent. However, another explanation might be that this island was colonized independently from the others as has been shown for the Canary Islands as well [22]. However, this hypothesis would require the assumption that both lineages converged substantially in morphology when adapting to the island habitats.

Conclusion
Our analyses support that the Galapagos endemic S. fuscoirroratus and the Caribbean endemic S. haitensis indeed belong to the tribe Sphingonotini and we therefore reject the hypothesis that
these species had been wrongly assigned to the Sphingonotini. The colonization is rather an-
cient which allows us to reject the hypothesis that the studied species were the result of anthrop-
ogenic translocation. However, we cannot infer with certainty if the populations are relics of 
a previously more widespread distribution or the result of long-distance, trans-Atlantic dis-
persal. In demonstrating a close phylogenetic relationship of Galapagos endemic species to Old 
World taxa, this study highlights the need to include geographically distantly distributed taxa 
in phylogeographic studies. Following the deep genetic splits detectable for our samples from 
Galapagos Islands, we assume that at least three to four distinct Sphingonotus species exist on 
the archipelago. It is likely that further genetic lineages are present on other islands that had 
not been studied here in concert with the original designation as species and subspecies [28].

Material and Methods

Study species

Grasshoppers of the genus Sphingonotus are widely distributed across major parts of the Palaearctic 
and Palaeotropic regions. A supposedly close relative, the genus Trimerotropis, can be found exclu-
sively in the Nearctic and Neotropic region [16, 17]. The genera Trimerotropis and Sphingonotus 
show strong morphological similarities; however, representatives of Trimerotropis are mostly larger 
[16, 24]. Both genera had been grouped in the tribe Sphingonotini for many decades, but recently 
the genus Trimerotropis was re-assigned to the previously erected Trimerotropini [23, 44]. Both 
genera are species-rich with 142 species for Sphingonotus and 52 Species for Trimerotropis [17].

Sampling

In total, 104 individuals belonging to 44 species from four continents were included in the anal-
yses (Table 1). Specimens were collected by hand or netted and subsequently frozen or stored 
in ethanol. Many samples were obtained from museums or colleagues. None of the collected 
species are protected and no sampling was performed on protected land aside from the Galapa-
gos. Sampling activities on Galapagos were performed by D. Otte (ANSP, Philadelphia) and 
S. B. Peck (Carleton University, Ottawa, Canada) under permission of the Galapagos National 
Park (F. Cepeda, A. Izurieta and E. Cruz, Superintendents, Department of Forestry, Ministry of 
Agriculture, Republic of Ecuador). The Gomphocerinae Cibolacris parviceps and the Oedipodini-
ae Chortophaga viridifasciata served as outgroups in all analyses. Details about all individuals 
collected and used for this study are given in Table 1.

Molecular analyses

Genomic DNA was extracted from dried or ethanol preserved hind leg muscle tissue using the 
Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer’s 
protocol for tissue samples. We amplified two mitochondrial and one nuclear gene fragment 
using a standard PCR protocol. Primers for the mitochondrial NADH Dehydrogenase subunit 5 
(ND5) were obtained from Su and colleagues [45] and for COI from Husemann and colleagues 
[23]. The primers for Histone 3 (H3) were taken from Colgan and colleagues [46]. PCR reactions 
were performed using the following setup: 36.6 μl of diH2O, 6 μl of 10 x PCR buffer (reaction 
concentration 1x), 4.8 μl of dNTP mixture (0.2 μM each), 0.6 μl of DyNAzyme DNA Polymerase 
(1.2 U, Finnzymes, USA), 3 μl of each primer (0.5 μM, Integrated DNA technologies, USA) and 
6 μl of DNA template adding up to a total volume of 60 μl. Amplification conditions were as fol-
lows: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min denaturation, 48–57°C 1 min an-
nealing and 72°C for 2 min elongation, with a final elongation step at 72°C for 10 min.
PCR products were visualized on a 1% agarose gel stained with Gel Red (0.1x, Biotium, USA and purified using Solid-phase Reversible Immobilization (SPRI) [47] with carboxylated magnetic beads (Bangs Laboratories, USA) and a 96-Ring SPRIplate (Agencourt, USA). The purified PCR products were sequenced at the Yale Sequencing Facility (New Haven, CT, USA). All sequences were deposited in Genbank; accession numbers are given in Table 1.

Phylogenetic analyses

Sequences were inspected, trimmed and aligned using the MAFFT algorithm in Geneious 5.0.3 [48]. Further we used sequences from previous studies [18–20, 22, 23]. All genes were subsequently analyzed as combined data set. In a first step we identified the best partitioning scheme treating codon positions separately and determined the most suitable substitution models using PartitionFinder v.1.1.1 [49]. We performed two runs of PartitionFinder, one including the models implemented in MrBayes and one including the models implemented in BEAST. We then analyzed the concatenated partitioned data set with MrBayes v.3.1.2 [50]. We ran MrBayes for 50 million generations sampling every 5000 generations. A burn-in of 25% of trees was discarded before constructing a consensus tree. In addition we used BEAST v. 1.8.0 [51] to analyze the data in a supertree framework. The input file for BEAST was setup with BEAUti v. 1.8.0 (implemented in the BEAST package). We used the partitioning scheme from PartitionFinder to link the substitution models. The clock models were linked for mitochondrial genes. The trees were linked for all data. We used the Yule prior as recommended for analyses at species and genus levels and ran the analyses for 100 million iterations sampling every 10,000 iterations. The log-files were checked in Tracer v.1.5 [52] to check for convergence. A burn-in of 1000 trees was discarded before generating a consensus tree. All trees were visualized using FigTree v.1.3.1 [53].

In addition we obtained coarse estimates of divergence dates by applying a molecular clock approach. We used published substitution rates of 0.0113 for ND5 [23] and 0.01 for COI estimating the rate for H3 and applied a strict clock in BEAST v.1.8.0 [51]. No better calibration was possible as no suitable fossil data is available and using island ages as calibration points appeared inappropriate considering that we intended to estimate the divergence times of island lineages. The analysis was run for 100 million generations sampling every 10,000 generations. Trees were summarized with TreeAnnotator and visualized with FigTree.

In a last step we obtained evidence for the origin of the Galapagos taxa by using statistical DIVA and Bayes-Lagrange analyses as implemented in RASP v.3.0 [54]. We used the trees generated by our BEAST run as input and defined the geographic areas as follows: A—N America, B—Africa (including Cape Verde), C—Europe (including the Canary Islands), D—Galapagos Islands, E—Caribbean, F—Asia, G—S America. The maximum areas per node were set as 2.

Supporting Information

S1 Fig. Results from S-DIVA analysis in RASP v.3.0 (Yu et al. 2010). We used the trees generated by our BEAST run as input and defined the geographic areas as follows: A—N America, B—Africa (including Cape Verde), C—Europe (including the Canary Islands), D—Galapagos Islands, E—Caribbean, F—Asia, G—S America. The maximum areas per node were set as 2. Values represent posterior probabilities.

S2 Fig. Divergence time estimates obtained from a molecular clock analysis in BEAST v.1.8.0 (Drummond et al. 2012). We used published substitution rates of 0.0113 for ND5 (Husemann et al. 2012) and 0.01 for COI estimating the rate for H3 and applied a strict clock. The analysis was run for 100 million generations sampling every 10,000 generations. Trees
were summarized with TreeAnnotator and visualized with FigTree. Numbers are divergence times in million years. The bars represent the 95% HPDs of age estimates.

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Author Contributions

Conceived and designed the experiments: MH AH DO PDD. Performed the experiments: MH SN AH. Analyzed the data: MH. Contributed reagents/materials/analysis tools: MH JCH AH DO PDD. Wrote the paper: MH AH. Collected samples from Galapagos: DO. Wrote the initial draft: MH. Contributed to the improvement of previous versions of the manuscript: MH JCH SN AH DO PDD.

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