Remarkably low host specificity in the bat fly *Penicillidia fulvida* (Diptera: Nycteribiidae) as assessed by mitochondrial COI and nuclear 28S sequence data

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Abstract

**Background:** The recognition and delineation of morphologically indistinguishable cryptic species can have broad implications for wildlife conservation, disease ecology and accurate estimates of biodiversity. Parasites are intriguing in the study of cryptic speciation because unique evolutionary pressures and diversifying factors are generated by ecological characteristics of host-parasite relationships, including host specificity. Bat flies (Diptera: Nycteribiidae and Streblidae) are obligate, hematophagous ectoparasites of bats that generally exhibit high host specificity. One rare exception is *Penicillidia fulvida* (Diptera: Nycteribiidae), an African bat fly found in association with many phylogenetically distant hosts. One explanation for *P. fulvida*'s extreme polyxeny is that it may represent a complex of host-specific yet cryptic species, an increasingly common finding in molecular genetic studies of supposed generalist parasites.

**Methods:** A total of 65 *P. fulvida* specimens were collected at 14 localities across Kenya, from bat species representing six bat families. Mitochondrial cytochrome c oxidase subunit 1 (COI) and nuclear 28S ribosomal RNA (rRNA) sequences were obtained from 59 specimens and used to construct Bayesian and maximum likelihood phylogenies. Analysis of molecular variance was used to determine how genetic variation in *P. fulvida* was allocated among host taxa.

**Results:** The 28S rRNA sequences studied were invariant within *P. fulvida*. Some genetic structure was present in the COI sequence data, but this could be more parsimoniously explained by geography than host family.

**Conclusions:** Our results support the status of *P. fulvida* as a rare example of a single bat fly species with primary host associations spanning multiple bat families. Gene flow among *P. fulvida* utilizing different host species may be promoted by polyspecific roosting behavior in bats, and host preference may also be malleable based on bat assemblages occupying shared roosts. The proclivity of generalist parasites to switch hosts makes them more likely to vector or opportunistically transmit pathogens across host species boundaries. Consequently, the presence of polyxenous bat flies is an important consideration to disease ecology as bat flies become increasingly known to be associated with bat pathogens.

**Keywords:** Bat flies, Chiroptera, Ectoparasites, Kenya, Nycteribiidae, Host specificity, Host-parasite interactions, Cryptic species, Molecular biology

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Introduction

Although the species is a basic unit of organization in biology and is of fundamental importance in the study of ecology and evolution, the definition of a species is famously contentious. At least 32 species concepts have been described [1], but these are often variations on a few themes [2]: (i) the ability to successfully reproduce (e.g. the Biological Species Concept [3]); (ii) distinctive morphology (e.g. the Morphological Species Concept [4]); and (iii) shared evolutionary descent (e.g. the Phylogenetic Species Concept [5]). An integrative approach to species delimitation addressing several species concepts is often desirable, but some criteria are difficult to satisfy in practice or categorically inapplicable to certain study systems [2].

The limited reach of species concepts is demonstrated by cryptic species that are morphologically indistinguishable but genetically and often ecologically distinct [6, 7]. The evolutionary processes associated with cryptic speciation are variable, with cryptic speciation known to occur in allopatry [8], sympatry [9] and parapatry [10]. Recent advances in molecular systematics have significantly increased the rate of discovery of cryptic species [6, 7]. The recognition and delimitation of cryptic species is not only critical to accurately quantifying biodiversity, but misidentification of cryptic species complexes as single species can have profound consequences for wildlife conservation and disease ecology [6, 7].

Parasites are compelling candidates for the study of cryptic speciation because they experience evolutionary pressure from their hosts, which may be strong enough to result in speciation [11–13]. The integration of molecular techniques in delimiting parasite species has uncovered substantial cryptic diversity, including the revision of 175 morphospecies of avian malaria parasites to an estimated 10,000 using mitochondrial (mt) DNA markers [14]. However, the division of nominal parasite species into cryptic species complexes has significance beyond the contribution to our knowledge of parasite biodiversity. Cryptic parasite species may inform our understanding of host-parasite coevolution and cospeciation [15], have different host-invasion pathways [16] and exhibit different degrees of host specificity [9, 17]. Where parasites are vectors of viruses or bacteria, resolving the host range of cryptic species can inform epidemiology [9].

Host specificity is a measure of the frequency with which a parasite species associates with a single host species [18]. Parasites limited to only one host species are host specialists, and less discriminate parasites found in association with multiple host species are host generalists. However, morphological conservatism looms as an alternative explanation for some host-generalist parasites identified solely by morphological attributes [9]. The value of using molecular genetic techniques to delimit levels of host specificity is increasingly recognized [19]. Genetic markers have shown several supposedly generalist parasite “species” to be complexes of cryptic, host-specific species [9, 20]. Accurately determining host specificity of parasite species is of wildlife conservation and human health concerns because generalist parasites...
may be more capable of vectoring pathogens to novel hosts [21]. Generalist parasites are also more competent invaders of new environments than specialists [21], a trait that warrants attention as anthropogenic change increasingly brings invasive parasites into contact with novel habitats and hosts [22].

Bat flies (Diptera: Streblidae and Nycteribiidae) are obligate, blood-feeding ectoparasites of bats. Species of Nycteribiidae are uniformly spider-like in appearance and wingless, restricting their ability to disperse independently of their hosts [23]. Bat flies reproduce via viviparous pupiparity, and gravid females generally leave the host only to adhere a single pupa to the roost substrate [24]. Bats are known reservoirs for an exceptionally diverse array of pathogens, including the ancestor of all mammalian malarias [25], as well as many zoonotic viruses relevant to human health [26–28]. As obligate parasites feeding exclusively on bat blood, bat flies warrant increased attention as potential vectors of these pathogens [29]. Bat flies have been identified as vectors of protozoan parasites [30] and bacterial pathogens in genus Bartonella [31, 32], and have recently been found to harbor bat-associated viruses related to medically impactful zoonoses [33–35].

Bat flies were historically understood to possess relatively low host specificity due to frequent host-switching opportunities, facilitated by polyspecific roosting behavior in bats [36]. They also spend a significant portion of their life-cycle off-host due to pupation on roost substrate [37]. However, most bat fly species are strictly monoxenous, or found in reliable association with only one bat species [29, 38]. In many parasites, patterns of host specificity are governed by host and parasite ecological characteristics facilitating reliable host-parasite encounters and eventual evolutionary associations. However, host specificity in bat flies seems to be unaligned with their host-independent dispersal ability (e.g. vagility) or other ecological associations that could provide host-switching opportunities [37, 39]. High host specificity in bat flies may therefore be maintained by host immunocompatibility or the decreased probability of flies encountering suitable mates on non-primary hosts (“reproductive filter” [37]). Bat flies associated with multiple host species are often steno- or oligoxenous, infesting only closely related or congeneric bats [37]. Previous studies examining genetic variation in oligoxenous bat flies using mitochondrial genetic markers have uncovered little geographic or host-structured population genetic structure [40, 41].

Penicillidia fulvida (Diptera: Nycteribiidae) is an African bat fly species that exhibits unusually low host specificity [42] and is thus apparently polyxenous. Collections summarized by Theodor in 1967 associated P. fulvida with 14 host species, and noted a wide range of host associations relative to other bat fly species [42]. Penicillidia fulvida specimens collected in Kenya and currently stored in the Field Museum of Natural History Collection of Hippoboscid Diptera (Field Museum of Natural History, Chicago, IL, USA) have been recovered from 14 putative host species spanning seven genera and six families of bat. In contrast to the general pattern of monoxenous or oligoxenous associations of bat flies with host bats [37], P. fulvida is known to parasitize bat families that diverged over 60 million years ago [43]. The rarity of polyxenous host associations among bat flies, in concert with the increasingly recognized prevalence and ecological importance of cryptic parasite species, suggests that P. fulvida may represent a complex of cryptic, and possibly more host-specific, bat fly species. Alternatively, P. fulvida may truly represent a single generalist species capable of colonizing distantly related hosts. The questions addressed in this study are: (i) do patterns of genetic differentiation exist within P. fulvida, potentially indicative of cryptic speciation? and (ii) is there genetic differentiation of P. fulvida parasitizing sympatric hosts, which might indicate cryptic host specificity?

Methods
Sampling
All P. fulvida specimens used in this study were collected during field expeditions of the “Bats of Kenya” project between 2006 and 2015. This survey was designed to inventory Kenya’s diverse habitats and rich faunas, and much remains to be learned about the limits and interrelationships of bat species. The project assayed genetic variation in 21 bat genera belonging to 10 families, identifying putative species using both mitochondrial and nuclear markers (e.g. [44–47]). To avoid adding to current taxonomic confusion, a conservative approach was purposefully taken regarding the assignment of names to clades in the analyses. Where a clade’s taxonomic identity was ambiguous or unknown, it was simply labeled as a numbered clade, and this convention is reflected in identifying P. fulvida hosts. Integrative taxonomic diagnoses of the various clades are needed to determine which (if any) existing names may apply to them.

Penicillidia fulvida were recovered from 14 localities, comprising mostly tropical broadleaf forests and savannas in the southern and western regions of Kenya (Figs. 1, 2). A total of 65 P. fulvida specimens were collected (Table 1); all are referenced in this study to describe host associations (Table 2), and 59 were sequenced. During collection, bats were captured in mist nets or harp traps during foraging/commuting or in hand nets at roosting sites, and all were subsequently stored individually in clean cloth bags to
minimize the risk of parasite disturbance transfers. Bats were either safely released or retained as species vouchers after processing. Vouchered bats were euthanized with halothane following guidelines by the American Society of Mammalogists [48], under the approval of the Field Museum’s Institutional Animal Care and Use Committee (2012-003). Voucher specimens were fumigated with ethyl ether to facilitate collection of their ectoparasites, but *P. fulvida* were also reliably detected and extracted from live bats because of their large size. Once extracted from the host, bat flies were immediately stored in 95% ethanol and later identified under a light microscope using species keys and descriptions from Theodor [42] as well as reference specimens from the Field Museum collection. All fly specimens are currently housed in the Field Museum of Natural History Collection of Hippoboscoid Diptera (currently on long-term loan to C. W. Dick at Western Kentucky University).

Two congeneric African bat flies, *Penicillidia pachymela* and *P. leptothrinax*, were also sequenced to better gauge “species-level” divergence in target genes. *Penicillidia pachymela* is posited to be closely related to *P. fulvida* based on morphological characteristics, and is also assigned to the *P. fulvida* group of species [42]. *Penicillidia pachymela* was collected in sympatry with *P. fulvida* but is exceedingly rare across their shared range in Kenya, with only a single specimen collected during this cumulative 9-year sampling effort.

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**Fig. 1** Map of Kenya featuring the 14 sampling localities yielding *Penicillidia fulvida*. A gazetteer of sampling localities is provided in Table 1.
Penicillidia leptothrinax is a more distantly related and smaller-bodied species endemic to Madagascar.

**DNA extraction, amplification and sequencing**

One or two legs were removed from each *P. fulvida* specimen for genetic analysis to allow retention of morphological vouchers. Prior to DNA extraction, each leg was lacerated with sterile forceps to expose the muscle tissue beneath the exoskeleton and transferred to a 1.5-ml microcentrifuge tube. Whole genomic DNA extractions were performed according to the manufacturer’s protocol using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), with the final elution divided into two steps at volumes of 35 and 65 μl, respectively, to optimize DNA concentration. All extractions were assessed for quality using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

A 658-bp fragment of the cytochrome c oxidase subunit I (COI) gene was amplified using the invertebrate-specific primer pair LCO1490 (5'-GGTCAAACAATATGATATTGG-3') and HCO2198 (5'-TAACTTCAGGGT GACCAAAAATCA-3') [49]. Each PCR assay was conducted in a total reaction volume of 25 μl containing 12.5 μl GoTaq MasterMix (Promega, Madison, WI, USA), 9 μl of nuclease-free water, 1 μl each of 10 μM forward and reverse primers and 1.5 μl of DNA template. A negative control, with nuclease-free water replacing the template DNA, was included with every set of reactions. Thermal cycling conditions for COI consisted of an initial denaturing at 95 °C for 1 min, followed by 35 cycles of 95 °C for 2 min, 50 °C and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

MtDNA has rapid substitution rates, making mitochondrial sequence data favorable for parsing relatively shallow, species-level phylogenetic relationships [50]. Although segments of COI have been proposed as “universal barcodes” suitable for species delimitation almost ubiquitously across taxa [50], sole reliance on mitochondrial markers disregards other modes of inheritance [51] and can obscure potential effects of introgression.
Table 1 Summary of Kenyan *Penicillidia fulvida* (*n*=65) collected, including host identity, collection locality and coordinates, specimens used for sequencing and cytochrome c oxidase subunit 1 haplotype groups

| Bat fly ID | Host family | Host species | Locality description | Locality | Latitude | Longitude | COI COI haplotype | 28S |
|------------|-------------|--------------|----------------------|----------|----------|-----------|-------------------|-----|
| FMNH 215683 | Miniopteridae | Miniopterus clade 10 | Kakamega Forest Station, Lirhanda Hill Cave | 1 | 0.218 | 34.897 | Yes | 7 | Yes |
| FMNH 215677 | Miniopteridae | Miniopterus clade 10 | Kakamega Forest Station, Lirhanda Hill Cave | 1 | 0.218 | 34.897 | Yes | 8 | Yes |
| FMNH 215707 | Miniopteridae | Miniopterus clade 10 | Kakamega Forest, Mahiakalo Cave | 2 | 0.244 | 34.907 | Yes | 1 | Yes |
| FMNH 215683 | Miniopteridae | Miniopterus clade 10 | Kakamega Forest, Mahiakalo Cave | 2 | 0.244 | 34.907 | Yes | 2 | Yes |
| NMK 184895 | Miniopteridae | Miniopterus clade 10 | Kakamega Forest, Mahiakalo Cave | 2 | 0.248 | 34.907 | No | - | Yes |
| FMNH 220168 | Rhinolophidae | Rhinolophus fumigatus | Fikirini, Pare Cave | 3 | −4.59 | 39.331 | Yes | 1 | Yes |
| FMNH 220543 | Miniopteridae | Miniopterus clade 2 | Fikirini, Pare Cave | 3 | −4.59 | 39.331 | Yes | 6 | Yes |
| FMNH 220544 | Miniopteridae | Miniopterus clade 2 | Fikirini, Pare Cave | 3 | −4.59 | 39.331 | Yes | 6 | Yes |
| FMNH 220519 | Miniopteridae | Miniopterus clade 2 | Fikirini, Three Sisters, Kisimani Cave | 4 | −4.615 | 39.353 | Yes | 6 | Yes |
| FMNH 220524 | Miniopteridae | Miniopterus clade 2 | Fikirini, Three Sisters, Kisimani Cave | 4 | −4.615 | 39.353 | Yes | 6 | Yes |
| FMNH 220254 | Hipposideridae | Triaenops afer | Fikirini, Three Sisters, Mbenyenyeye Cave | 4 | −4.61 | 39.354 | Yes | 6 | Yes |
| FMNH 220517a | Miniopteridae | Miniopterus clade 2 | Fikirini, Three Sisters, Mbenyenyeye Cave | 4 | −4.61 | 39.354 | Yes | 6 | Yes |
| FMNH 220517b | Miniopteridae | Miniopterus clade 2 | Fikirini, Three Sisters, Mbenyenyeye Cave | 4 | −4.61 | 39.354 | Yes | 6 | Yes |
| FMNH 220371 | Emballonuridae | Taphozous hildegardeae | Mwaluganje Community Elephant Sanctuary, Ngomeni Cave | 5 | −4.082 | 39.483 | Yes | 3 | Yes |
| FMNH 220560 | Miniopteridae | Miniopterus clade 3 | Mwaluganje Community Elephant Sanctuary, Ngomeni Cave | 5 | −4.082 | 39.483 | Yes | 6 | Yes |
| FMNH 220464 | Nycteridae | Nyctereutes thebaica | Shimba Hills National Reserve, Sable Bandas | 6 | −4.215 | 39.451 | Yes | 9 | No |
| NMK 184220 | Rhinolophidae | Rhinolophus cf. landeri | Marsabit National Park and Reserve, southeast | 7 | 2.32 | 37.994 | Yes | 10 | Yes |
| NMK 184392 | Rhinolophidae | Rhinolophus fumigatus | Marsabit National Park and Reserve, southeast | 7 | 2.309 | 37.994 | Yes | 10 | Yes |
| NMK 184197 | Rhinolophidae | Rhinolophus fumigatus | Marsabit National Park and Reserve, southeast | 7 | 2.32 | 37.994 | Yes | 10 | Yes |
| NMK 184246 | Rhinolophidae | Rhinolophus fumigatus | Marsabit National Park and Reserve, southwest | 8 | 2.283 | 37.954 | Yes | 10 | Yes |
| NMK 184251 | Rhinolophidae | Rhinolophus fumigatus | Marsabit National Park and Reserve, southwest | 8 | 2.283 | 37.954 | Yes | 10 | Yes |
| NMK 184242a | Rhinolophidae | Rhinolophus fumigatus | Marsabit National Park and Reserve, southwest | 8 | 2.283 | 37.954 | Yes | 10 | Yes |
| NMK 184242b | Rhinolophidae | Rhinolophus fumigatus | Marsabit National Park and Reserve, southwest | 8 | 2.283 | 37.954 | Yes | 2 | Yes |
Table 1 (continued)

| Bat fly ID | Host family | Host species | Locality description | Locality | Latitude | Longitude | COI | COI haplotype | 28S |
|------------|-------------|--------------|----------------------|----------|----------|-----------|-----|---------------|-----|
| NMK 182293 | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| NMK 182292a | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 12            | Yes |
| NMK 182292b | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 1             | Yes |
| NMK 184700 | Miniopteridae | *Miniopterus* clade 1 or 4 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 4             | Yes |
| NMK 184688 | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| NMK 184669 | Rhinolophidae | *Rhinolophus clivosus* 2 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| NMK 184792 | Miniopteridae | *Miniopterus* clade 1 or 4 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| NMK 184689a | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| NMK 184689b | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| NMK 184757 | Miniopteridae | *Miniopterus* clade 1 | Gilgil, Kariandusi Mines | 9        | −0.451   | 36.282    | Yes | 6             | Yes |
| FMNH 234882 | Miniopteridae | *Miniopterus* clade 1 | Soysambu Conservancy, Diatomite Cave | 11 | −0.430 | 36.174 | Yes | 11            | Yes |
| FMNH 225833 | Vespertilionidae | *Myotis tricolor* | Soysambu Conservancy, Monkey Bridge Campsite | 11 | −0.392 | 36.211 | Yes | 6             | Yes |
| NMK 184679 | Vespertilionidae | *Myotis tricolor* | Menengai crater, Mau Mau cave | 12 | −0.217 | 36.055 | Yes | 6             | Yes |
| NMK 184709 | Miniopteridae | *Miniopterus* clade 1 or 4 | Menengai crater, Mau Mau cave | 12 | −0.217 | 36.055 | Yes | 6             | Yes |
| NMK 184717a | Miniopteridae | *Miniopterus* clade 1 or 4 | Menengai crater, Mau Mau cave | 12 | −0.217 | 36.055 | Yes | 6             | Yes |
| NMK 184717b | Miniopteridae | *Miniopterus* clade 1 or 4 | Menengai crater, Mau Mau cave | 12 | −0.217 | 36.055 | Yes | 6             | Yes |
| FMNH 234771 | Vespertilionidae | *Myotis tricolor* | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | 1             | Yes |
| FMNH 225826a | Vespertilionidae | *Myotis tricolor* | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | 6             | Yes |
| FMNH 225826b | Vespertilionidae | *Myotis tricolor* | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | 6             | Yes |
| FMNH 225828a | Vespertilionidae | *Myotis tricolor* | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | 1             | Yes |
| FMNH 225828b | Vespertilionidae | *Myotis tricolor* | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | 1             | Yes |
Table 1 (continued)

| Bat fly ID | Host family | Host species | Locality description | Locality | Latitude | Longitude | COI | COI haplotype | 28S |
|-----------|-------------|--------------|----------------------|----------|----------|-----------|-----|--------------|-----|
| FMNH 225829 | Vespertilionidae | Myotis tricolor | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| FMNH 225907 | Miniopteridae | Miniopterus clade 1 or 4 | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| FMNH 225830 | Vespertilionidae | Myotis tricolor | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| FMNH 225825 | Vespertilionidae | Myotis tricolor | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| FMNH 225824 | Vespertilionidae | Myotis tricolor | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| NMK 184705 | Miniopteridae | Miniopterus clade 1 or 4 | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| NMK 184712 | Miniopteridae | Miniopterus clade 1 or 4 | Menengai crater, Mau Mau cave | 12 | −0.252 | 36.055 | Yes | Yes |
| FMNH 216012-013a | – | Pipistrellus sp. or Coleura afra | Marungu Cave | 13 | −0.252 | 36.055 | Yes | Yes |
| FMNH 216012-013b | – | Pipistrellus sp. or Coleura afra | Marungu Cave | 13 | −0.252 | 36.055 | Yes | Yes |
| FMNH 215994 | Emballonuridae | Coleura afra | Marungu Cave | 13 | −0.252 | 36.055 | Yes | Yes |
| FMNH 216021 | Emballonuridae | Coleura afra | Marungu Cave | 13 | −0.252 | 36.055 | Yes | Yes |
| FMNH 216015 | Emballonuridae | Coleura afra | Marungu Cave | 13 | −0.252 | 36.055 | Yes | Yes |
| FMNH 216023 | Emballonuridae | Coleura afra | Marungu Cave | 13 | −0.252 | 36.055 | Yes | Yes |

COI: Cytochrome c oxidase subunit 1, FMNH: Field Museum of Natural History Collection, NMK: National Museums of Kenya

Table 2 Concise summary of 63 P. fulvida host associations in Kenya

| Host family | No. of P. fulvida specimens | Host species | No. of P. fulvida | Percentage total associations |
|-------------|-----------------------------|--------------|------------------|------------------------------|
| Miniopteridae | 34 | Miniopterus clade 1 | 7 | 11.1 |
| | | Miniopterus clade 1 or 4 | 11 | 17.5 |
| | | Miniopterus clade 10 | 6 | 9.5 |
| | | Miniopterus clade 2 | 6 | 9.5 |
| | | Miniopterus clade 3 | 1 | 1.6 |
| | | Miniopterus clade 4 or 7 | 2 | 3.2 |
| | | Miniopterus clade 8 | 1 | 1.6 |
| Rhinolophidae | 12 | Rhinolophus clivosus 2 | 2 | 3.2 |
| | | Rhinolophus fumigatus 2–3 | 1 | 1.6 |
| | | Rhinolophus fumigatus 3 | 5 | 7.9 |
| | | Rhinolophus fumigatus 4 | 1 | 1.6 |
| | | Rhinolophus fumigatus 8 | 1 | 1.6 |
| | | Rhinolophus cf. landeri | 2 | 3.2 |
| Vespertilionidae | 10 | Myotis tricolor | 10 | 15.9 |
| Emballonuridae | 5 | Coleura afra | 4 | 6.3 |
| | | Taphozous hildegardae | 1 | 1.6 |
| Rhinonycteridae | 1 | Triaenops afer | 1 | 1.6 |
| Nycteridae | 1 | Nyctenys thebaica | 1 | 1.6 |

* Two specimens with uncertain host associations (BDP4273-4a and b) are excluded

* Proportionally, Miniopterus “clade 1 or 4” and Myotis tricolor hosted the most P. fulvida (17.5% and 15.9% respectively)
or infection with Wolbachia, an arthropod-associated bacterial endosymbiont capable of disrupting patterns of mitochondrial inheritance [52]. For a multilocus approach, we also amplified 28S ribosomal RNA (rRNA), a nuclear gene commonly used alongside COI for species-level analyses in arthropods [53].

The D2 region of the nuclear 28S gene was amplified using the primers F2 (5'-AGAGAGATTCAAGAGTAGT - CGTG-3') and 3DR (5'-TAGTTCAACCATCTTTCCGG GTC-3') [54]. Reaction components and volumes were identical to those used to amplify COI. Thermal cycling conditions for 28S were consisted of an initial denaturing at 98 °C for 2 min, followed by 35 cycles of denaturing at 94 °C for 3 min, annealing at 51 °C for 30 s, with a first extension at 72 °C for 2 min and then a final extension at 72 °C for 8 min.

PCR products were verified for size and quality via electrophoresis on a 1.5% agarose gel stained with SybrSafe (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and visualized under a blue LED light. Sanger sequencing was performed at the North Carolina State Genomic Sciences Laboratory (Raleigh, NC, USA) using forward and reverse primers for both genes.

Phylogenetic analyses

Sequences were trimmed and assessed for ambiguous bases by eye, then aligned using the MUSCLE algorithm [55] as implemented in Geneious 6.0 [56]. A hippoboscid fly, Pseudolynchia canarensis, was included as an out-group (sequence data retrieved from GenBank). Haplotype groups were identified using DnaSP 6 [57], and a minimum-spanning haplotype network was constructed with PopART [58].

Substitution models for Bayesian and maximum likelihood analyses were estimated with jModeltest [59]. Optimized substitution models based on Akaike’s information criterion were GTR+G for COI and HKY+I for 28S. Both concatenated and single-gene trees were created, with concatenated trees partitioned by locus. Bayesian analysis was performed in MrBayes 3.2 [60] using the default burn-in of 25% and 10 million Markov chain Monte Carlo generations. Average standard deviation in split frequencies fell below 0.01 at 380,000 then at 650,000 generations and remained under this threshold until the final generation, indicating stationarity was reached. Posterior probabilities for clade support were calculated using the trees remaining after burn-in. A maximum likelihood phylogeny was created using RAxML 8.0 [61]. The starting tree was obtained by searching for the best-scoring maximum likelihood tree in a single program run. Branch support was calculated using a random seed and 1000 rapid bootstrap replicates.

Analysis of molecular variance

A hierarchical analysis of molecular variance (AMOVA) was used to determine the extent to which genetic variation in P. fulvida is allocated among host taxa. Because AMOVA requires groups to be defined a priori, haplotypes were conservatively pooled by host family. Host families with insufficient sample sizes for statistical analysis (Emballonuridae, Nycteridae and Hipposideridae, from which only 1–3 P. fulvida specimens were recovered or sequenced) were excluded; therefore, only specimens from Miniopteridae (n = 33), Rhinolophidae (n = 11) and Vespertilionidae (n = 9) were compared. AMOVA was performed in Arlequin 3.5.2.2 [62] using a locus-by-locus method to accommodate missing data. The significance of pairwise fixation indices (Fst, an F-statistic measuring variance in allele frequency among populations) was calculated using 1023 permutations, each assigning parasite haplotypes to host families at random to generate a null distribution.

Results

Phylogenetic analyses

A total of 12 COI haplotypes were present within P. fulvida, with 11 variable sites (Fig. 3). One specimen (FMNH216012-013) could not be assigned a COI haplotype due to an ambiguous base at a variable site, and was excluded from the haplotype network. Only a single 28S haplotype was recovered from P. fulvida, but 28S haplotypes were distinct among the three described species within Penicillidia (1.8% pairwise uncorrected distance [p-distance] between P. leptothrinax and the most common fulvida haplotype, 1.6% p-distance between P. pachymela and P. fulvida and 3.2% p-distance between P. leptothrinax and pachymela). The concatenation of 28S with COI in phylogenetic analyses yielded poorly resolved relationships among the three putative species and was too conservative to inform population genetic structure within P. fulvida. Because P. pachymela and P. leptothrinax had only single specimens included for comparative purposes and, in addition, resolving relationships among putative species within Penicillidia is outside the scope of this study, only COI-based trees were included.

Bayesian and maximum likelihood analyses yielded incongruent topologies within P. fulvida (Figs. 4, 5). The clades that are moderately well-supported but recovered using only a single method lack apparent host-based or geographic structure, and therefore do not appear to warrant discussion. One clade was supported by both Bayesian and maximum likelihood analyses, comprising six specimens from the host family Rhinolophidae and from a single, distant locality (Marsabit; Table 1; Figs. 4,
Neither analysis recovered outgroups within the ingroup of *P. fulvida*.

### Analysis of molecular variance

Hierarchical AMOVA rejected the null hypothesis of haplotype homogeneity among host families (*P* < 0.001; Table 3). The significance of pairwise *F*ₚₛ values indicated that allele frequencies were unique in *P. fulvida* haplotype groups from rhinolophid bats (Yinpterochiroptera: Rhinolophoidea) when compared to haplotype groups from both miniopterid and vespertilionid bats (Yangochiroptera: Vespertilionoidea) (Table 4). However, this taxonomic association appears artefactual when regarded alongside phylogenetic structure and sampling composition. Genetic structure associated with Rhinolophidae aligns more reliably with sampling locality (Table 1; Fig. 4); 7 of 11 *P. fulvida* recovered from rhinolophid bats were recovered from Marsabit, and 6/6 of the “rhinolophid” clade were from Marsabit bats. Viewed in this light, the results do not suggest host-based genetic differentiation is present within *P. fulvida*.

### Discussion

Parasites are susceptible to placement in artificial species groups because of their morphological conservatism relative to their hosts [9]. The advent of molecular genetic techniques has provided a valuable strategy for scrutinizing morphologically indistinguishable but ecologically differentiated parasites, and can result in the division of host-generalist nominal species into more host-specific cryptic species complexes [9, 14, 15, 17]. Using two genetic markers (mitochondrial *COI* and nuclear 28S), we have uncovered no such evidence of host-mediated genetic structure in the polyxenous bat fly *P. fulvida*. This finding is concordant with other studies evaluating patterns of population genetic structure in non-monoxenous (oligoxenous) nycteribiid bat flies [40, 41], but to our knowledge represents the first such investigation of a truly polyxenous bat fly species. These results suggest inter-host gene flow may be occurring in *P. fulvida*, and support its recognition as a single species under both morphological and phylogenetic species concepts. Unlike most bat flies, it is capable of parasitizing phylogenetically disparate bat species, and in Kenya parasitizes both suborders (Yinpterochiroptera and Yangochiroptera) and six different families of bats.

Although no genetic structure could be reliably attributed to host identity, there is some evidence *P. fulvida* is not uniformly panmictic across Kenya. Both maximum likelihood and Bayesian phylogenetic analyses supported a clade of 6/7 *P. fulvida* from a single remote sampling site in Marsabit Forest (Figs. 3, 4).
Isolation by distance alone is insufficient to explain this geographic differentiation, as *P. fulvida* is essentially panmictic (with respect to the localities sampled) across the comparable 700 km distance between Kakamega and Fikirini (Figs. 1, 2). Kenya is composed of diverse biomes, and Marsabit Forest is well-representative of this mosaic: a tropical broadleaf forest sustained by volcanic soil and orographic precipitation that is completely surrounded by swaths of rocky desert (Fig. 2). Further, all *P. fulvida* from Marsabit Forest were collected from horseshoe bats (5/6 from *Rhinolophus fumigatus* [clade 2–3; see [45]], and 1/6 from *Rhinolophus cf. landeri*); all have wings with relatively low aspect ratios, marking them as relatively weak fliers [63]. Marsabit Forest's geographic isolation may stymie gene flow in *P. fulvida* by restricting the dispersal of its bat hosts. However, given the small sample size and the lack of intervening sampling sites that might firmly implicate the surrounding desert as a barrier to
**Fig. 5** Maximum likelihood tree \((P. \text{fulvida})\) labeled with voucher IDs, congeners \(P. \text{pachymela}\) and \(P. \text{leptothrinax}\) and outgroup \(Pseudolynchia \text{canarensis}\) generated using COI and a GTR+G substitution model. Branch support was calculated using 1000 rapid bootstrap replicates, and only support values > 60% are displayed.

**Table 3** Analysis of molecular variance of genetic structure in \(P. \text{fulvida}\) as it corresponds to host family

| Source of variation | Sum of squares | Variance | Percent variation | F-statistic | P-value |
|---------------------|----------------|----------|------------------|-------------|---------|
| Among host families | 5.804          | 0.08851  | 19.42387         | 0.19424     | < 0.001 |
| Within host families| 37.818         | 0.36717  | 80.57613         |             |         |
gene flow, the origin of this site-related genetic structure remains speculative.

One specimen of Rhinolophus fumigatus from Marsabit hosted two distinct haplotypes of *P. fulvida*: the typical Marsabit-associated clade and a haplotype also found on a Miniopterus host at Kakamega Forest over 400 km distant. This suggests lack of barriers between Marsabit and other populations, but absent additional data this finding cannot be explored in more detail.

An alternative explanation for the lack of genetic structure in *P. fulvida* is insufficient variety and variability in the genes used. Nuclear 28S was conservative among the three *Penicillidia* species sequenced and invariant in *P. fulvida*. Consequently, only mitochondrial COI was used to assess patterns of intraspecific, inter-host variation in *P. fulvida*. Drawing conclusions from one mitochondrial gene can be precarious, as potential mito-nuclear discordance and the small proportion of the overall genome represented in single-gene phylogenies can cause incongruence [64]. Further, reliance on mtDNA is unfavorable in some insect taxa, including bat flies, due to possible infection with bacterial Wolbachia [65]. Wolbachia is maternally transmitted and can cause disruptions in mitochondrial inheritance, resulting in the overrepresentation of certain haplotypes in a population or the absence of polymorphism altogether [52, 66]. The use of more variable nuclear genetic markers in future studies, alongside increased research efforts investigating the ability for Wolbachia to influence inheritance in nectaribid bat flies, could help clarify the possibility of Wolbachia infection obscuring genetic structure in *P. fulvida*.

Understanding host specificity and the processes responsible for its evolution and maintenance across parasite communities is crucial to understanding the evolutionary context of host-parasite associations and the role of parasites in disease transmission. High host specificity in parasites has historically been regarded as the default trend in parasite evolution; specialization is also regarded as an evolutionary “dead end” because morphological specialization required for a highly specific parasite to efficiently exploit its host makes a specialist lineage’s “return” to a generalist strategy improbable [67]. However, the evolution of ecological resource specialization is not characterized by fixed directionality; different degrees of host specificity can arise multiple times in the evolutionary history of a parasite, with generalist species sometimes emerging from specialist lineages [67–69]. Extensive biodiversity surveys have found bat flies to possess considerably high host specificity as a group, and some instances of low specificity in bat flies may be attributable to poorly understood species boundaries [29]. Using molecular techniques, this study demonstrates that, although apparently rare, polyxenous bat fly species do exist. If *P. fulvida* evolved from specialist ancestors, the ecological precedent for a decrease in specificity across evolutionary time may be resource breadth-associated performance trade-offs [70, 71] or interspecific competition [69].

Host ecology serves as an important evolutionary driver of patterns of host specificity in parasite communities. Multiple bat species often aggregate closely in a single roost, a behavior which may confer anti-predator benefits or simply reflect limited availability of suitable roosts [72, 73]. Mixed-species groups present host-switching opportunities for parasites that depend on their hosts for dispersal; as a result, polyspecific bat roosts were a historical precedent for proposing universally low host specificity in bat flies [36, 74]. *Penicillidia fulvida* was collected from multiple host species in eight of 14 roosts and from multiple host families in seven of 14 roosts in Kenya, indicating polyspecific roosting behavior may be a proponent of *P. fulvida*’s broad host range and high genetic structure within hosts. Further, although it is now recognized that specialist bat flies can maintain their high specificity despite host-switching opportunities [37], bat assemblages in shared roosts may influence host preference in generalist bat flies [75]. Accordingly, there is some indication that parasitism by *P. fulvida* is based on host availability. *Penicillidia fulvida* occasionally parasitizes the African sheath-tailed bat *Coleura afra* (4/63 total host associations in the Bats of Kenya survey; Table 2), but associations with *C. afra* were only recorded in the absence of potential miniopterid and rhinolophid hosts (Table 1). *Coleura afra* was present in three shared roosts containing Miniopterus hosts of *P. fulvida*, but *P. fulvida* was never recovered from *C. afra* when these alternative hosts were available. This pattern suggests that although host selection in *P. fulvida* is unconstrained by phylogenetic distance, *P. fulvida* may still demonstrate tiers of host preference, which could function to increase fitness by mitigating local competition or selecting optimally nutritious or compatible host blood [29].

Vector ecology is a valuable determinant of pathogen spread and potential zoonotic spillover [76]. Low host specificity in parasites may promote exposure to a wider range of infectious agents and facilitate disease transmission to new hosts and geographic areas [22]. Bats are

| Table 4: Pairwise Fst values among host families of *P. fulvida* |
|---------------------------------------------------------------|
|                          | Rhinolophidae | Miniopteridae | Vespertilionidae |
|--------------------------|--------------|--------------|-----------------|
| Rhinolophidae            | 0.26579*     | 0.27479*     |                 |
| Miniopteridae            | 0.26579*     | 0.01145      |                 |
| Vespertilionida          | 0.27479*     | 0.01145      |                 |

*Significant at P < 0.05 in permutation test.
reservoirs for a broad spectrum of pathogens, a product of immunological or ecological predisposition [77] or a reflection of their high species diversity [28]. Nycteribiid bat flies, including *P. fulvida*, can serve as vectors facilitating the transmission of several bat pathogens: hemoparidian parasites in genus *Polychromophilus* [25, 30] and bacterial bartonellae [31, 32]. Moreover, research linking nycteribiid bat flies to impactful bat-associated viral zoonoses is expanding [33, 35, 78]. Within the scope of this study, Kenyan hosts of *P. fulvida* are known reservoirs of paramyxoviruses (*Miniopterus* spp. [79]), SARS coronaviruses (*Rhinolophus fumigatus* and *R. landeri* [80]) and lyssaviruses (*Miniopterus* spp. [81]).

Because this study failed to detect significant genetic differentiation among *P. fulvida* parasitizing bats across three superfamilies in both suborders, the results clearly demonstrate that *P. fulvida* is a rare polypcynous species of bat fly. However, it is important to acknowledge this study is not comprehensive with respect to *P. fulvida*’s geographic and host range. *Penicillidia fulvida* has a broad range outside Kenya, comprising much of sub-Saharan Africa [42] and possibly Madagascar (C.W. Dick, unpublished data). Additionally, *P. fulvida* was collected from three host families in Kenya with relatively uninformative sample sizes (Emballonuridae, Rhinonycteridae and Nycteridae) and has previously been recorded from Pteropodidae, although this association is tenuously based on a single record (*Eidolon helvum* in South Africa; [42]). Based on associations recorded in this study and past surveys, it seems probable that bats from Miniopteraidae, Rhinolophidae and Vespertilionidae represent *P. fulvida*’s primary host range, whereas other, infrequently observed hosts are non-primary associations indicative of natural host-switching events. Increased, targeted sampling efforts may be useful for further parsing host associations and for testing hypotheses associated with geographic differentiation in *P. fulvida*.

Conclusions

Host specificity is one of the most basic ecological characteristics of parasites and likely subject to strong selection. Accurate measurement of host specificity is vital to understanding parasite biodiversity, vulnerability to population decline and the role of parasites in disease transmission. Moreover, accurately estimating host specificity relies on acknowledging the presence of cryptic diversity and using integrative approaches to delimiting parasite species. This study provides molecular genetic evidence that the nycteribiid bat fly *P. fulvida* does not exhibit cryptic host specificity, and instead represents a single species with a wide range of phylogenetically distant bat hosts.

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Authors contributions

TBV contributed to the study design, conducted laboratory work and systematic analyses and drafted the initial manuscript. CWD contributed to the study design, specimen collection, bat fly identification and manuscript writing. BDP and PWW contributed to the original Bats of Kenya Survey, specimen collection, bat identification and manuscript writing. All authors read and approved the final manuscript.

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Availability of data and materials

All sequence data are available in the GenBank repository (COI accession numbers ON704663-ON70421, 28S accession numbers ON693298-ON693355).

Declarations

Ethics approval and consent to participate

All protocols for bat work were conducted under the approval of the Field Museum’s Institutional Animal Care and Use Committee (2012-003).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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