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Some Like It Hot: Evolution and Ecology of Novel Endosymbionts in Bat Flies of Cave-Roosting Bats (Hippoboscoidea, Nycterophiliinae)

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We investigated previously unknown associations between bacterial endosymbionts and bat flies of the subfamily Nycterophiliinae (Diptera, Streblidae). Molecular analyses revealed a novel clade of *Gammaproteobacteria* in *Nycterophilia* bat flies. This clade was not closely related to *Arsenophonus*-like microbes found in its sister genus *Phalconomus* and other bat flies. High population infection rates in *Nycterophilia* across a wide geographic area, the presence of the symbionts in pupae, the general codivergence between hosts and symbionts, and high AT composition bias in symbiont genes together suggest that this host-symbiont association is obligate in nature and ancient in origin. Some *Nycterophilia* samples (14.8%) also contained *Wolbachia* supergroup F (*Alphaproteobacteria*), suggesting a facultative symbiosis. Likelihood-based ancestral character mapping revealed that, initially, obligate symbionts exhibited association with host-specific *Nycterophilia* bat flies that use a broad temperature range of cave environments for pupal development. As this mutualism evolved, the temperature range of bat flies narrowed to an exclusive use of hot caves, which was followed by a secondary broadening of the bat flies' host associations. These results suggest that the symbiosis has influenced the environmental tolerance of parasite life history stages. Furthermore, the contingent change to an expanded host range of *Nycterophilia* bat flies upon narrowing the ecological niche of their developmental stages suggests that altered environmental tolerance across life history stages may be a crucial factor in shaping parasite-host relationships.

Endosymbiotic relationships are commonly documented in insects that utilize specialized and nutritionally deficient foods, such as blood. Blood feeding has developed many times throughout invertebrate evolution, but despite their diversity, potential roles as vectors of pathogens, and impacts on human health, the ecology and evolution of symbiotic systems in blood-feeding insects remain poorly understood. For example, it has long been known that tsetse flies are associated with the obligate intracellular microbe *Wigglesworthia glossinidia* (1, 18), but only recently have researchers identified associations between the human louse *Pediculus humanus* and its obligate endosymbiont "*Candidatus* Riesia pediculicola" (49) or between the bedbug *Cimex lectularius* and *Wolbachia* (20). Evidence suggests that these symbionts are mutualists, providing B-complex vitamins and other nutrients that are deficient in a blood diet (20, 36, 41).

One important but still understudied group of blood feeders is the bat flies (Streblidae, Nycteribiidae), which are ectoparasitic, viviparous dipterans exclusively adapted to bats. They belong to the Hippoboscoidea, a group that also encompasses tsetse flies (Glossinidae, see above) and louse flies (Hippoboscidae). Previous studies have identified bacterial symbionts associated with the nycteribiid genera *Basilia*, *Nycteribia*, *Penicillidia*, and *Phthiridium* (21, 28), and the streblid genus *Trichobius* (31, 56). Some of these symbionts are vertically transmitted, and sequences seem to form a monophyletic clade associated with the presumed mutualist bacteria found in sucking lice ("*Candidatus* Riesia") (37). Other bat fly-associated symbionts fall within an *Arsenophonus* clade that is widely distributed across a diversity of insects, apparently the result of rampant horizontal transmission (37). However, given the diversity of bat flies, current symbiont records are superficial at best, and no comprehensive studies across monophyletic groups are available.

Poor sampling has hindered our understanding of the functional role that these bacteria play in both the ecology and the evolution of their invertebrate hosts. For instance, the identification of cospeciation and vertical transmission would suggest an ancient, obligate relationship between partners, as may be typical of nutritional mutualists documented in other insects (5, 6, 50). On the other hand, the identification of symbiont replacement events within and among lineages may help to identify key transitions in their hosts' evolutionary ecology.

To elucidate some of the evolutionary and ecological dynamics of bat fly symbioses, we studied a little-known subfamily of Neotropical bat flies, the streblid subfamily Nycterophiliinae Wenzel 1966, using populations across their host and geographic distributions. Extant Nycterophiliinae comprise the rare and poorly known genus *Phalconomus* Wenzel 1984 and the genus *Nycterophilia* Ferris 1916. Currently, the genus *Phalconomus* contains three species (two are undescribed). *Phalconomus puliciformis* is known only from *Lonchophylla robusta*, while *Phalconomus* species B sensu Wenzel 1976 is recorded only from *Platalina genovensium*. Both of these nectar-feeding bat species belong to the phyllostomid subfamily Lonchophyllinae and have a restricted distribution in Andean South America (51). *Phalconomus* species A sensu Wenzel 1976 is known only from *Natalus stramineus* (Natalidae) in Guatemala. The genus *Nycterophilia* comprises five described species (Fig. 1), which are associated with bats belonging

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FIG 1 Adult female *Nycterophylia coxata* group specimen from the Dominican Republic (Cueva de Peter, Baoruco).

to the Mormoopidae, Natalidae, and several species within the Phyllostomidae. *Nycterophilia* bat flies are widely distributed from the southwestern United States and Mexico through northern South America and the Caribbean (57, 58). Evolutionary relationships of Nycterophiliinae to other Neotropical bat flies are unexplored, and no information exists regarding the evolutionary relationships within this subfamily.

Both *Phalconomus* and *Nycterophilia* occur exclusively on cave-roosting bats, yet the latter may occur on, and often prefers, "hot roosting" bats. Hot roosts are characterized by roost temperatures between 28 and 40°C and relative humidity exceeding 90% (47). It is thought that hot roosts are the result of the metabolic activity of bats, aided by a specific cave topology that facilitates heat entrapment (47). Typical of all bat flies, female nycterophiliines leave their bat hosts multiple times throughout their lives to deposit a single third-instar larva onto a substrate in the bat roost (i.e., cave wall). There, they immediately pupate and remain immobile and exposed to the roost environment until eclosion. Newly eclosed, unfed flies (teneral flies) have to survive exposure to the roost environment until they find a suitable host bat.

In this project, we were particularly interested in exploring the roles of bacterial associates in the evolution and ecology of *Nycterophilia* bat flies. Specific questions relate to the following. (i) What is the relationship of bacterial associates in Nycterophiliinae to known bacterial associates in other insects and bat flies? Based on prior evidence from bat flies, it can be expected that Nycterophiliinae harbor bacterial symbionts and that these are likely related to symbionts within the *Gammaproteobacteria* (21, 31, 56). (ii) Is there evidence for obligate associations of bacteria with particular species of Nycterophiliinae? Processes such as vertical transmission and high infection rates across populations would suggest a potential obligate association between symbiont and host (2, 34). (iii) How are bacterial associates of nycterophiliine species related to each other, and how congruent is their evolutionary history to that of their invertebrate hosts? Monophyly of microbes and patterns of codivergence across geographic distributions would suggest a stable evolutionary association and point toward the possibility of a cooperative (i.e., mutualistic) relationship. (iv) How does symbiont evolution in Nycterophiliinae relate to their ecological niche specificity? Tracing the evolutionary transitions of ecological characters of parasites in the context of symbiont evolution may provide further insight into the relative role of endosymbionts in driving parasite evolution.

MATERIALS AND METHODS

Samples for molecular studies. Adult *Nycterophilia* bat flies were collected for molecular studies from host bats in Mexico, the Dominican Republic, and Puerto Rico by using previously described methods (Table 1) (Institutional Animal Care and Use Committee [IACUC] numbers: SUNY BIO21098N, WKU 09-06, and FMNH 06-9) (16). *Phalconomus* species B was obtained from *Platalina genovensium* in coastal Peru. To test for vertical transmission, pupae were collected in Cueva de los Culebrones, Puerto Rico, from the general cave environment (i.e., cave walls) or by using glue boards (15). Glue-board collecting ensured the capture of the female and her offspring, enabling direct comparisons of their microbiomes. Samples were collected in 96% ethanol and stored at −80°C.

Molecular studies. Before extraction, adult flies and pupae were washed in sterile water; pupal cases were dissected from pupae. The abdomen of each adult fly sample was pierced with a sterilized dissecting pin to allow tissue extraction while maintaining exoskeleton integrity to allow for mounting and identification postextraction. Total genomic DNA was extracted using the Qiagen animal tissue kit and protocol. DNA concentration was measured using a Nanodrop spectrophotometer (Fisher Scientific).

A multilocus sequencing approach was applied for the microbial associates, targeting 16S rRNA and 16S-23S internal transcribed spacer (ITS) sequences, as well as the chaperonin *groL* genes. A 1.3-kb fragment of the eubacterial 16S rRNA gene (small-subunit [SSU] rRNA) was amplified using general eubacterial 16S primers according to a standard PCR protocol (18). The 16S-23S ribosomal ITS region (~600 bp) was amplified with the 16S forward primer ArsITSf (5′-CCG TAG GGG AAC TGC GGT TG-3′) and the 23S reverse primer ArsITSr (5′-TTR GAG GAT GGY CCC CCC AT-3′), using PCR protocols outlined in the work of Sorfová et al. (52). An 850-bp region of the chaperonin *groL* gene was amplified according to the protocols outlined in the work of Hosokawa and Fukatsu (19) as well as the work of Hosokawa et al. (21). Additional custom primers were designed for challenging samples, NgroeL61f (5′-AAG CWG TTG CAG CTG GWA TGA ATC-3′) and NgroEL840r (5′-YTT TGC AAC TCT TTC TTG YAA TTT TTC-3′), resulting in ~750-bp sequences. Positive bacterial PCR samples were subjected to cloning using a Topo TA kit (Invitrogen) and Sanger sequenced from both ends.

Because no molecular phylogeny is currently available for Nycterophiliinae, host bat flies were sequenced along with their microbial associates. To elucidate the evolutionary relationships of *Nycterophilia* species to each other, we sequenced the cytochrome oxidase II (COII) mitochondrial gene (~755 bp) and the nuclear 18S rRNA (~1,828-bp) and CAD protein (~650-bp) genes using the primers and protocols outlined in the work of Dittmar et al. (16). These genes have been shown to resolve relationships among species within dipteran genera (42, 59).

Molecular and phylogenetic analyses. Raw sequences were edited and assembled using Geneious Pro 5.6. NCBI's BLASTn search was used to identify taxonomic affinities of the sequences. QIIME 1.5.0 was used to check for chimeric sequences. Select Diptera were used as outgroups for the *Nycterophilia* bat flies (Fig. 2). As outgroups for the bacterial associates, we chose endosymbionts previously detected in blood-feeding invertebrates, including bat flies, such as *Wigglesworthia*, "*Candidatus* Riesia,"

TABLE 1 Bat fly samples used in the evolutionary analyses of this study (58[f]), including species identification, country of origin, general location, cave, sex (where known), age (adult or pupa), host bat species (where known), and GenBank accession number for both host fly and endosymbiont genes

Species and specimen identifier	Country	Location	Cave	Sex[a]	Age[b]	Host species[c]	GenBank accession no.			
							COII	16S	*groL*	ITS
Phalconomus species B sensu Wenzel 1976										
BDP5030	Peru	Arequipa, Atiquipa	Unnamed mine	U	A	*Platalina genovensium*	JX853071			JX857164
Nycterophilia parnelli										
MEXA4.1	Mexico	Tamaulipas	Cueva Taninul I	F	A	*Pteronotus parnellii*	JX853110	JX853062	JX853125	JX853008
MEXA4.2	Mexico	Tamaulipas	Cueva Taninul I	F	A	*Pteronotus parnellii*	JX853111	JX853063	JX853126	JX853009
MEXA4.3	Mexico	Tamaulipas	Cueva Taninul I	U	A	*Pteronotus parnellii*	JX853112		JX853127	JX8530010
Nycterophilia natali										
MEX09	Mexico	Tamaulipas	Cueva Taninul I	U	A	*Natalus mexicanus*	JX853109		JX853146	JX853007
Nycterophilia n. sp.										
DR05084	Dominican Republic	Los Haitises NP[e]	Cueva Kavirma	U	A	*Macrotus waterhousii*	JX853076	JX853027	JX853128	JX852974
DR05086	Dominican Republic	Los Haitises NP	Cueva Kavirma	F	A	*Macrotus waterhousii*	JX853077	JX853028	JX853129	JX852975
DR05119.1	Dominican Republic	Barahona	Cueva de los Tainos	F	A	*Macrotus waterhousii*	JX853078	JX853029	JX853130	JX852976
DR05119.2	Dominican Republic	Barahona	Cueva de los Tainos	F	A	*Macrotus waterhousii*	JX853079	JX853030	JX853131	JX852977
DR05139.1	Dominican Republic	Pedernales	Cueva Verna	F	A	*Macrotus waterhousii*	JX853080	JX853031	JX853132	JX852978
DR05139.2	Dominican Republic	Pedernales	Cueva Verna	F	A	*Macrotus waterhousii*	JX853081	JX853032	JX853133	JX852979
DR05142.0	Dominican Republic	Pedernales	Cueva Verna	U	A	*Macrotus waterhousii*		JX853033		
DR05142.1	Dominican Republic	Pedernales	Cueva Verna	M	A	*Macrotus waterhousii*	JX853082	JX853034	JX853134	JX852980
DR05145[d]	Dominican Republic	Pedernales	Cueva Verna	U	A	*Macrotus waterhousii*		JX853035		
DR05146.0[d]	Dominican Republic	Pedernales	Cueva Verna	U	A	*Macrotus waterhousii*	JX853083	JX853036	JX853135	JX852981
DR05146.1	Dominican Republic	Pedernales	Cueva Verna	M	A	*Macrotus waterhousii*	JX853084	JX853037	JX853136	JX852982
DR05161.1	Dominican Republic	Pedernales	Cueva Verna	F	A	*Macrotus waterhousii*	JX853085	JX853038	JX853137	JX852983
DR05161.2	Dominican Republic	Pedernales	Cueva Verna	F	A	*Macrotus waterhousii*	JX853086	JX853039	JX853138	JX852984
DR05177	Dominican Republic	Baoruco	Cueva de Peter	U	A	*Macrotus waterhousii*	JX853087	JX853040	JX853139	JX852985
DR05193	Dominican Republic	Baoruco	Cueva de Peter	U	A	*Macrotus waterhousii*	JX853088	JX853041	JX853140	JX852986
DR05203.1	Dominican Republic	Baoruco	Cueva de Peter	F	A	*Macrotus waterhousii*	JX853091	JX853044	JX853141	JX852989
DR05203.2	Dominican Republic	Baoruco	Cueva de Peter	F	A	*Macrotus waterhousii*	JX853092	JX853045	JX853142	JX852990
DR05233.0	Dominican Republic	Baoruco	Cueva de Los Murcielagos de la Cabeza del Rio Guayabal	U	A	*Macrotus waterhousii*	JX853093	JX853046	JX853143	JX852991
DR05233.1	Dominican Republic	Baoruco	Cueva de Los Murcielagos de la Cabeza del Rio Guayabal	F	A	*Macrotus waterhousii*	JX853094	JX853047	JX853144	JX852992
DR05242	Dominican Republic	Baoruco	Cueva de Los Murcielagos de la Cabeza del Rio Guayabal	U	A	*Macrotus waterhousii*	JX853095	JX853048	JX853145	JX852993
Nycterophilia coxata group										
DR05037	Dominican Republic	El Seibo	El Seibo, Los Haitises	U	A	*Pteronotus parnellii*	JX853072	JX853024	JX853147	JX852970
DR05045.1	Dominican Republic	El Seibo	El Seibo, Los Haitises	M	A	*Monophyllus redmani*	JX853073		JX853148	JX852971
DR05045.2	Dominican Republic	El Seibo	El Seibo, Los Haitises	U	A	*Monophyllus redmani*	JX853074	JX853025	JX853149	JX852972
DR05081	Dominican Republic	Los Haitises NP	Cueva Kavirma	U	A	*Pteronotus quadridens*	JX853075	JX853026	JX853150	JX852973
DR05201.1	Dominican Republic	Baoruco	Cueva de Peter	F	A	*Pteronotus quadridens*	JX853089	JX853042	JX853151	JX852987
DR05201.2	Dominican Republic	Baoruco	Cueva de Peter	U	A	*Pteronotus quadridens*	JX853090	JX853043	JX853152	JX852988
DR05256.1	Dominican Republic	Baoruco	Cueva de Los Murcielagos de la Cabeza del Rio Guayabal	F	A	*Pteronotus parnellii*	JX853096	JX853049	JX853153	JX852994
DR05258.1[d]	Dominican Republic	Baoruco	Cueva de Los Murcielagos de la Cabeza del Rio Guayabal	F	A	*Pteronotus parnellii*	JX853097	JX853050	JX853154	JX852995
DR05258.2[d]	Dominican Republic	Baoruco	Cueva de Los Murcielagos de la Cabeza del Rio Guayabal	F	A	*Pteronotus parnellii*	JX853098	JX853051	JX853155	JX852996

(Continued on following page)

TABLE 1 (Continued)

Species and specimen identifier	Country	Location	Cave	Sex[a]	Age[b]	Host species[c]	GenBank accession no.			
							COII	16S	*groL*	ITS
MEXB2	Mexico	Tamaulipas	Cueva Florida	U	A	*Pteronotus davyi*	JX853113	JX853064	JX853157	JX853011
MEXF2	Mexico	Tamaulipas	Cueva Florida	F	A	*Pteronotus davyi*	JX853114		JX853156	JX853012
MEXG3	Mexico	Tamaulipas	Cueva Florida	F	A	*Pteronotus* sp.	JX853115	JX853065	JX853158	JX853013
KD02151102.1	Puerto Rico	Isabela	Cueva Cucaracha	F	A	Cave wall	JX853104	JX853057	JX853159	JX853002
KD02151102.2[d]	Puerto Rico	Isabela	Cueva Cucaracha	F	A	Cave wall	JX853105	JX853058	JX853160	JX853003
KD02151102.3	Puerto Rico	Isabela	Cueva Cucaracha	F	A	Cave wall	JX853106	JX853059	JX853161	JX853004
KD17070809	Puerto Rico	Arecibo	Cueva Culebrones	F	A	*Monophyllus redmani*	JX853107	JX853060	JX853163	JX853005
KD17070811	Puerto Rico	Arecibo	Cueva Culebrones	M	A	*Monophyllus redmani*	JX853108	JX853061	JX853162	JX853006
SM10052103.1	Puerto Rico	Arecibo	Cueva Culebrones	U	P	Cave wall	JX853118			JX853016
SM10052103.3	Puerto Rico	Arecibo	Cueva Culebrones	U	P	Cave wall		JX853067		JX853017
SM10052103.4	Puerto Rico	Arecibo	Cueva Culebrones	U	P	Cave wall				JX853018
FE19[d]	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853099	JX853052	JX853164	JX852997
FE20[d]	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853100	JX853053	JX853165	JX852998
FE21[d]	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853101	JX853054	JX853166	JX852999
FE23	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853102	JX853055	JX853167	JX853000
FE24	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853104	JX853056	JX853168	JX853001
PU20	Puerto Rico	Arecibo	Cueva Culebrones	U	P	Glue trap	JX853116	JX853066	JX853169	JX853014
PU23	Puerto Rico	Arecibo	Cueva Culebrones	U	P	Glue trap	JX853117		JX853170	JX853015
SM10052138.1	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853119	JX853068	JX853171	
SM10052138.2	Puerto Rico	Arecibo	Cueva Culebrones	M	A	Glue trap	JX853120		JX853172	JX853019
SM10052138.3	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853121		JX853173	JX8530120
SM10052138.4	Puerto Rico	Arecibo	Cueva Culebrones	M	A	Glue trap	JX853122	JX853069	JX853174	JX8530121
SM10052138.5	Puerto Rico	Arecibo	Cueva Culebrones	F	A	Glue trap	JX853123		JX853175	JX8530122
WCL015	Puerto Rico	Mona Island	Cueva Caballos	M	A	*Pteronotus parnellii*	JX853124	JX853070	JX853176	JX8530123

[a] F, female; M, male; U, sex unknown (referring to bat fly).
[b] A, adult; P, pupa (referring to bat fly).
[c] Where known; several samples were captured directly from the cave wall by using forceps or glue traps (see Materials and Methods.).
[d] *Wolbachia* bacteria were also detected for these samples. An additional pupa for which only *Wolbachia* was detected is not listed.
[e] NP, National Park.
[f] One additional pupal sample yielded only *Wolbachia* sequences, and another pupal sample was not included in the phylogenetic analysis due to sequence ambiguities.

and *Arsenophonus*. Sequences were aligned with MAFFT (26). Because the bacterial 16S rRNA gene sequences contained highly conserved regions interspersed with divergent regions, we aligned them using the E-INS-i algorithm, as it is optimized for aligning sequences with multiple conserved domains and long gaps; ITS and *groL* sequences were aligned using the G-INS-i algorithm, which is optimized for aligning sequences with global homology (25). ITS and *groL* were unambiguously aligned, while the 16S rRNA gene alignments had ambiguous and highly divergent regions. These areas were removed using GBLOCKS, allowing for less strict blocks, gaps within the final blocks, and less strict flanking positions (55). Geneious Pro 5.6 was used to manually correct the alignments. Codonw was employed to calculate the GC content (40). Evolutionary models were selected for each gene using the Akaike information criterion (AIC) as implemented in jModelTest 2 (44). Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian methods. ML trees were obtained from single and concatenated data sets for symbiont and bat fly genes (separately) through RAxML 7.0.4 (53). Node support was assessed by rapid bootstrap analyses with 1,000 bootstrap replicates. Bayesian topologies were obtained through MrBayes 3.1.2 (48); posterior probabilities were used to gauge nodal support. Resulting trees were visualized and compared using Dendroscope 3 (23).

Network analyses of endosymbionts. Previous studies on vertically transmitted endosymbionts have shown that these bacteria often possess highly divergent, strongly AT-biased genomes due to genetic drift and relaxed selection imposed on them by small population sizes and frequent population bottlenecks. These genome characteristics may result in long-branch attraction artifacts and can limit resolution of phylogenetic relationships. We therefore conducted a network analysis, employing SplitsTree with the GTR evolutionary model in conjunction with the agglomerating NeighborNet algorithm (22). For comparative purposes, data were combined with select GenBank entries of invertebrate symbionts and environmental (free-living) microbes. Because recent studies have shown a higher within-group resolution for housekeeping genes than for the ubiquitously available 16S rRNA gene sequences (24), we concentrated our analyses on the *groL* gene.

Cophylogenetic analyses of *Nycterophilia* bat flies and their endosymbionts. Cophylogenetic analyses were conducted on bat fly and endosymbiont topologies obtained from the ML analyses, because both ML and Bayesian topologies were congruent among bat fly and symbiont analyses. The NN-tanglegram method was used as implemented in Dendroscope 3.0 to compare rooted, resolved phylogenetic trees based on single genes. This approach visualizes similarities and differences of topologies by auxiliary lines between corresponding terminals (4). In the case of a host-parasite tree comparison, disruptions of the coevolutionary continuum are highlighted.

Host (*Nycterophilia*) and endosymbiont trees were furthermore used in a reconciliation approach that explains the previously observed incongruence between host and parasite trees in terms of duplications, host switches, and losses. Reconciliation analyses were conducted in Jane 3.0 (8). This reconciliation software finds optimal solutions in polynomial time via a dynamic programming algorithm and is thus better able to deal with larger data sets (>25 paired tips). Event costs in our analysis were modified from the cost scheme suggested in the work of Charleston and Perkins (4) and set as 0 for cospeciation, 1 for duplication, 5 for host switching, and 2 for loss. Failure to diverge was left at the default value. The high cost (5) for host switching in our analysis effectively disallowed this event based on the following rationale. The viviparous development of the flies results in a nonfeeding pupal stage, thus making (horizontal) uptake of endosymbionts from the environment unlikely. The only food source for adult flies is bat blood, which does not harbor fly endosymbionts. Therefore, this is a naturally contained system, which is unfavorable to host switches (35). According to the experimental results for midsize trees (8), the data set was run with the number of iterations (*G*) set at 45 and with the population size (*S*) set at 23. Analyses were repeated 20 times, to check for consistency of the results. Randomized tree simulations were used to check for significance of the resolved cost matrix.

Mapping of ecological niche characters. The ecological niche of bat flies includes their host bats and the surrounding bat roost environment. Therefore, we describe general bat fly ecology via two characters: "host specificity" (adult flies only) and "roost specificity" (pupae and teneral

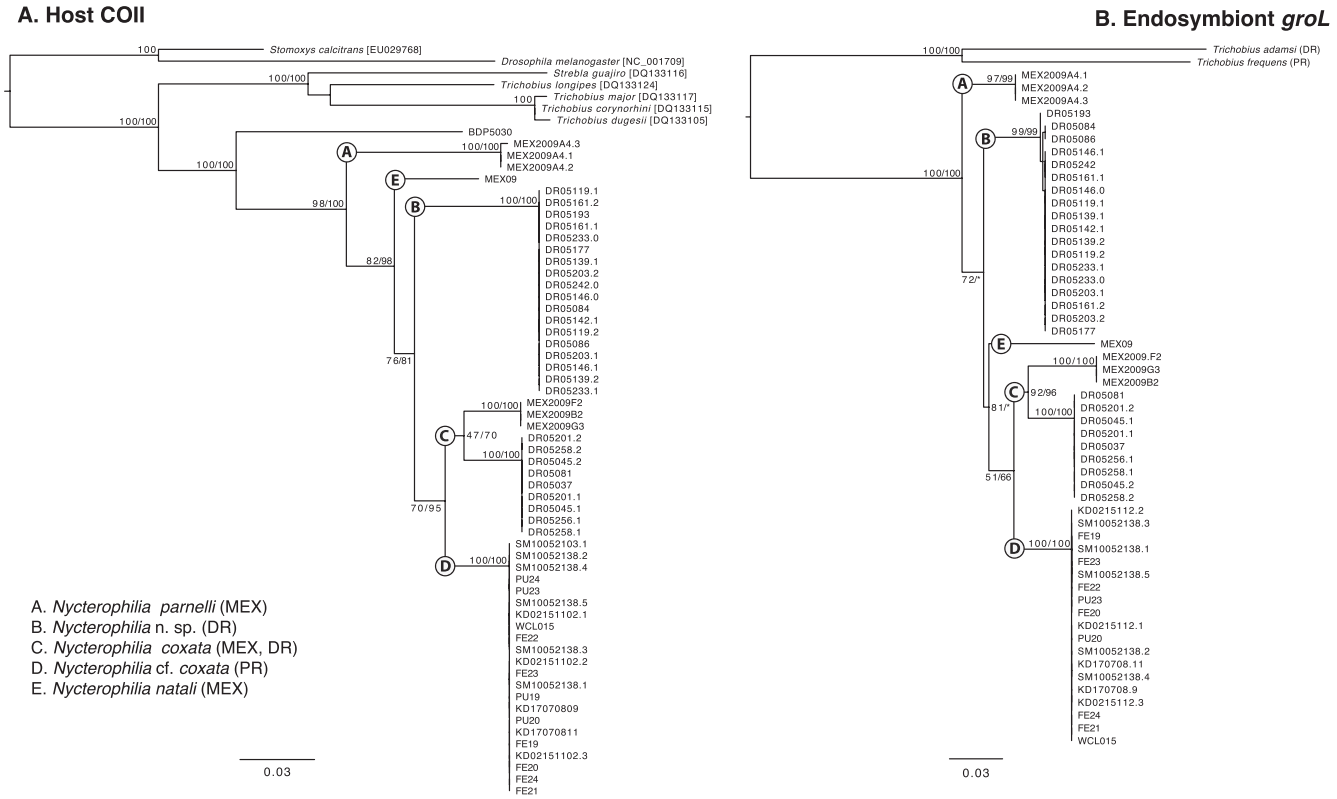

FIG 2 Phylogenetic trees (ML). Geographic location of samples is as follows: DR, Dominican Republic; PR, Puerto Rico; MEX, Mexico. (A) *Nycterophilia* bat flies as inferred from the mitochondrial cytochrome oxidase II (COII) gene. Numbers at nodes represent bootstrap values/Bayesian posterior probabilities. Major *Nycterophilia* subclades are labeled A to E (see the text). (B) *Nycterophilia* endosymbionts as inferred from the *groL* gene, with endosymbionts of *Trichobius* spp. as outgroups. Numbers at nodes represent bootstrap values/Bayesian posterior probabilities. Major *Nycterophilia* symbiont subclades are labeled A to E (see the text.).

flies). The host specificity of *Nycterophilia* species was classified in terms of the number of host genera utilized and was estimated from data collected by the authors, as well as from verified host association records found in previously published sources (see Table S1 in the supplemental material). Terminals were coded as 0 where parasite associations were restricted to a single bat genus (high host specificity) and as 1 where parasites associated with more than one host genus (low host specificity). Roost specificity was assigned to bat flies according to temperature gradients in the general roost environment (see the introduction), taking into account roost temperature preference of the bats and preferred pupal deposition microhabitats. Gradient classification was guided by the work of Rodríguez-Durán (47), who divided hot tropical caves by temperature into hot main chambers (HMC; 28 to 40°C) and hot cave foyers (HCF; <28°C, described as ambient in most other literature). The bat fly species included in this study were described as obligate hot cave users (HMC only) and facultative hot cave users (use of HCF and sometimes HMC) (see Table S2). Binary character coding assigned character states as 0 and 1, respectively. Host specificity and roost specificity were traced via likelihood-based ancestral state reconstruction on the endosymbiont ML topology, using the asymmetric Markov k-state 2-parameter model (AssymmMK, root frequencies same as equilibrium; Mesquite). The potential correlation of evolutionary change in roost specificity and host specificity along endosymbiont evolution was explored with Pagel's 94 method as implemented in Mesquite, using the ML topology (38).

RESULTS

Molecular studies. GenBank database searches confirmed a taxonomically restricted bacterial fauna in Nycterophiliinae, composed primarily of *Enterobacteriaceae* (*Gammaproteobacteria*). All *Nycterophilia* endosymbiont populations were dominated by a novel microbial clade. BLASTn scores (16S rRNA) indicated 90% similarity with *Providencia* spp. (E value = 0.0) and 87% similarity with *Arsenophonus* spp. (E value = 0.0), both members of the *Enterobacteriaceae*. Our results show association with 5 *Nycterophilia* species and 60 specimens from five widely separated geographic locations. We detected the same *Nycterophilia* symbiont in all 3 pupae collected directly from a cave wall and in three of four pupae collected from glue traps following deposition by captured females. Alignments of pupa-derived symbiont sequences (and subsequent phylogenetic analyses) confirm their congruence (100%) to sequences isolated from the adult flies. We also detected *Wolbachia* in nine specimens (14.8% of all adult flies) comprising three species (*Nycterophilia* n. sp. [Dominican Republic], *Nycterophilia coxata* [Dominican Republic], and *Nycterophilia* cf. *coxata* [Puerto Rico] [a *Nycterophilia* sp. that looks like *N. coxata*). The single pupa for which we did not detect the *Nycterophilia* endosymbiont was infected with *Wolbachia*; this pupa's maternal parent was coinfected with both *Wolbachia* and the *Nycterophilia* endosymbiont. All *Wolbachia* sequences were similar to known genotypes from the *Wolbachia* supergroup F. We recovered only the bacterial ITS gene from *Phalconomus* species B. Unlike all *Nycterophilia* spp., this sequence shared 98.3% pairwise similarity with *Arsenophonus* spp. (*Enterobacteriaceae*, *Gammaproteobacte-*

ria; E value = 0.0; tree not shown), which has been previously detected in other bat flies (21, 31, 56).

AT bias in the 16S rRNA gene sequences for the symbionts of the *Nycterophilia* exhibited values ranging around 51 to 52%. Likewise, the AT bias in *groL* sequences for *Nycterophilia* endosymbionts is extremely high, at nearly 70%. ITS/23S rRNA gene sequences exhibited 65 to 66% AT, and the *Nycterophilia* ITS sequences contained no embedded tRNA regions, as are present in *Arsenophonus*, *Proteus*, and other *Enterobacteriaceae*.

Phylogenetic analyses. (i) Nycterophiliinae (invertebrate host). For all genes and analyses (ML and Bayesian), Nycterophiliinae (*Nycterophilia* plus *Phalconomus*) formed a well-supported monophyletic clade, with *Phalconomus* in a sister-group position to the remaining clade. Nuclear and mitochondrial host genes showed phyletic congruence, and COII trees were used for subsequent analyses, as they provided complete specimen coverage. Within *Nycterophilia*, five clades (A to E) were recovered with moderate to strong bootstrap support. Clade A (Fig. 2A) included only *N. parnelli*, from Mexico. Clade B (Fig. 2A) was exclusively composed of samples collected from the bat host *Macrotus waterhousii* in the Dominican Republic. These flies likely represent an undescribed species, and for the purposes of this publication, we designated them *Nycterophilia* n. sp. Clade C (Fig. 2A) included *N. coxata* complex from Mexico and the Dominican Republic. This group fell into two well-supported subclades based on geography. Clade D (Fig. 2A) allied closely with clade C and included samples morphologically similar to the *N. coxata* group but may constitute a new subspecies prevalent in Puerto Rico. For the purpose of this publication, we designated these specimens *Nycterophilia* cf. *coxata* (29). Lastly, clade E comprised a single specimen of *N. natali* from *Natalus stramineus* subsp. *mexicanus* (12, 51) collected in Mexico, grouped at the base of clades B, C, and D with high support (clade E, Fig. 2A).

(ii) Microbial associates (endosymbionts). Sequence analysis of the 16S, *groL*, and ITS sequences of the *Nycterophilia* endosymbionts recovered a monophyletic clade, with 5 subclades, each also supported with moderate to high support values (ML and Bayesian) (Fig. 2B). These subclades generally mirrored their respective bat fly host clades [see "Phylogenetic analyses. (i) Nycterophiliinae (invertebrate host)"]. The only discernible difference was the position of the endosymbiont lineage from *N. natali* (clade E, Fig. 2B), which occupied a sister-group relationship with clades C and D, placing clade B in a more basal position with respect to clades C, D, and E (Fig. 2B). Additionally, the endosymbionts of *Nycterophilia* were not closely allied to those of other bat flies and invertebrates and were separated from them by a long branch. Conversely, based on an ITS gene phylogeny (not shown), the endosymbiont DNA isolated from *Phalconomus* species B grouped with *Arsenophonus*, the bacterial genus identified as an endosymbiont in other New World bat flies (56).

Network analyses. Network analysis of 78 *groL* sequences, including 53 *Nycterophilia* symbiont sequences, showed the bacterial associates of *Nycterophilia* as a distinct long branch, which was clearly set apart from other invertebrate symbionts (Fig. 3). This is consistent with results from the phylogenetic analyses. Moreover, *Nycterophilia* associates clustered apart from other known bacterial associates of bat flies, such as New World Streblidae and Nycteribiidae, again supporting results from our phylogenetic analyses. Within the cluster of *Nycterophilia* symbionts, five subclusters were discernible, which clearly mirrors the previously described phylogenetic tree structure, suggesting robustness of these clades across analytical approaches. Each of these clusters (A to E, Fig. 3) received moderate to high bootstrap support (70 to 100%). Within-cluster sequence similarity was higher (100 to 99.8%) than between-cluster similarity (92.4 to 96.9%).

Cophylogenetic analyses. Comparative mapping of endosymbiont (ML-based, concatenated data) and bat fly (ML-based) topologies in Dendroscope resulted in a tanglegram highlighting the incongruent position of *N. natali* (Fig. 4A, clade E). Incorporating the previously described cost structure (see Materials and Methods) into a timed ML topology-based analysis, Jane 3.0 recovered 11 optimal solutions with a minimal cost scenario of one duplication and three losses to resolve the incongruence (after the root node), with a total cost of 7 (see Materials and Methods). This result was consistent over 20 runs. Randomized tip mapping with 1,000 samples resulted in a mean simulated cost of 21.1 ($\pm$5.13), with 1.9% of the samples at or more extreme than the original cost computed in the solve mode. This means that results from the solve mode are significantly nonrandom ($P < 0.05$; one-tailed). The duplication event was inferred for the ancestral symbiont branch leading to clades B to E (Fig. 4B). While one symbiont lineage subsequently associated with *N. natali* (clade E) and the *N. coxata* group (clades C and D), representatives of the other (duplicated) lineage associated with *Nycterophilia* n. sp. (clade B) from *Macrotus waterhousii* (Fig. 4B).

Character mapping—ecological niche. Results from the ancestral character mapping of roost and host specificity are presented in Fig. 5a and b, respectively, in the context of endosymbiont phylogeny (ML, concatenated data). These results suggested that, initially, symbionts were associated with bat flies that were facultative hot cave users, frequenting both hot cave foyers and hot cave main chambers. Such bat flies can be found on the extant families Mormoopidae (i.e., *Pteronotus parnellii*) and Phyllostomidae (i.e., *Macrotus waterhousii*). The association of *Nycterophilia* bat flies and their symbionts with exclusively hot cave habitats (HCM, obligate hot cave users) developed secondarily (Fig. 5a). Likewise, symbiont association with less-host-specific bat flies appears to be a derived character, with a transition occurring at the base of the symbionts of the *N. coxata* group (Fig. 5b), following the switch to obligate hot cave use. Tests of correlation of roost and host specificity were significant ($P = 0.032$; 500 simulations), indicating that the model allowing correlation of characters fit the data significantly better than did the model assuming independence. Further testing involving contingent changes showed significant support for the hypothesis that a change in host specificity depends on the state of roost specificity (likelihood ratio = 15.28; 1 degree of freedom [df], $P < 0.0005$).

DISCUSSION

Evolutionary origin of nycterophiliine symbiosis. Molecular analysis revealed a taxonomically restricted microbiome in Nycterophiliinae, including only three groups of *Gammaproteobacteria* and *Alphaproteobacteria*. Two different groups of primary microbial associates (*Gammaproteobacteria*) occur within the Nycterophiliinae. The bat fly genus *Phalconomus* is associated with an *Arsenophonus*-type microbe, similar to that of other bat flies (21, 56). Unlike its sister genus, *Nycterophilia* invariably harbored a novel, robustly supported monophyletic clade of symbiotic *Gammaproteobacteria* (*Enterobacteriaceae*) across a broad geographic distribution. Relationships of this novel clade within

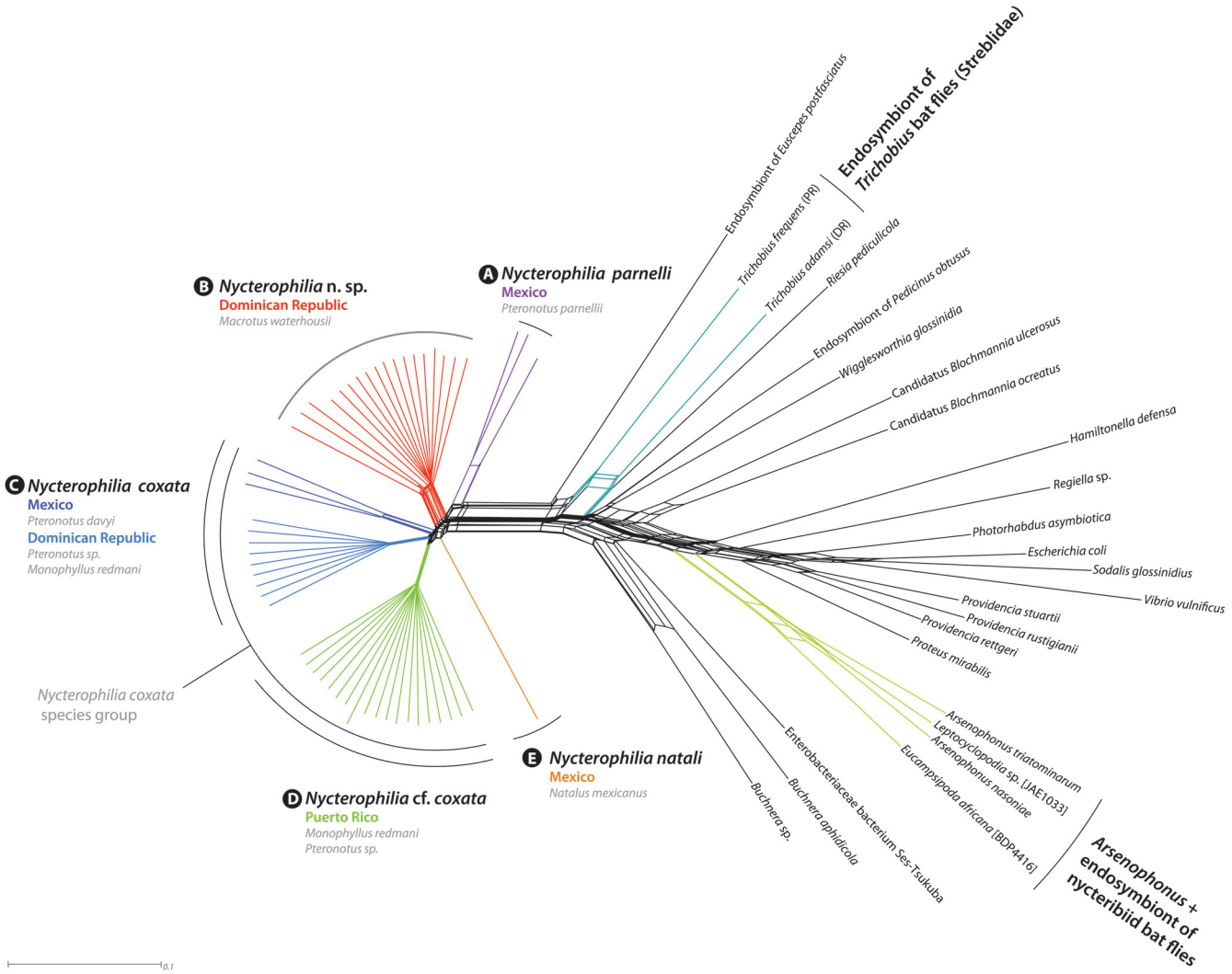

FIG 3 A neighbor net including *Nycterophilia* symbionts (*groL* sequences), symbionts of other arthropods, and various free-living bacteria. *Nycterophilia* symbiont clades are labeled A to E (see the text).

Gammaproteobacteria remain uncertain, and the *Nycterophilia* symbionts do not seem to be closely related to other endosymbiotic mutualists of blood-feeding insects, such as "*Candidatus* Riesia" or *Wigglesworthia* (Fig. 3). Moreover, this clade is apparently not a close relative to the endosymbiont lineages that have been detected in most other bat fly lineages (e.g., *Arsenophonus*-like bacteria). Based on similarity scores, this new clade of symbionts falls into the *Enterobacteriaceae*, but its evolutionary origin within this group remains unclear.

The extreme AT bias and the long branch length leading to the clade of *Nycterophilia* symbionts suggest that their endosymbiotic lifestyle is ancient, evolution was particularly rapid, or both (34). In fact, the AT bias in *groL* sequences for *Nycterophilia* symbionts (69 to 70%) is among the highest recorded for *Enterobacteriaceae*, compared to 60 to 67% for other symbiont *groL* sequences obtained from GenBank, with the exception of the endosymbiont of the weevil *Euscepes postfasciatus*, which exhibited an AT bias of 71% (19). The recently described Dominican amber fossil *Enischnomyia stegosoma* is morphologically closer to extant *Nycterophilia* than to *Phalconomus*, suggesting that *Nycterophilia* and its symbiont may not be older than the Early Miocene (~20 million years ago [mya]) (43). The basal position of *Nycterophilia parnelli*, associated exclusively with the host *Pteronotus parnellii* (Mormoopidae), suggests an origin coinciding with, or slightly younger than, that of *Pteronotus* bats. Recent molecular divergence time analyses estimate the origin of *Pteronotus* bats into the Pliocene (~5 mya) (11).

The low diversity of sequences within each endosymbiont clade (A to E) suggests high clonality of endosymbiont populations and an evolutionarily sustained selective specificity of symbionts to their respective bat fly host species (13). This is further supported by the fact that symbionts from geographically distant populations of closely related hosts (i.e., within the *N. coxata* group) are more closely related to each other than are symbionts from sympatric populations of distantly related host species. For instance, symbionts within clades B and C (Fig. 2B), which are found in two species of bat flies that parasitize different host bats, occur in sympatry within the same cave in the Dominican Repub-

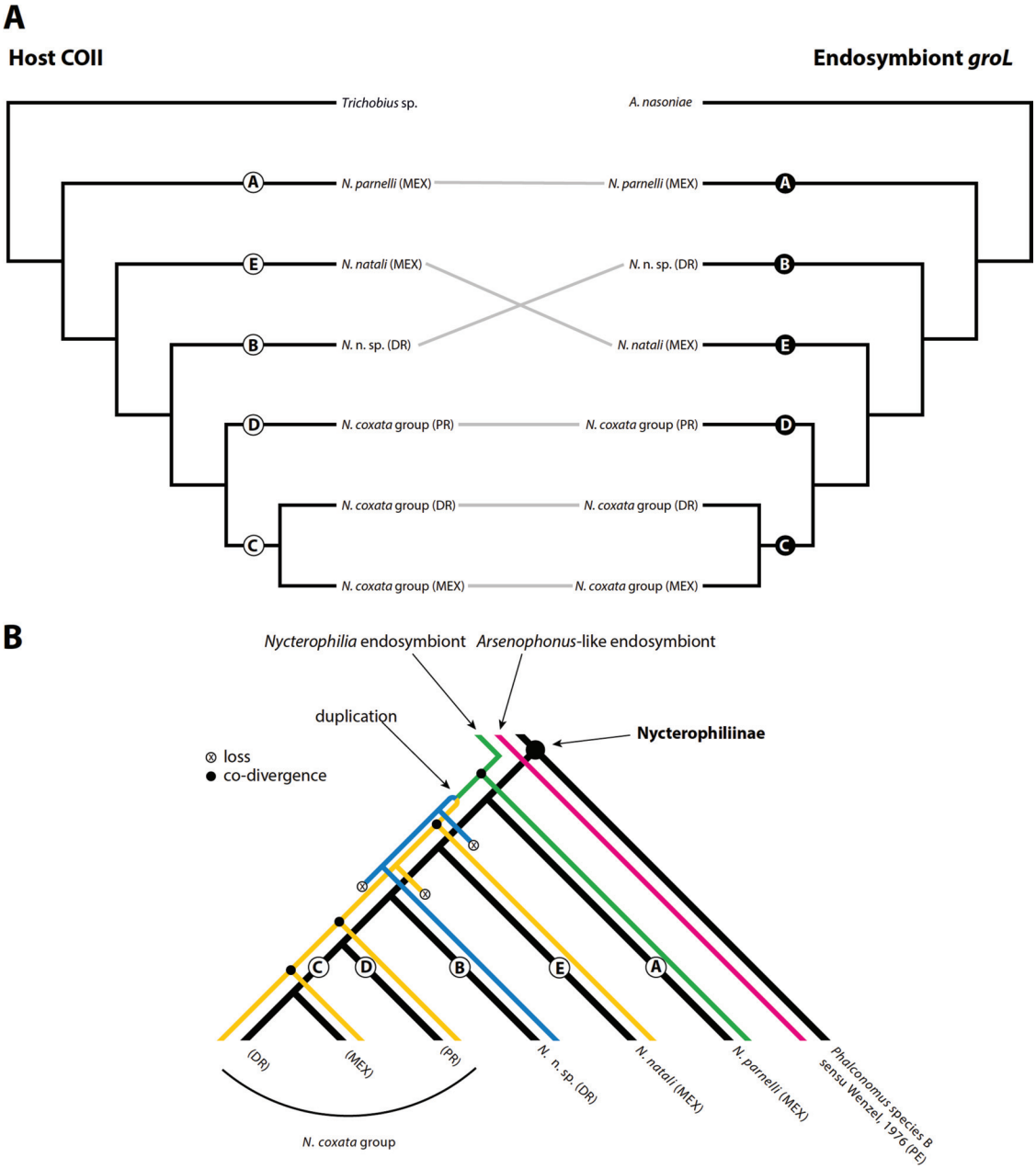

FIG 4 (A) Tanglegram of *Nycterophilia* bat flies and their endosymbionts, showing major clades. Host COII (left) and endosymbiont *groL* (right) trees shown. Lines connecting bat flies and symbionts indicate associations and incongruences. Host and bacterial clades labeled A to E (see the text). Geographic location of samples is as follows: DR, Dominican Republic; PR, Puerto Rico; MEX, Mexico. (B) *Nycterophilia* and symbiont cophylogenetic history (ML) of major clades estimated using Jane 3.0 with no host switching. The black lines represent the host topology; colored lines represent the symbiont topology. Duplications, losses, and codivergences are indicated in the figure. Host and bacterial clades labeled A to E (see the text). Geographic location of samples is as follows: DR, Dominican Republic; PR, Puerto Rico; PE, Peru; MEX, Mexico.

lic. The higher-than-expected diversity of *Nycterophilia* bat flies in the Caribbean (i.e., new species were discovered) suggests a pivotal role of this region in the diversification of this genus and, by extension, that of their endosymbionts. However, because of their basal association with *Pteronotus* bats (from Mexico), the ultimate origin of Caribbean *Nycterophilia* flies and their symbionts may have been continental. In fact, all three lineages of *Pteronotus* bats in the Antilles are thought to be of continental origin (11). However, congruent with the suggestion of Dávalos (11, 12) that Caribbean islands act both as sources and sinks for bat colonists, mainland bat flies in the *N. coxata* group (and their symbionts) likely have a Caribbean origin (Fig. 2A, clades C and D).

Nature of *Nycterophilia* symbioses. The presence of a novel monophyletic clade of microbes in *Nycterophilia*, as well as its high prevalence per adult population (100%), suggests an obligate association. This is further supported by the detection of endosymbiont DNA in *Nycterophilia* pupae, which demonstrates that transmission occurs vertically from maternal parent to offspring.

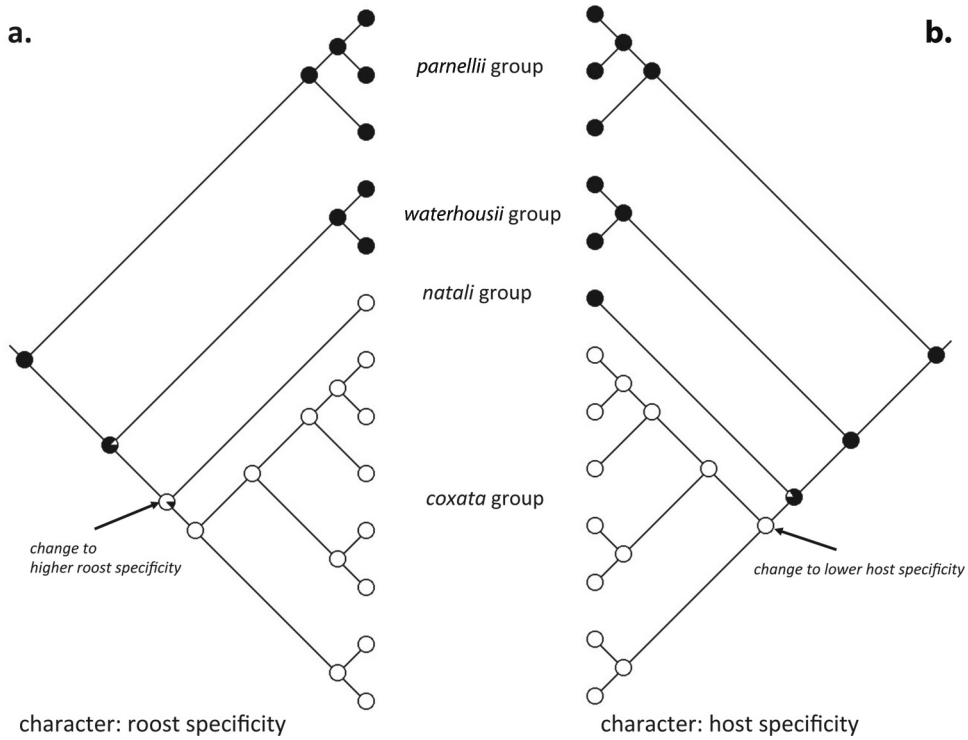

FIG 5 Ancestral ML character state reconstruction of "roost specificity" and "host specificity" along the *Nycterophilia* symbiont topology (see the text for definition). Circles are partitioned according to probability of the reconstructed character state. (a) Black represents facultative hot cave use of bat flies; white represents obligate hot cave use. (b) Black represents high host specificity of bat flies; white denotes low host specificity.

The Hippoboscoidea, which include tsetse flies (Glossinidae), louse flies (Hippoboscidae) and bat flies, share many characteristics, such as viviparity and the presence of a specialized accessory gland (the so-called "milk gland") that produces proteins consumed *in utero* by the developing larvae. In tsetse flies, endosymbionts have been detected in the milk gland and in pupae, suggesting that the larvae are infected during feeding within the body of the female (33, 39). In nycteribiid bat flies, recent research has shown the localization of microbial associates by *in situ* hybridization; infected tissues included bacteriomes located in the periphery of the lower abdomen and within the milk gland tubules. While the exact localization of the *Nycterophilia* endosymbionts remains unknown, their detection in freshly deposited pupae with minimal exposure to the general cave environment suggests infection of pupal tissues prior to deposition (i.e., within the female's body), similar to the process known from other hippoboscid flies (21, 56).

Given the sporadic nature of the molecular detection of *Wolbachia* (14.8%, supergroup F) in specimens related to this effort, a facultative association with *Nycterophilia* is hypothesized for these microbes. The detection of *Wolbachia* in a pupa and its maternal parent suggests vertical transmission, which has been observed for other *Wolbachia* organisms. At this point, no obvious congruence was observed between *Wolbachia* and *Nycterophilia* fly phylogeny (trees not shown). Supergroup F *Wolbachia* organisms have been detected in a range of invertebrates, including filarial nematodes (27) and blood-feeding insects such as bedbugs (45), lice (9), hippoboscid flies (10), and fleas (17), and have recently been identified as a mutualist in some bedbug populations (20).

Symbiont-*Nycterophilia* evolution. Many previous studies on endosymbionts have shown that vertical transmission usually facilitates cospeciation. Indeed, cophylogenetic analyses conducted in this effort are clearly dominated by congruence among the symbiont and *Nycterophilia* bat fly phylogenies (Fig. 3B). This result further supports the idea of a mutualistic and obligatory relationship, after initial successful establishment of this symbiosis.

The duplication event with subsequent losses inferred early in the evolution of *Nycterophilia* (after *N. parnelli*) is likely explained as the result of lineage sorting in an initially diverse pool of symbiont lineages in ancestral *Nycterophilia* bat fly populations. Over time, with further diversification of their bat fly hosts, particularly advantageous lineages became fixed in a population, while others were lost. Some of the inferred initial endosymbiont diversity in bat fly populations may have been (or may still be) driven by the dispersal and migration of bats (which are host to the *Nycterophilia* bat flies). In particular, the contemporary and historical movement of bats through the Neotropics (including the Caribbean), as well as their fission-fusion population structure, may remove reproductive barriers for previously isolated bat fly and endosymbiont populations along a geographic and ecological cline (3, 11). In the context of this argument, it is important to note that the samples analyzed in this effort do not encompass the full genetic diversity of symbionts or *Nycterophilia* bat flies. Many details about *Nycterophilia*-endosymbiont evolution likely are yet to be discovered.

Endosymbiosis across ecological tiers. The distribution of unique endosymbiont clades across two closely related bat fly genera indicates a symbiont replacement within the Nycterophiliinae. Because of the sister-group relationship of *Phalconomus* and *Nycterophilia* and the unresolved phylogenetic position of Nyctero-

philiinae within the bat flies (16), the direction of this replacement is ambiguous. However, given our current state of knowledge, the most parsimonious explanation would involve a replacement from a likely ancestral endosymbiotic association (i.e., all other streblid bat flies known have an *Arsenophonus*-like association). Symbiont replacements are often hypothesized to result from competition among symbiont populations and/or selective pressure on the symbiont host in connection with ecological shifts (7, 30, 32). For blood-feeding insects, this is commonly understood as a trophic shift to a nutrient-poor diet (blood) and may have been a driving factor for the initial establishment of symbiosis in bat flies. It is interesting, however, that *Nycterophilia* flies colonize the same trophic resources (i.e., bat species) as do other Neotropical bat flies (e.g., *Trichobius* spp.) while harboring their own, vastly divergent clade of microbial associates. Hence, this replacement is at odds with a strictly trophic causality linked to diet. The significant correlation of host specificity and roost specificity along the symbiont phylogeny may provide a clue for alternative considerations (see "Character mapping—ecological niche" in Results). Specifically, the novel *Nycterophilia* endosymbiont clade is exclusively associated with parasites that not only feed on facultative or obligate hot-cave-roosting bats but may also deposit and rear their pupae in the hot main chamber of tropical caves. In this they are unique—Neotropical bat flies in association with *Arsenophonus*-like endosymbionts can parasitize hot-cave-roosting bats in the adult stage but have to deposit their pupae in exclusively ambient or hot cave foyer environments (14), sometimes quite distant from the bats. In fact, preliminary rearing experiments with *Arsenophonus*-associated *Trichobius* sp. under controlled conditions at 34°C and 90 to 100% relative humidity resulted in 100% pupal mortality (16 pupae [unpublished data]). Therefore, ecological dynamics within bat fly species and communities, such as competition for suitable pupal deposition microhabitats and predator avoidance during deposition, should be recognized as potential facilitating factors of symbiont replacements and evolution, in addition to the colonization of novel bat hosts as an outcome of competition for trophic resources. Subsequently, microhabitat selection for pupal deposition may explain the observed niche partitioning of bat fly communities along a developmental gradient, but further studies are necessary to elucidate this process. In some holometabolous insects, obligate endosymbiosis has been shown to be more important for developmental stages than for adults. For instance, "*Candidatus* Blochmannia floridanus," the obligate mutualist of carpenter ants, is known to increase in population size during pupal development of its host (54), as does *Wigglesworthia* in tsetse flies (46). Although nonfeeding, pupae are metabolically very active due to processes of metamorphosis associated with holometabolism. In the case of carpenter ants, endosymbiont activity produces essential amino acids and provides nitrogen recycling for breakdown products accumulated during pupal development. Although the biological function of the novel *Nycterophilia* symbiont is unknown at this point, similar adaptive mechanisms have to be considered. Most importantly, the contingent change to expanded host use of *Nycterophilia* bat flies upon narrowing the ecological niche of their developmental stages suggests that environmental tolerance across life history stages is an understudied but crucial factor in shaping parasite-host relationships.

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