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More than meets the eye: decrypting diversity reveals hidden interaction specificity between frogs and frog-biting midges

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Abstract. 1. Female frog-biting midges (Diptera: Corethrellidae) eavesdrop on the nocturnal mating calls of their blood hosts – male frogs. Available data suggest variable degrees of specialisation among *Corethrella*-host associations, with limited information on the mechanisms involved in host selection and partitioning on a community level.

2. Our study provides a first comprehensive analysis of host interactions for a neotropical community of frog-biting midges, based on both morphological and molecular genetic species delimitation. We used quantitative bipartite interaction networks to investigate host specificity among the midge-frog community of La Gamba, Pacific lowland Costa Rica.

3. Midges that were collected directly from frog hosts (16 frog species) showed more pronounced levels of specificity (network-wide degree of specialisation: $H2' = 0.3$) than those caught with acoustic traps broadcasting their calls (12 frog species; $H2' = 0.08$). This indicates that, despite a rather generalist acoustic foraging behaviour, frog-biting midges discriminate between potential hosts by using additional close-range recognition cues.

4. Based on COI and ITS2 sequencing data, we identified considerable levels of cryptic diversity within our five *Corethrella* morphotypes, with at least 17 distinct MOTUs of *Corethrella* in La Gamba. Including these MOTUs in bipartite network analyses produced higher resolution in species interactions, and increased estimators of network specificity ($H2' = 0.42$).

Key words. Bipartite network, coevolution, Corethrellidae, haematophagy, host specificity.

Introduction

Biotic interactions are a major driving force of evolution and play a crucial role in the origin and maintenance of biodiversity (Jordano, 2016; Zhang *et al.*, 2018). This might be particularly true for antagonistic interactions, including the varied forms of predation, which can exert strong directional and potentially disruptive selection pressures on prey populations (Johnson & Belk, 2020). Organisms are intertwined in a mosaic of interactions, forming complex multidimensional interaction networks, the resolution of which poses a major challenge to modern-day ecologists (see Cushman & Huettmann, 2010). Whereas full trophic networks ('foodwebs', see Pimm *et al.*, 1991) are used to depict all trophic links within a community, quantitative bipartite interaction networks (see Memmott, 1999) illustrate interdependence of two sets of interacting organisms, e.g. taxonomic groups or ecological guilds (Poulin, 2010). In this study, we used quantitative bipartite interaction networks to investigate host specificity among a tropical community of haematophagous frog-biting midges and their anuran hosts.

Haematophagy (i.e. blood-feeding) is a common consumer strategy that independently evolved among a wide range of organisms, spanning from protist endoparasites to higher metazoan phyla, such as plathelminths, nematodes,

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annelids, arthropods, and even vertebrates (e.g. Balashov, 1984; Mostovski, 2003; Gnocchi & Srbek-Araujo, 2017; Korytář *et al.*, 2020). Blood can be either utilised as a sole nutrition source (obligate haematophagy; see Waage, 1979) or supplementarily (facultative haematophagy) – often linked to reproductive cycles in the female sex (e.g. Lehane, 2005; Davey, 2007). The life histories of haematophagous organisms fall into two main categories: parasitism and predation. 'True' parasites are closely associated with only a single-host individual during a certain stage of their life cycle. Free-living haematophagous organisms, in contrast, are considered (micro) predators if the realised number of blood hosts is >1 (Lafferty & Kuris, 2002). Both types of haematophages can be highly specialised, with the degree of host species specificity determined by ecological conditions and phylogenetic constraints (see Poulin, 2011a). Parasite–host interactions are bidirectionally interdependent (Solomon *et al.*, 2015); phylogeny and host characteristics determine parasite community structures (Dallas & Presley, 2014), which in turn reciprocally affect host behaviour and trait evolution (Ezenwa *et al.*, 2016). Here we investigate the specificity of host associations among a community of frog-biting midges and their blood hosts, and analyse the mechanisms involved in host selection and blood-resource partitioning on a community level.

Female frog-biting midges (Diptera: Corethrellidae) eavesdrop on the nocturnal mating calls of their blood hosts, male frogs, being attracted by a combination of spectral and temporal call properties (Meuche *et al.*, 2016; Toma *et al.*, 2019; Virgo *et al.*, 2019). Costs imposed by frog-biting midges on blood hosts could be substantial, ranging from irritation (indicated by defensive behaviours) and loss of blood (possibly substantial, see Camp, 2006) to an increased risk of infection with pathogens (Johnson *et al.*, 1993; Meuche *et al.*, 2016). Like other blood feeders from the suborder Nematocera, frog-biting midges are best regarded as micropredators rather than true parasites although the number of blood hosts per individual midge is certainly low, and presumably often one (the host). Calls of different species of frogs (McKeever & French, 1991; Grafe *et al.*, 2008; Virgo *et al.*, 2019), and also calls of different complexity of the same species (Bernal *et al.*, 2006; Aihara *et al.*, 2016), have been shown to attract variable numbers of midges. A study conducted by Grafe *et al.*, 2019 used bipartite interaction networks to analyse midge-frog associations at different sites in Brunei Darussalam, showing strong differences in specialisation between two research sites. This indicates that *Corethrella*-frog interactions vary depending on species composition and habitat, likely due to adaptation to local frog communities. Although experiments with acoustic traps have yielded insights on attractive call properties, the complete mechanism of host discrimination, and especially the importance of nonacoustic close-range cues, remains unknown.

Presently, there are 111 extant *Corethrella* spp. described worldwide (Amaral *et al.*, 2019), of which 35 (+5 undescribed) were reported from Costa Rica (Borkent, 2014). An increasing interest in frog-biting midge research over the last years has led to novel species descriptions (Amaral & Pinho, 2015; Caldart *et al.*, 2016; Kvifte & Bernal, 2018), indicating that substantial diversity remains to be uncovered. However, so far species delimitation for *Corethrella* were exclusively based on morphological traits (but see Miller *et al.*, 1997). Despite a vast and increasing number of studies, the species richness of many insect communities remains highly uncertain, partly due to a large degree of cryptic, i.e morphologically indistinguishable but genetically distinct, species (Bickford *et al.*, 2007), especially among many parasitic taxa (e.g. Poulin, 2011a; Pérez-Ponce de León & Nadler, 2016; Benda *et al.*, 2021), with potential implications for epidemiology, diagnostics, and our understanding of trophic network topology.

To our knowledge, the present study is the first to include molecular genetic characters to assess frog-biting midge diversity and host specificity for an entire frog/midge community. Our study is based on multiyear collections from the forested surroundings of the La Gamba research station in the Golfo Dulce area, Pacific Costa Rica. We collected midges directly from frog hosts and by using acoustic traps broadcasting advertisement calls of frog species identified as blood hosts. We asked the following questions: 1) How host-specific are frog-biting midges at La Gamba? 2) Does midge attraction to acoustic traps represent the patterns of specificity observed when sampling midges directly from male frogs? 3) Does taking into account midge cryptic diversity alter the observed interaction network structure?

Methods

Study area

Sampling was conducted at La Gamba research station (8°42′N, 83°12′W) in Puntarenas, southern Costa Rica (www.lagamba.at). The station is located near the Pacific coast at the edge of the Piedras Blancas National Park, one of Central Americas last remaining areas of primary lowland tropical rainforest and one of the most diverse forests in the world (Huber *et al.*, 2017). Amphibian diversity at the study site is high, with at least 36 species of anurans being encountered in close vicinity of the station (Franzen & Kollarits, 2018). Most experiments were performed during the onset of the rainy season (March–May), with additional sampling periods during peak and offset of the rainy season (June–December).

Sampling of frog-biting midges

Frog-biting midges were collected at the study site by collecting midges directly from frog hosts via aspirators and by using acoustic traps (Fig. 1). The data presented show cumulative catch data for the years 2013–2019. Frog hosts were identified directly in the field and were not harmed in any way. We collected female frog-biting midges that were found actively feeding on frogs, as well as midges resting or walking on the frogs.

For acoustic trap experiments, we used two different trap setups as described in Virgo *et al.*, 2019. Traps broadcasting specific anuran advertisement calls (1 species per trap from a total of 12 species) were deployed at different amphibian perch sites within the area, e.g. ponds within the garden of the station, a larger artificial swamp at the edge of the forest ('Laguna')

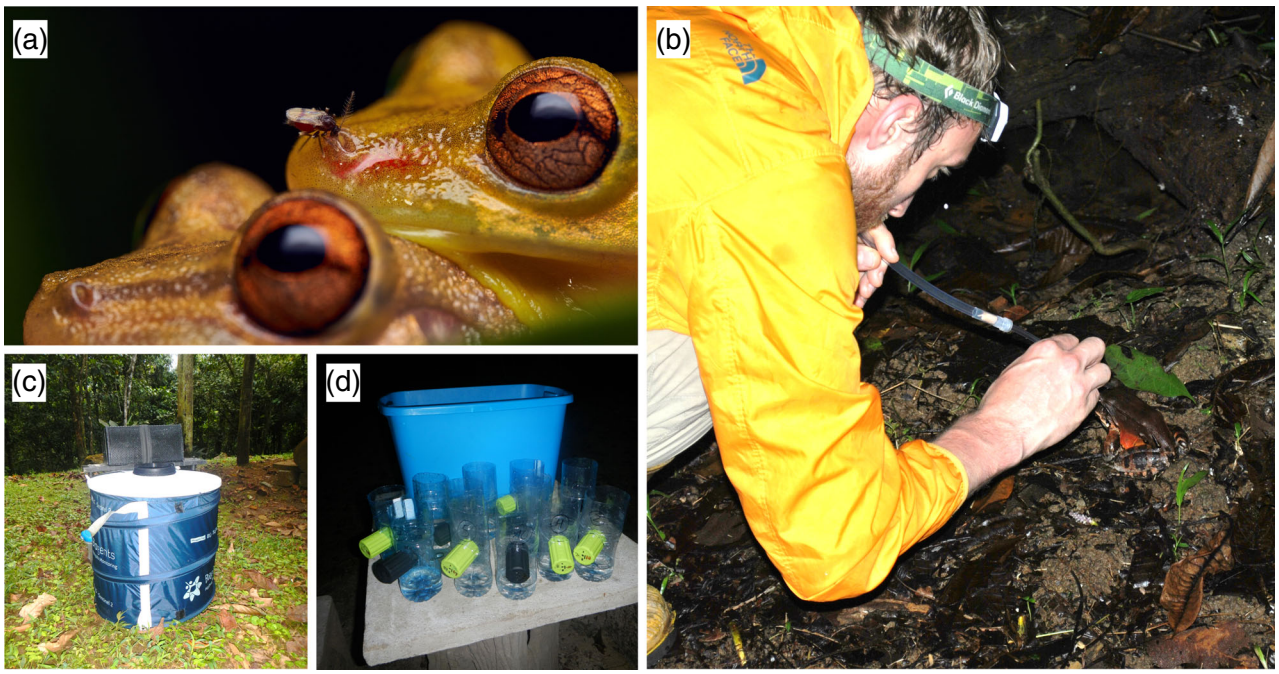

Fig. 1. Sampling of frog-biting midges (*Corethrella* spp.). (a) *C. ranapungens* sucking blood from an amplectant male *Scinax elaeochrous* (photo: A. Ruppert), (b) midges are collected from a male *Leptodactylus savagei* with an aspirator, (c) fan-operated mosquito trap (Biogents Sentinel 2) equipped with a loudspeaker, (d) self-made 'bottle traps' filled with water and equipped with small loudspeakers (see Virgo *et al.*, 2019).

and along the Quebrada Negra, a small river bordering the garden. All traps were deployed on ground level. Between trials, runtimes (5–60 min) and sound pressure levels (78–84 dB at 1 m, dB re 20 μP, flat weighted, fast response setting) were varied, depending on trap type and test design (recognition vs. choice experiments, see Virgo *et al.*, 2019). For the twelve calls tested, the coverage of sites and seasons as well as the number of repetitions per species were very similar. All frog calls used in the experiments were recorded at the study area with a Marantz PMD-561 portable digital recorder (.wav, 48 kHz/24 bit) and a Rode NTG4 directional condenser microphone (Rode Microphones, Sydney, Australia). Sound files for each target species were generated with Reaper (Vers. 5.311, Cockos Inc.) by extracting single calls of these recordings. For replicate trials ($N = 4–10$), we used different call recordings from different individual frogs of each target species, to avoid effects of pseudoreplication (see Kroodsma *et al.*, 2001). Standardised sound files of 1 min were generated with 25 consecutive calls, allowing for cross-comparability between frog species. All midges were euthanised by freezing (−20 °C), immediate transfer to EtOH (p.a.) or by overexposure to Triethylamine (99.5%), and stored in >70% EtOH. *Corethrella* spp. were categorised based on morphological features using the characters in the key to new world species of Corethrellidae (Borkent, 2008). Representative individuals were mounted on microscopic slides using Entellan® rapid mounting medium (Merck Millipore, Billerica, Massachusetts, USA) and identified to species by A. Borkent, Salmon Arm, British Columbia, Canada. From midges caught in acoustic traps, only a subset was used for morphological identification with a maximum of 100 midges per sample (= individual trap per trial); samples with fewer than 100 midges were identified completely. To avoid observer bias, all midge subsamples were picked blindly from the main samples.

Bipartite interaction networks

We used the bipartite package (Dormann *et al.*, 2008) presented in R (Vers. 3.4.0) to generate quantitative bipartite interaction networks for midges and frog hosts. Networks were generated separately for midges collected directly from frog hosts and those captured with acoustic traps. The presence of a particular *Corethrella* species found on an individual host/in a given trap was counted as one interaction, regardless of the number of individuals (compare Grafe *et al.*, 2019). Network structure was analysed based on the following metrics, described by Dormann *et al.*, 2009: Quantitative weighted specialisation index H2′ as an estimate for the network-wide degree of specificity; species-level specialisation index d′ for each midge (d′m) and frog (d′f) species separately. Values for both H2′ and d′ range from 0 (= no specificity) to 1 (= maximum specificity). We calculated connectance (C) as a qualitative measure for the proportion of realised links. To test for deviation from chance-based networks, obtained H2′ values were tested against those of null models of randomly assorted networks, while maintaining the marginal totals and connectance (10 000 permutations, t-test).

At first, networks were generated based on morphological species identification. Preliminary molecular genetic analyses, however, revealed high levels of genetic differentiation among our *Corethrella* morphotypes, indicating cryptic species

diversity and thus leading to potentially under-resolved network structure. To test for this, an additional network was generated using a subsample of midge specimens from the direct-sampling network. We picked a representative subset of 382 midges, covering as many *Corethrella*-frog interactions as possible, with a total maximum of 10 identical (randomly selected) interactions per frog species per year. For interactions represented by multiple midge individuals, specimens were chosen randomly. The subnetwork was then reconstructed for both morphological species identification, as well as novel species delimitation based on COI-MOTU-clustering results.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted using the GeneReleaser (BioVentures Inc.) reagent using a protocol described by Weigand 2013. DNA extraction is done using the whole specimen, leaving the exoskeleton intact for slide making and morphological investigation. The extraction protocol is provided in Appendix S1. We used primers HCO2198/LCO1490 (Folmer *et al.*, 1994) to amplify a ∼750 bp region of the mitochondrially encoded Cytochrome C Oxidase I (COI) gene, and primers ITS2A/ITS2B (Foley *et al.*, 2007) to amplify a ∼320 bp region of the rRNA Internal Transcribed Spacer 2 (ITS2). PCR reactions of 12.5 µl were setup as follows: 1 µl DNA template, 4.75 µl H_2O, 6.25 µl GoTaq Colourless Master Mix (Promega, Fitchburg, Wisconsin, USA), 0.25 µl forward/reverse primers, with the following thermocycling protocols used. Initial denaturation (hot start) at 94 °C for 3 min, followed by (COI:) 40 cycles of 94 °C for 20 s, 50 °C – 20 s, and 72 °C – 40 s; final elongation at 72 °C for 5 min; (ITS2:) 45 cycles of 94 °C – 40 s, 56 °C – 30 s, 72 °C – 50 s; final elongation at 72 °C for 5 min. All PCR products were purified using Exo1/FastAP (Thermo Scientific), and sequencing was performed on a CE-sequencer (Applied Biosystems 3130xl Genetic Analyser, Waltham, Massachusetts, USA) at Ruhr-University Bochum, Department of Receptor Biochemistry. For the ITS2 locus, smaller indels and SNPs prevented direct sequencing. Therefore, amplicons of the ITS2 gene were ligated to a pGEM-T vector (Promega) and transformed into *E. coli* JM109 high-efficiency competent cells (Promega), following protocol. About3 µl of PCR product were used for setting up the ligation reaction, which were incubated at room temperature for 1 h. Plates (LB/ampicillin/IPTG/X-Gal) were incubated over night at 37 °C and stored at 5 °C for 90 min, to intensify colouration of non-recombinant (blue) colonies. For each sample, 10 recombinant (white) colonies were picked and transferred to prepared PCR-premix. PCR was performed following the same protocols as before. Preliminary tests indicated that intra-individual allelic variation was low (<1.5%). Therefore, we randomly picked one allele for subsequent phylogenetic analyses.

Species delimitation

Editing and processing of nucleotide sequences was conducted using GeneiousPrime® software (version 2019.2.1). Forward and reverse sequences were trimmed according to quality, with a cut-off value of >5% error probability. For COI, sequences were aligned using the MAFFT plugin (Katoh, 2013). For the hyper-variable ITS2 sequences, alignments were constructed based on RNA transcripts: ITS2 often shows a high divergence in sequence, but a conservation in secondary structure (Schultz *et al.*, 2005; Zhang *et al.*, 2015). Secondary structure was predicted using the LocARNA online tool (http://www.bioinf.uni-freiburg.de/Software/LocARNA/#webserver; Will *et al.*, 2012). Folding and manually aligning the input sequences produced a more accurate alignment (i.e. less gaps, higher identity score) than using standard alignment algorithms alone. The obtained output alignment was imported into Geneious for further evaluation and analysis. All alignments were visually inspected, with manual correction of sequencing errors, gaps, and inserts. Sites containing >75% gaps were stripped from the analyses.

We performed Bayesian analyses using Mr Bayes (version 3.2.6; Huelsenbeck & Ronquist, 2001) for Geneious. Following Abadi *et al.*, 2019, we skipped a-priori model selection and instead chose the most parameter-rich model GTR + I + G (4 gamma categories) as our substitution model. Four MCMC chains (3 hot/1 cold) were run in a duplicate for 1 100 000 generations with a subsampling frequency of 200 generations, using default temperatures and default prior distributions with unconstrained branch lengths. The first 250 000 generations were discarded as burn-in, and a majority rule consensus tree was constructed. The convergence of run parameters was assessed by visual inspection of trace/density plots and effective sample size estimates (ESS threshold >200). The COI tree (full dataset) was rooted using BLAST-Hit Genbank sequences of Dipteran Phlebotominae (MT644252), *Anopheles galvaoi* (MF381669), and *Anopheles donaldi* (MT669939) as outgroups. Phylogenetic trees were visualised using the iTOL online tool (https://itol.embl.de/; Letunic & Bork, n.d.).

In addition to the tree-based (visual) species delimitation, we used the ASAP-web tool (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html; Puillandre *et al.*, 2021) to calculate a barcoding gap. ASAP partitions species based on an ascending hierarchical clustering algorithm of pairwise genetic distances. Partitions are ranked based on a scoring algorithm ('asap-score'), combining partitioning probabilities and gap-width. We ran the web application using the Kimura-2 parameter distance model with default parameter settings and chose partition output (i.e. number and composition of genetic clusters) with the lowest asap-score and/or best-fitting threshold distance (see Puillandre *et al.*, 2021).

For both methods, we analysed COI and ITS2 gene datasets both separately and in a concatenated supermatrix. For COI, analyses were first performed on the full dataset (382 sequences), and subsequently on a subset of 42 sequences, representing the COI -clusters. The same subset was used for the ITS2 and combined COI/ITS2 analyses. For naming of MOTUs, we followed nomenclature guidelines proposed by Morard *et al.*, 2016. MOTU-assignments and GeneBank-accession numbers for generated COI and ITS2 sequences are provided in Table S1.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All field experiments and collections were conducted under permissions granted by the Costa Rican National System of Conservation Areas (SINAC) and the National Commission for the Management of Biodiversity (Conagebio) (permission IDs: INV-ACOSA-036–2015; R-007-2016-OT-CONAGEBIO; SINAC-ACOSA-PI-PC-078-18).

Results

Sampling of frog-biting midges

We collected a total of 2545 *Corethrella* specimens directly from 744 individual frog hosts (17 species). All of the midges were morphologically identified and included in the network analysis, representing 815 midge-frog interactions (see below). Midges were found usually on or near male frogs, and in some cases also on amplectant females (compare Bernal and Pinto 2016) and on males, that were not observed calling. All collected midges were female. Frog hosts that were sampled during or immediately after calling were often infested, with multiple midges (>50 in *Incilius coniferus* and *Leptodactylus savagei*), whereas hosts that had not shown immediate prior calling activity had fewer midges (JV, pers. obs.). Note that on some occasions observed midge infestation on hosts was considerably higher than realised catches, as not all feeding individuals could be collected. Further, certain frog species were difficult to sample due to concealed calling sites or flight-proneness (e.g. *L. savagei*), leaving these species underrepresented in our analyses.

Acoustic traps were highly efficient in catching frog-biting midges. A total of 11 662 trap-caught midges were morphologically identified, representing 502 observed midge-frog call (trap) interactions.

We grouped all midges based on morphological traits visible under a dissecting scope and assigned them to 5 distinct morphotypes. Based on microscopic inspection of slide-mounted specimens, and using additional characters given in Borkent, 2008, two of those morphotypes were identified as the described species *C. ranapunges* and *C. peruviana*, one was tentatively identified as *C. cf. quadrivittata*, one contains the two very similar species *C. amazonica* and *C. ramentum* (which can be distinguished based on a more detailed microscopic investigation), and one could not be assigned to published species and is called *Corethrella sp. LG1*, (LG1 = La Gamba 1). Morphological bipartite network analyses were performed based on these five morphotypes.

Abundance distributions of collected midges varied greatly. For midges collected directly from frog hosts, *Corethrella ranapungens* was most abundant, representing 63% of interactions, followed by *Corethrella peruviana* (27%), *Corethrella amazonica/ Corethrella ramentum* (6%), *Corethrella* sp. LG1 (3%), and *Corethrella* cf. *quadrivittata* (1%). In acoustic traps, *C. ranapungens* was most abundant, representing 49% of interactions, followed by *C. amazonica/ C. ramentum* (30%) and *C. peruviana* (14%). The more rarely collected *Corethrella* sp. LG1 and *C.* cf. *quadrivittata* represented 4% and 3% of interactions, respectively.

Bipartite interaction networks (morphological species ID)

For midges collected directly from frog hosts, the overall degree of network specialisation was $H2' = 0.3$, indicating low to moderate network specificity (Fig. 2a). $H2'$ was significantly higher than expected from null models ($P = 0.02$). Individual degrees of specialisation ranged from $d'm = 0.13–0.48$ and $d'f = 0–0.26$ for midges and frogs, respectively (for summary of network statistics, see Tables 1 + 2). For *Corethrella*, the number of realised links varied from 2 (*C.*cf. *quadrivittata*, 10 interactions) to 16 (*C. ranapungens*, 503 interactions) with a mean of 7.6 links. Realised links for the host side ranged from 1 (4 frog species, 1–8 interactions) to 5 (*Dendropsophus ebraccatus*, 142 interactions), with a mean of 2.1 links per host. Connectance for this network was 0.53.

The overall degree of specialisation for the trap-based network (Fig. 2b) was low ($H2' = 0.08$), showing no deviation from null models ($P = 0.38$). Individual degrees of specialisation ranged from $d'm = 0.03–0.33$ and $d'f = 0.01–0.18$ for midges and frogs, respectively. For *Corethrella*, the number of realised links varied from 4 (*C.* cf. *quadrivittata*, 13 interactions) to 12 (*C. ranapungens*, 244 interactions) with a mean of 8.4 links. Realised links for frog hosts ranged from 1 (*Dendropsophus microcephalus*, 5 interactions) to 5 (*Smilisca phaeota*, 103 interactions), with a mean of 3.5 links per host. Connectance for this network was 0.7.

Molecular genetic species delimitation

To assess levels of cryptic diversity, we sequenced 382 representative specimens from all five *Corethrella* morphotypes. Mitochondrial COI barcoding revealed 17 distinct haplotype clusters (Fig. 3a), supported by high Bayesian posterior probabilities (>0.95), and a distinct barcoding gap, based on K2P-distances (intraspecific: <0.1–2.3%; interspecific: 7.9–31.6%; for ASAP-output histograms see Fig. S1a). Three clusters were represented only by singleton midges, whereas the largest cluster contained 146 specimens. Cryptic diversity was found in all 5 morphotypes, however, to a different extent. *C. peruviana* and *C. cf. quadrivittata* both formed monophyletic clades with their respective haplotypes (2 each). For the other morphotypes phylogeny was not fully resolved on higher levels, showing polytomies and indicating paraphyletic morphotype-relationships. The morphotype *C. amazonica/ C. ramentum* showed the highest level of cryptic diversity, branching into seven distinct clusters. Besides the cluster that contained the reference specimens identified by A. Borkent (*C. amazonica* and *C. ramentum*) we found five additional clusters labelled as '*C.amazonica/C.ramentum* 1–5' represented by 1–31 specimens. For the most abundant morphotype *C. ranapungens*, COI delimitation resulted in three distinct clusters, of which the most abundant one (146 specimens) included the reference specimen of *C. ranapungens*. The two additional clusters were labelled as '*C. ranapungens* 1' (30 specimens)

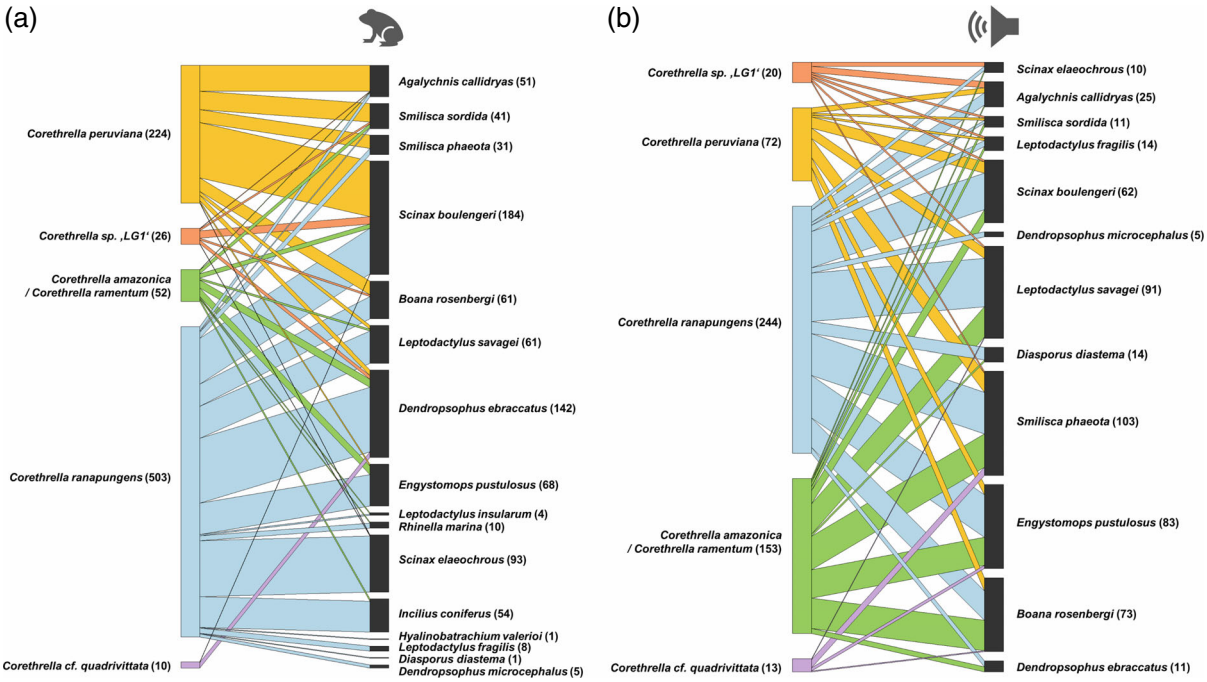

Fig. 2. Quantitative bipartite interaction networks of frog-biting midges (*Corethrella* spp.) and frog hosts in La Gamba, Costa Rica. (a) Midges collected directly from frogs, (b) midges attracted to acoustic traps broadcasting frog advertisement calls. The presence of a particular *Corethrella* species found on an individual host/ in a given trap was counted as one interaction, regardless of the number of individuals. Box/line width indicates interaction frequency; numbers of total per-species interactions in brackets. Networks were generated based on morphological species categorisation; sequence of species with minimised crossing of lines.

and '*C. ranapungens* 2' (singleton specimen). *C. peruviana* (102 specimens) was split into an additional cluster, labelled as '*C. peruviana* 1'. The more rarely collected morphotype *C. cf. quadrivittata* and the yet unidentified *Corethrella* sp. LG1, formed two distinct clusters each, including 1–15 specimens. As both were not morphologically referenced, we labelled them as '*C. cf. quadrivittata* 1 and 2', and '*Corethrella* sp. LG1 1 and 2'.

Subsequently, nuclear ITS2 sequence data were used to verify the COI-clustering results. For this, we analysed a subsample of 42 specimens, representing the 17 COI clusters. Species delimitation for both markers separately produced mostly congruent results with regard to terminal clusters (see Fig. S2). However, basal branching patterns and branch lengths differed, and the number of total clusters increased to 19 for the ITS2-tree (three additional splits, one lump). K2P-distance for the clusters was between <0.1–2.3% (COI) and <0.1–1.2% (ITS2), with an interspecific diversity of 7.9–31.9% and 9.3–80.4%, respectively. Although polytomies were fewer in the ITS2-subtree, overall branch support (posterior output) for this tree was considerably lower than for the COI tree (see Fig. S2).

Information from both genetic markers was integrated in a concatenated COI/ITS2 tree (Fig. 3b). Here, cluster composition was mostly congruent with the outgroup-rooted COI tree (full dataset). Three additional (low-level) splits occurred in *C. ranapungens* 1, *C. amazonica*/*C. ramentum* 1, and *Corethrella* sp. LG1 1 – resulting in a total of 20 clusters for the tree-based delimitation, without introducing paraphylies. For this clustering result, K2P-diversity was <0.1–2.4% (intraspecific) and 3.5–32.5% (interspecific; for ASAP-output histograms see Fig. S1b). Following a hierarchical naming procedure (Morard *et al.*, 2016), the additional clusters were labelled as e.g. *C. ranapungens* 1a/1b.

Tree topology was in part congruent with morphological species delimitation and confirmed the monophyletic origin of *C. peruviana*. Evolutionary history was less straight forward in the other morphotypes, indicating possible polyphyletic origins of *C. ranapungens* and C. sp. 'LG 1'. Overall branch support was high, rendering the concatenated subtree as the most reliable representation of *Corethrella* phylogeny for our dataset. Given the overall consensus of COI and concatenated COI + ITS2-clustering results, we defined the broader (more conservative) COI-delimitated clusters as MOTUs, on which we performed the network analysis. The hierarchical naming procedure allows for a future further refinement of MOTUs, if necessary.

Impact of cryptic midge diversity on network structure

To allow direct comparison between morphotype-based versus MOTU-based network topologies, we first constructed a morphotype-based subnetwork from the initial direct-sampling-network (Fig. 2a) including only the 382 midge-frog interactions for which we also had the midge COI haplotypes. Overall, network topology was approximately maintained following subsampling (Fig. 4a), resulting in only

Table 1. Summary of bipartite network statistics for *Corethrella*/frog interactions.

	N interactions	N hosts	H2′	d′
Traps	**502**	**12**	**0.08**	**0.16**
C. quadrivittata	13	4		0.14
C. sp. LG1	20	7		0.33
C. peruviana	72	8		0.05
C. amazonica/C. ramentum	153	11		0.05
C. ranapungens	244	12		0.25
Direct-sampling	**815**	**16**	**0.3**	**0.26**
C. quadrivittata	10	2		0.29
C. sp. LG1	26	6		0.13
C. peruviana	224	9		0.43
C. amazonica/C. ramentum	52	8		0.19
C. ranapungens	503	16		0.25
Direct-sampling: Subnetwork	**382**	**14**	**0.29**	**0.29**
C. quadrivittata	7	1		0.34
C. sp. LG1	18	5		0.26
C. peruviana	105	8		0.42
C. amazonica/C. ramentum	64	9		0.22
C. ranapungens	188	13		0.2
Direct-sampling: Subnetwork MOTUs (COI)	**382**	**14**	**0.42**	**0.35**
C. cf. quadrivittata 1	1	1		0
C. cf. quadrivittata 2	6	1		0.32
C. sp., LG1′ 1	3	1		0.2
C. sp., LG1′ 2	15	5		0.32
C. peruviana	102	8		0.44
C. peruviana 1	3	1		0.2
C. amazonica	10	4		0.36
C. ramentum	9	2		0.46
C. amazonica/C. ramentum 1	10	2		0.42
C. amazonica/C. ramentum 2	31	3		0.46
C. amazonica/C. ramentum 3	1	1		0.75
C. amazonica/C. ramentum 4	1	1		0.55
C. amazonica/C. ramentum 5	2	1		0.45
C. ranapungens	146	13		0.38
C. ranapungens 1	30	6		0.3
C. ranapungens 2	1	1		0
C. ranapungens 3	11	3		0.28

Corethrella-side. (For frog-side, see Table 2; bold values: N interactions/N hosts: total; H2′/d′: mean).

slight deviation in overall network specificity (H2′ = 0.29/0.3) and connectance (0.51/0.53), compared to the original network. Note that the more rarely collected *Corethrella* morphotypes were proportionally overrepresented in the subnetwork, to allow for a more comprehensive investigation of cryptic diversity within these groups. Also, species-level degrees of specialisation d′ were slightly altered by the subsampling process, with an increased median specificity of 0.01/0.07 on the midge and frog-side, respectively (see Tables 1 + 2).

Bipartite network analysis based on the COI species delimitation (Fig. 3a), produced a more diversified network with an increased resolution on the midge side (Fig. 4b), and an increased network-wide degree of specialisation of H2 = 0.42. For both midges and frogs, individual degrees of specificity (d′) were overall higher than for the morpho-based networks, and ranged from d′(midge) = 0–0.75 and d′(frog) = 0–0.63 (Tables 1 + 2). Note that the d′ data-range was broader, indicating both generalists and specialists within the midge-frog community (Fig. 5). For midges, the number of realised links varied from 1 (9 MOTUs, 1–6 interactions) to 13 (*C. ranapungens*, 146 interactions) with a mean of 3.1 links. Realised links for the host side ranged from 1 (*D. microcephalus*, single interaction) to 11 (*D. ebraccatus*, 79 interactions), with a mean of 3.9 links per host. Connectance for this network was 0.22. (For a network-wide comparison of network statistics, also see Fig. 5.)

Discussion

Our study provides a first comprehensive analysis of host interactions for a neotropical community of frog-biting midges, based on both morphological and molecular genetic species delimitation. Through extensive collection of midges from frog hosts and acoustic trap experiments, we assessed levels of specificity within this fascinating antagonistic network. With five morphologically distinct *Corethrella* morphotypes our research locality, La Gamba, has similar morphospecies diversity of Corethrellidae compared to other tropical forest

Table 2. Summary of bipartite network statistics for *Corethrella*/frog interactions.

	N interactions				N *Corethrella* spp.				d′			
	T (mor)	DS (mor)	DS-sub (mor)	DS-sub (MOTU)	T (mor)	DS (mor)	DS-sub (mor)	DS-sub (MOTU)	T (mor)	DS (mor)	DS-sub (mor)	DS-sub (MOTU)
Agalychnis callidryas	25	51	27	27	4	4	4	5	0.13	0.24	0.18	0.21
Boana rosenbergi	73	61	38	38	4	3	3	3	0.04	0.04	0.17	0.23
Dendropsophus ebraccatus	11	142	79	79	2	5	5	11	0.05	0.11	0.12	0.63
Dendropsophus microcephalus	5	5	1	1	1	1	1	1	0.14	0.07	0	0
Diasporus diastema	14	1				1			0.1	0		
Engystomops pustulosus	83	68	45	45	4	3	2	4	0.03	0.12	0.2	0.42
Hyalinobatrachium valerioi		1				1				0		
Incilius coniferus		54	37	37		2	2	3		0.14	0.2	0.3
Leptodactylus fragilis	14	8	8	8	4	1	1	1	0.02	0.09	0.17	0.2
Leptodactylus insularum		4	2	2			1	2		0.15	0.05	0.62
Leptodactylus sovogei	91	61	20	20	5	3	3	4	0.01	0.06	0.02	0.19
Rhinella marina		10	7	7		2	2	3		0.09	0.1	0.27
Scinax boulengeri	62	184	58	58	4	4	4	6	0.03	0.09	0.05	0.13
Scinax elaeochrous	10	93	18	18	3	2	2	3	0.18	0.19	0.17	0.17
Smilisca phaeota	103	31	22	22	5	2	2	4	0.03	0.12	0.19	0.21
Smilisca sordida	11	41	20	20	4	4	3	3	0.04	0.26	0.26	0.39
	502 (Total)	815	382	382	3.6 (Mean)	2.5	2.5	3.8	0.07 (Mean)	0.11	0.13	0.28

Frog-side. (For *Corethrella*-side, see Table 1).

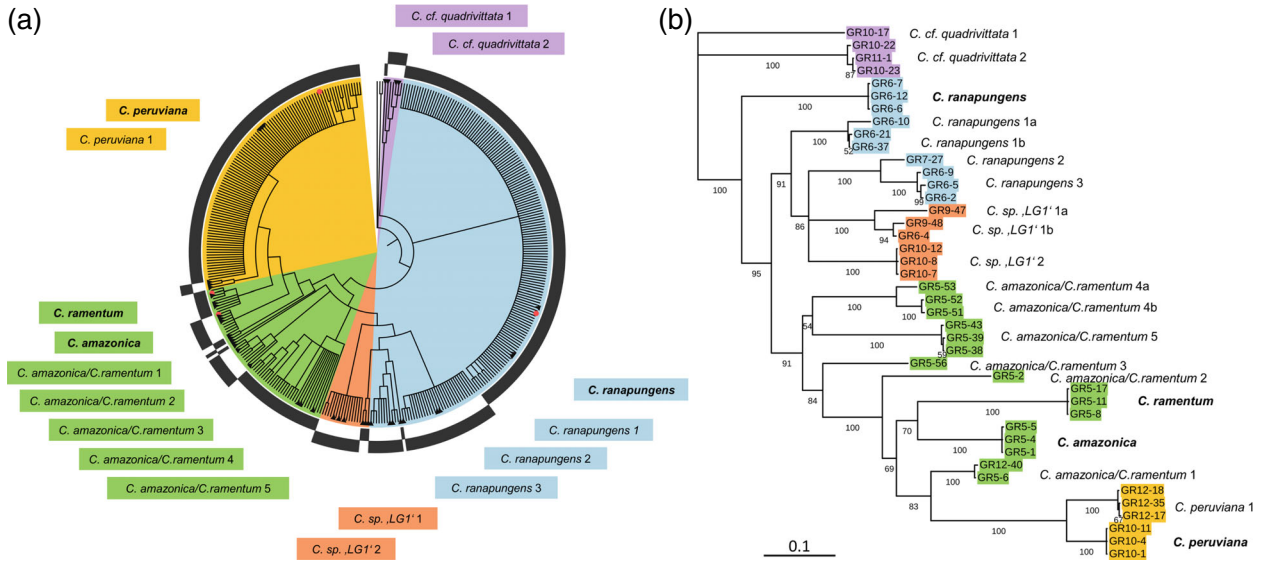

Fig. 3. Phylogenetic reconstruction of frog-biting midges (*Corethrella* spp.) from La Gamba, Costa Rica. Bayesian phylogeny was inferred based on a representative subset of 382 midges collected directly from frog hosts (interaction network Fig. 2a); clade colours represent morphological species categorisation. Identification of novel MOTUs was based on K2P-divergence. MOTUs containing morphological reference specimens in bold. (a) Cladogram view of COI haplotypes inferred from the full dataset. Midge specimens were divided into 17 distinct MOTUs (black outer bars). Red dots indicate morphological reference specimens (Id: A. Borkent), black triangles indicate specimens used for the subsampling-network (b). (b) Tree view of concatenated COI/ITS2 supermatrix, performed on a subset of 42 *Corethrella* specimens. Integration of the ITS2-marker resulted in mostly concurrent tree topology and MOTU-clustering, with three additional splits – resulting in a total number of 20 MOTUs. Branches are supported by overall high posterior output values. (Voucher specimen Id, Genbank Accession Numbers, and metadata information for MOTUs provided in Table S1. Nomenclature MOTUs: 'Genus species 1a' = Morphospecies + Arabic numeral referring to COI species delimitation, followed by a letter indicating further subsplits derived from COI/ITS2 concatenated analysis.)

sites, e.g. in Panama (eight species, Legett *et al.*, 2018) and Brunei (4–7, Grafe *et al.*, 2019). It should be noted that, while some *Corethrella* morphospecies are widespread (see below), studies across Costa Rica showed high ß-diversity in *Corethrella* communities even on small geographic scales (Borkent, 2008), suggesting a regional mosaic of midge/frog interactions. In comparison with other sites, La Gamba appears to have above average abundance of frog-biting midges all year round, with hundreds of individuals congregating on individual calling frogs, and sometimes more than a thousand midges in 5-min

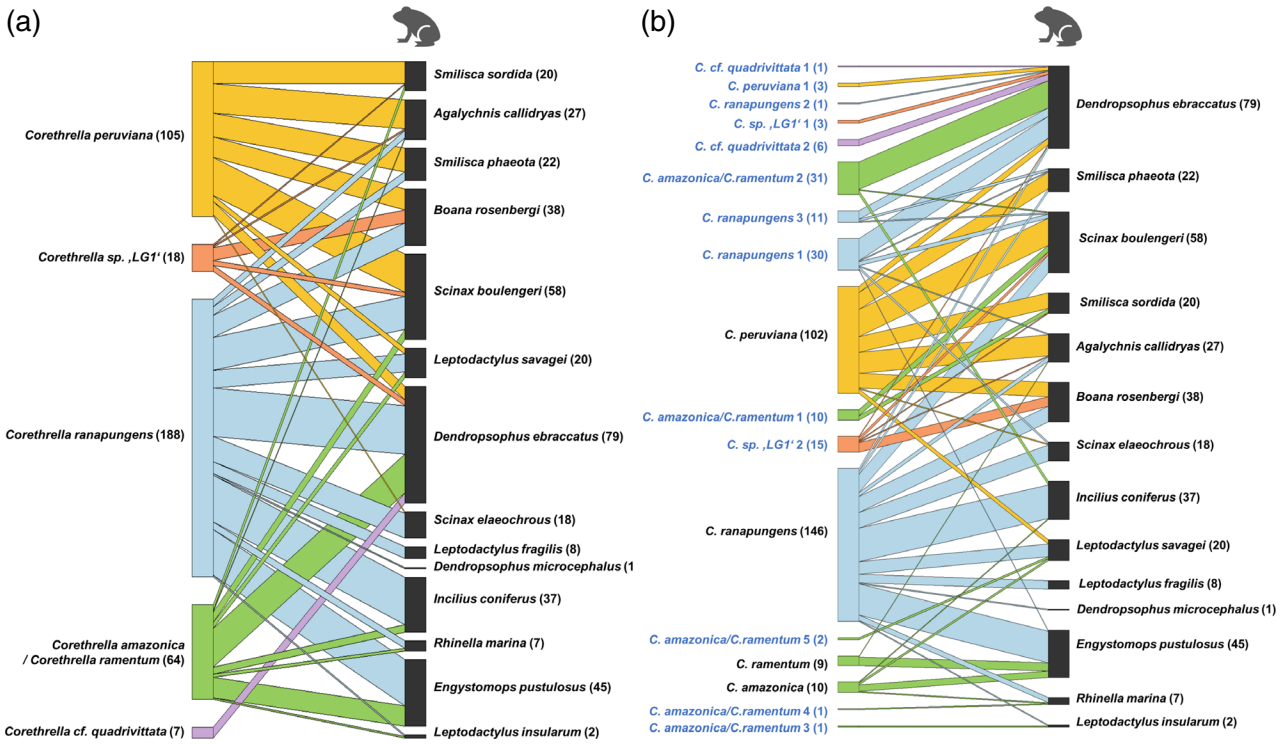

Fig. 4. (a) Subset of morphotype-based quantitative bipartite interaction network of *Corethrella* – frog associations from Fig. 2a. (b) Network of same subset based on molecular genetic species delimitation (COI sequence data). Code of name assignments as in Fig. 3a; novel MOTUs in blue. Clade colours represent morphological species categorisation; box/line width indicates interaction frequency; numbers of total per-species interactions in brackets; sequence of species with minimised crossing of lines.

acoustic trap catches. Climate and habitat structure might be especially favourable at the site, with high precipitation levels (~6000 mm p.a.; compare Weissenhofer *et al.*, 2008) and a variety of natural and artificial perennial waterbodies, providing year-round access to breeding sites and frog hosts. High catch numbers are mostly based on the exceptional abundance of *C. ranapungens*, which represented >78% and > 94% of collected individuals in La Gamba, for direct-sampling and acoustic traps, respectively (see also Virgo *et al.*, 2019). This species has a large geographic range occurring from southern Mexico to Brazil, and was regularly encountered among the most abundant species in acoustic trap experiments across Costa Rica and Panama (Borkent, 2008; Legett *et al.*, 2018). The regional dominance of *C. ranapungens* might be mediated by its generalist feeding behaviour (see below) and/or other (underexplored) life-history traits. With regard to blood resources, it should be noted that one of its preferred hosts, *L. savagei*, is very common in the La Gamba clearings and along forest edges (Virgo *et al.*, 2019). Concerning the breeding niche preliminary data suggest that larvae of *C. ranapungens* develop in a broad range of aquatic habitats, including phytotelmata (*Calathea lutea*, inflorescence; JV, pers. obs.), small ponds (JV, pers. obs.), and even stream margins (Borkent, 2008). However, as life-history data for *Corethrella* spp. is generally sparse, the micro- and macroecological rules determining their abundance and distribution remain largely unknown. Also note that this 'species' has been found to contain substantial cryptic diversity (this paper; see below), limiting the validity of morphology-based observations.

The remaining morphotypes were found in substantially lower numbers, both in direct-sampling and acoustic trap experiments. Note that interaction frequencies illustrated in our bipartite networks do not accurately reflect variation in abundances, and that interaction frequencies are much smaller than the total number of individual midges collected.

Cryptic midge diversity of La Gamba

Molecular species delimitation showed substantial cryptic diversity in all of our morphotypes, increasing putative local *Corethrella* species diversity by a factor of 3–4, with 17 or 20 putative species, depending on the genetic marker and delimitation algorithm (i.e. barcoding-gap threshold). For COI we used a conservative barcoding gap of >2.3%, similar to cut-off values used for closely related mosquitoes (Tahir *et al.*, 2016a,b) and a broad range of other Dipteran families (compare Morinière *et al.*, 2019). MOTUs based on ITS2 were largely congruent with those based on the more comprehensive COI analysis and provided additional resolution within some morphotypes. Consequently, we suggest that a concatenated supertree (COI + ITS2) at this time provides the most reliable hypothesis of *Corethrella* phylogeny for the La Gamba community. In general, molecular analysis did not

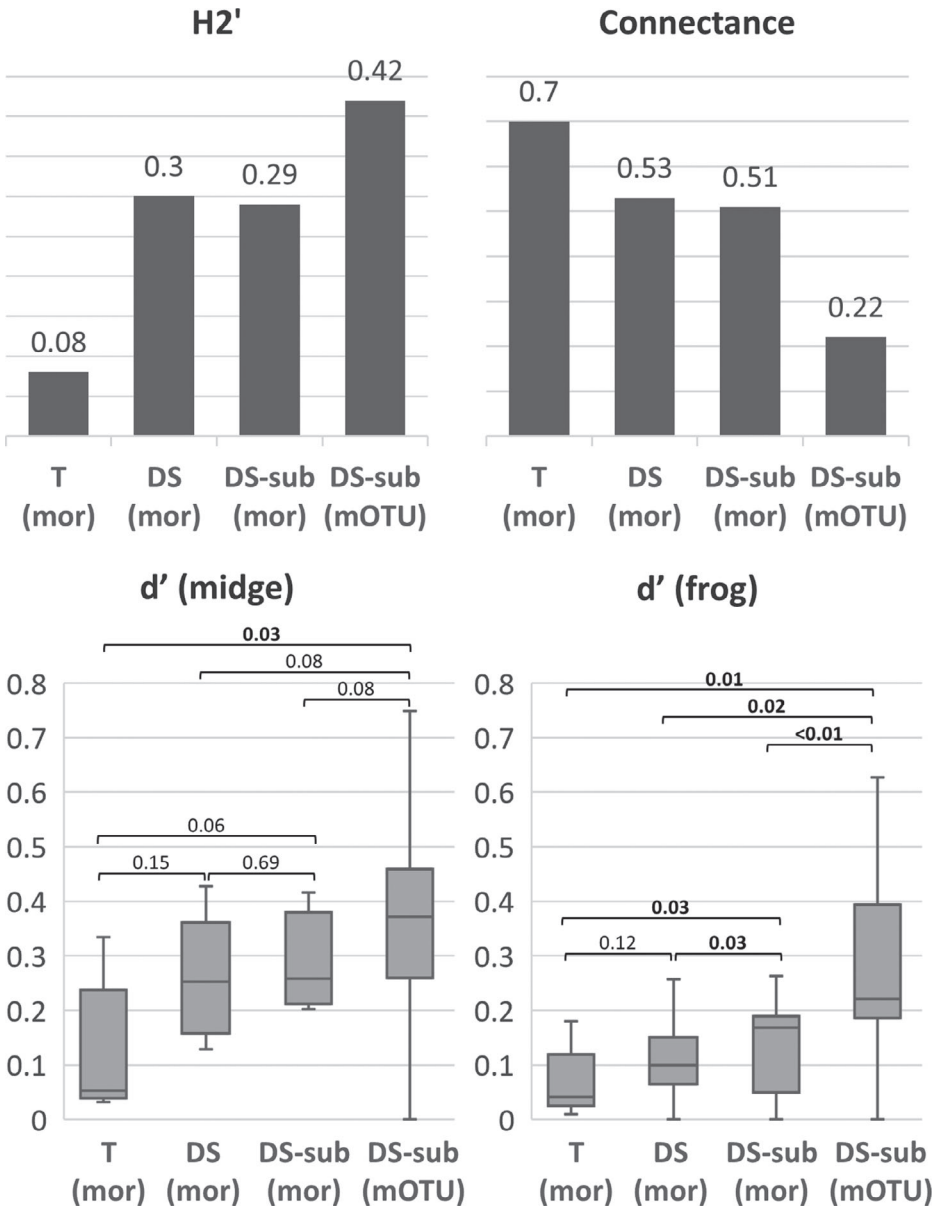

Fig. 5. Values of network metrics for the presented quantitative bipartite interaction networks of frog-biting midges (*Corethrella* spp.) and frog hosts (Figs 2 and 4). T: Trap-based network, DS: Direct sample-network; sub: Subnetwork; (mor): based on morphological species identification; (mOTU): based on molecular genetic species delimitation. For species-level specialisation indices d′, *P*-values for pairwise comparisons (Mann–Whitney-U/Wilcoxon nonparametric tests) are shown. (For MWU/Wilcoxon test statistics, see Table S1).

contradict morphological species categorisation overall but mostly increased within-morphotype species diversity.

Host specificity

Our data indicate that frog-biting midges do partition the available frog host resources, but the degree of partitioning that was evident depended on the method/depth of analysis. First, when samples of midge morphotypes were considered that were collected with acoustic traps, the degree of host specificity was very low to absent (mean morphotype d′ = 0.16). This is in agreement with previous studies suggesting that auditory tuning of morphotyped *Corethrella* to frog calls is quite broad. E.g., in a previous analysis of acoustic trap catches in La Gamba we had found that all *Corethrella* morphotypes were attracted to all broadcast frog calls (Virgo *et al.*, 2019). Significant quantitative differences in preferences among morphotypes were only found using individual-based analyses across very large sample sizes (Virgo *et al.*, 2019). Second, when midges were considered that were collected directly from frogs using aspirators (direct-sampling) midge host associations were

clearly more specialised (mean morphotype $d' = 0.26$) This suggests that host specificity in *Corethrella* is either based on acoustic cues not transmitted by our acoustic traps, or, more likely, it requires additional nonacoustic cues (further discussion see below). Finally, host specificity was found to be highest when we considered midges collected directly from frog hosts and used DNA-based species delimitation (mean MOTU $d' = 0.35$). Hereby, overall levels of specificity were higher on the midge side, with a considerable proportion of specialised links. Frog hosts, in turn, were parasitised only by a subset of the available *Corethrella* species, suggesting that frogs have evolved mechanisms to avoid exploitation by certain midge species (compare Grafe *et al.*, 2019). Observed structural differences in mouthparts of *Corethrella* spp. appear to reflect differences in host type or/and feeding site (compare Borkent, 2008; de Silva *et al.*, 2014), indicating that midge species are not functionally identical. Generally, our analysis demonstrates that substantial specificity in midge-frog interactions is hidden by the difficulty of distinguishing frog-biting midges by morphological characters alone.

In general, more specialist MOTUs were found on frog species with high midge species richness, whereas more generalist midges were also found on frog species infested by fewer *Corethrella* species. This form of specialisation-asymmetry has been reported for *Corethrella* (Grafe *et al.*, 2019) and by other studies investigating parasite–host-interactions (e.g. Vázquez *et al.*, 2005). For the more abundant *Corethrella* MOTUs we can differentiate between 'generalists', e.g. *C. ranapungens*, exploiting many host resources in similar proportions, oligophagous (weak) specialists such as *C. peruviana* that were almost exclusively (97%) found on treefrogs of the family Hylidae, and near monophagous (strong) specialists, e.g. *C.amazonica/ C. ramentum* 2, showing strong preferences for a single frog species. Cluster-specific interactions were not linked to sampling years (compare metadata presented in Table S1), rendering seasonal shifts in genotype-abundances as a main cause for observed specificity patterns unlikely.

We only have limited information on the factors mediating midge specificity as well as the relevant cues eliciting host choice (see below). Midge specificity may evolve in response to certain host properties, such as the body size (compare Virgo *et al.*, 2019) and life-history traits (e.g. longevity, phenology, dispersal; also see Caira, 1994). Preliminary data indicate, that realised on-host feeding sites are both midge and frog-specific (also see de Silva *et al.*, 2014), potentially corresponding to differences in frog calling behaviour and defence reactions (Virgo et al. in prep.). For ectoparasites/micropredators with high dispersal capability, higher specificity is likely related to adaptive constraints (see Dick & Patterson, 2007). Realised specificity can also be largely determined by the presence and abundance of suitable hosts (Poulin, 2011a) and their encounter probability (Combes, 1991), governed by spatiotemporal dynamics (e.g. Krasnov *et al.*, 2004; Bodawatta *et al.*, 2020).

Frog species-assemblages show strong variation across habitat types, climate/elevational gradients, and between seasons (e.g. Santos-Pereira *et al.*, 2011; Khatiwada *et al.*, 2019; Libke, 2019). As yet, there are no comprehensive studies on frog-biting midge phenology (but see Legett *et al.*, 2018), but a synchronous occurrence of host and parasite/predator may also reflect a high degree of specialisation. In La Gamba, *Corethrella cf. quadrivittata* was almost exclusively (9 out of 10 individuals) collected from the treefrog *D. ebraccatus*. Although this specialisation was not reflected by acoustic trap data, all trap catches of *C. cf. quadrivittata* coincided with the peak calling period of *D. ebraccatus* at the beginning of the rainy season (J. Virgo, unpublished data).

Further, only little is known about dispersal capabilities in *Corethrella* and how these enable colonisation or geographic host switching. Host specificity in frog-biting midges, therefore, has to be investigated as a continuous variable governed by both micro- and macroevolutionary processes. A deeper understanding of *Corethrella* life history, species distributions and host associations, as well as a more comprehensive phylogeny are mandatory for further exploring *Corethrella*-frog coevolution.

Cues used in host finding

There remains little doubt that frog-biting midges rely on acoustic cues for locating hosts from a distance. Frog-biting midges can be attracted to appropriate acoustic stimuli in large numbers and within surprisingly short time intervals (minutes, sometimes seconds) (JV pers. obs.). The exact distance from which they can be attracted remains uncertain (Borkent, 2008), but judging from the numbers that arrive it is clearly in the range of metres rather than centimetres (compare Bartlett-Healy et al., 2008; Feugère et al., 2020; Menda et al., 2019). It is also evident that certain acoustic properties, e.g. the frequency range of the sound and a pulsed sound structure, are necessary to enable midge attraction (Meuche *et al.*, 2016; Virgo *et al.*, 2019). However, as the specificity of host associations was clearly increased when we considered midges collected directly from frogs vs. midges collected by acoustic trapping, it seems likely that additional cues are needed for close-range host recognition. Unfortunately, the nature of potential additional cues remains unknown. Bernal and Silva (2015) pursued the very plausible hypothesis of carbon-dioxide-based host attraction, but found that added CO2 did neither increase the attractiveness of active sound traps broadcasting frog calls, nor did CO2 alone attract any *Corethrella* when it was dissipated from silent traps (Bernal & Silva, 2015). They concluded that CO2 has no role in host attraction. However, the possibility remains that low concentrations of CO2 might mediate host recognition upon very close contact. The fact that frog-biting midges often congregate around the nasal openings (Bernal *et al.*, 2006; de Silva *et al.*, 2014) appears to support this possibility. Other olfactory, or gustatory, cues could also be involved. Bernal and Silva (2015) speculated that skin peptides might be recognition cues, and certain skin secretions could also have a repellent effect (Williams *et al.*, 2006). Both of these possibilities remain to be explored in future experiments.

The use of additional cues is congruent with the behaviour of host-seeking midges. Based on our observations, midges mostly do not land directly on the sound source but in a small radius

of <20 cm near the frog, or loudspeaker (JV, pers. obs.). On some occasions, midges landed directly on the host and walked directly to a particular feeding site (e.g. the hindlegs in *S. boulengeri*, or the nostrils in *Scinax elaeochrous*; also compare de Silva *et al.*, 2014). These anecdotal observations could also suggest the integration of visual cues, as reported for foraging mosquitoes (e.g. Van Breugel *et al.*, 2015; Vinauger *et al.*, 2019), even under low-light conditions (Hawkes & Gibson, 2016; also see Warrant, 2017). Finally, it is also possible that, at close distance, i.e. after having landed in close proximity (in near field) to the frog, the midges are able to scrutinise other acoustic properties of frog calls than during long-range (airborne) phonotaxis. This could explain the higher specificity of midges attracted to true frogs vs. acoustic traps. Such acoustic parameters may even be perceived with a different sensory structure than those responsible for long-range attraction. However, the organs and mechanisms of sound perception in *Corethrella* are still not identified.

Conclusion and outlook

Despite an overall generalist acoustic foraging behaviour, *Corethrella* spp. partitioned frog host resources at La Gamba, Costa Rica, and our findings support the presence of both generalist and specialist midge species. The use of molecular barcoding markers has been instrumental for disentangling the realised food web specificity. It increased the local richness of *Corethrella* by a factor of 3–4 and produced better resolution in bipartite network analyses. Unfortunately, the proximate recognition cues used for host discrimination remain unknown.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supporting information

Table S1. Supporting information

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