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Off-target capture data, endosymbiont genes and morphology reveal a relict lineage that is sister to all other singing cicadas

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Phylogenetic asymmetry is common throughout the tree of life and results from contrasting patterns of speciation and extinction in the paired descendant lineages of ancestral nodes. On the depauperate side of a node, we find extant 'relict' taxa that sit atop long, unbranched lineages. Here, we show that a tiny, pale green, inconspicuous and poorly known cicada in the genus *Derotettix*, endemic to degraded salt-plain habitats in arid regions of central Argentina, is a relict lineage that is sister to all other modern cicadas. Nuclear and mitochondrial phylogenies of cicadas inferred from probe-based genomic hybrid capture data of both target and non-target loci and a morphological cladogram support this hypothesis. We strengthen this conclusion with genomic data from one of the cicada nutritional bacterial endosymbionts, *Sulcia*, an ancient and obligate endosymbiont of the larger plant-sucking bugs (Auchenorrhyncha) and an important source of maternally inherited phylogenetic data. We establish **Derotettiginae subfam. nov.** as a new, monogeneric, fifth cicada subfamily, and compile existing and new data on the distribution, ecology and diet of *Derotettix*. Our consideration of the palaeoenvironmental literature and host-plant phylogenetics allows us to predict what might have led to the relict status of *Derotettix* over 100 Myr of habitat change in South America.

ADDITIONAL KEYWORDS: Amaranthaceae – anchored hybrid enrichment –Argentina – Derotettiginae – *Derotettix* – hybrid capture bycatch – palaeobiology – phylogenomics – *Sulcia* – South America.

INTRODUCTION

Phylogenetic tree asymmetry is a phenomenon that has captivated evolutionary biologists for a number of reasons. Some biologists focus on the expectation of asymmetrical trees in phylogenetic tree construction (Raup *et al.*, 1973; Farris, 1976; Kirkpatrick & Slatkin,

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1993; Mooers & Heard, 1997; Blum & François, 2006); others search for 'key innovations' that might have resulted in descendant lineages that differ dramatically in species richness (Sanderson & Donoghue, 1994; Ree, 2005; Rabosky *et al.*, 2007; Nicholson *et al.*, 2014; Simões *et al.*, 2016; Branstetter *et al.*, 2017), and still others focus on the long unbranched lineages, or relict taxa [e.g. horseshoe crabs (Lamsdell, 2013, 2016), coelacanths (Takezaki & Nishihara, 2016), tuataras (Jones *et al.*, 2009), ginkgoes (Wang *et al.*, 2017) and spotted wren babblers (Alström *et al.*, 2014)]. Long branches in asymmetrical phylogenies can result from trivial but common causes, such as lack of taxon sampling (Hedtke *et al.*, 2006), or from evolutionary processes, such as extinction of species (Crisp & Cook, 2005), different rates of cladogenesis in different lineages (e.g. Ellis & Oakley, 2016; Janicke *et al.*, 2018), long periods of time when cladogenesis does not happen (Vaux *et al.*, 2016) or combinations of the above. By studying depauperate lineages, we may learn as much about the ability of species to adapt to changing climates and landscapes as by studying more species-rich lineages (Rabosky, 2017).

Cases of phylogenetically asymmetrical living diversity are found in many well-studied taxonomic groups; for example, caecilians (212 species), which split from the remaining extant amphibians > 350 Mya and make up only 3% of current amphibian diversity (Amphibiaweb, 2019; Roelants *et al.*, 2007). Among insects, ancient depauperate relict lineages are also common, e.g. the myxophagan and archostematan beetles (McKenna *et al.*, 2015), myerslopiid leaf hoppers (Hamilton, 1999; Dietrich *et al.*, 2017) and Gondwanan moss bugs (Coleorrhyncha; Yoshizawa *et al.*, 2017). Examples from cicadas include the two extant species of hairy cicadas (Tettigarctidae) that are sister to ~3000 living species of singing cicadas (Cicadidae) (Moulds, 2018; Kaulfuss & Moulds, 2015) and, in the cicada subfamily Cicadettinae, the now depauperate tribe Pictilini (four known species), which at ~60 Mya split from the tribe Cicadettini (500 species; Marshall *et al.*, 2016). Asymmetry is not restricted to deep time. A more recent example is two single species of New Zealand shade-singing cicada lineages in the genus *Kikihia* (Cicadettinae), which ~6–7 Mya split successively from the remaining 28 *Kikihia* species and subspecies (Marshall *et al.*, 2008, 2011; Ellis *et al.*, 2015; Banker *et al.*, 2017). The same asymmetry can be found in hundreds of other clades of animals.

THE STUDY ORGANISM

Cicadas (Box 1) belong to the superfamily Cicadoidea that, along with the spittle bug superfamily Cercopoidea, make up the sap-feeding bug infraorder Cicadomorpha in the suborder Auchenorrhyncha (large plant-sucking bugs) of the order Hemiptera. The age of Auchenorrhyncha can be traced by fossils to 250 Mya and by molecular dating to > 300 Mya (Misof *et al.*, 2014; Johnson *et al.*, 2018). Cicadoidea comprise two families: the largely extinct hairy cicadas (Tettigarctidae, one extant genus) and the modern, singing cicadas (Cicadidae, ~450 genera) (Marshall *et al.*, 2018). Fossils of hairy cicadas are rare in the Cenozoic geological record but relatively abundant in the Mesozoic and date back to 200 Mya (Shcherbakov, 2009; Moulds, 2018; Lambkin, 2019). The fossil record of the family Cicadidae places modern cicadas with some doubt in the Cretaceous (~99 Mya) and with certainty in the Palaeocene (~59.2–56 Mya; Moulds, 2018).

Cicadas have been studied taxonomically since the time of Linnaeus, and their subfamily structure has been a subject of continuous debate, having gone through at least seven substantial revisions (Fig. 1) since Distant's (1906) original classification scheme. Many of the early classification schemes were based on convergent characters associated with sound-producing structures, including covered or uncovered ribbed timbal membranes, resonating chambers of various morphologies, stridulatory organs and wings designed for snapping. Marshall *et al.* (2018) produced the first scheme based on both molecular and morphological phylogenetic data, but despite their sampling 46 of the 53 tribes, there are still gaps in our understanding of the evolutionary history of cicadas.

Here, we broaden the taxonomic and environmental scope of our worldwide survey of cicadas with the addition of a tiny, pale green, inconspicuous cicada, *Derotettix mendosensis* Berg, 1882, which we found living in degraded salt-plain habitats in the 'Monte de Llanuras y Mesettas' (plateaus/plains) and the 'Dry Chaco' regions of central Argentina (Fig. 2) (Pometti *et al.*, 2012). A recent mitochondrial phylogeny of > 100 members of the family Cicadidae (Łukasik *et al.*, 2019) suggested that *Derotettix* (represented by two living Argentine species) might be the only surviving genus in a lineage that is the sister group to all other subfamilies in the family Cicadidae. By exploiting reduced representation genome sequencing, we were able to generate nuclear, mitochondrial and symbiont phylogenies to test this hypothesis. Along with a morphological phylogeny, also presented here, these data support and strengthen this sister-group relationship and support the monophy of all other subfamilies *sensu* Marshall *et al.* (2018). We create a new, fifth, cicada subfamily, Derotettiginae subfam. nov., which we propose split from the rest of Cicadidae ~100–60 Mya in the transition between the Mesozoic and Cenozoic eras. We discuss the factors that might have led to the relict status of *Derotettix* over 100 Myr of habitat change in South America.

BOX 1.

Cicadas occupy a broad range of habitats and are distributed on all continents except Antarctica (Marshall *et al.*, 2018). Cicadas are unique among non-diapausing Hemiptera in having a typical life cycle (egg to adult) that, with few exceptions, spans 3 years or more (Table S3 in Campbell *et al.* [2015]). Life cycles longer than 1 year allow the development of synchronized episodic life cycles (Duffels, 1988; Heliövaara *et al.*, 1994; Lehmann-Ziebarth *et al.*, 2005; Hajong & Yaakop, 2013; Sota *et al.*, 2013; Chatfield-Taylor & Cole, 2017; Cooley *et al.*, 2018). Despite the difficulty of captive rearing, cicadas offer useful study systems by virtue of their acoustic sexual signals, ease of collection and widespread distribution. Songs of cicadas are highly species specific and facilitate rapid gathering of distributional data and identification of cryptic species (e.g. Marshall & Cooley, 2000; Puissant & Sueur, 2001; Marshall *et al.*, 2011; Hertach *et al.*, 2016). Short-lived adults are known for low dispersal rates (e.g. Duffels, 1988; de Boer & Duffels, 1996; Duffels & Turner, 2002) and high levels of phylogeographical structure within species (e.g. Hill *et al.*, 2009; Marshall *et al.*, 2009; Ellis *et al.*, 2015; Hertach *et al.*, 2016; Liu *et al.*, 2018). Although dispersal rates are generally low, over the span of tens of millions of years, occasional long-distance dispersal has resulted in colonization of distant islands and continents worldwide (Arensburger *et al.*, 2004; Marshall *et al.*, 2016). Low dispersal also enhances the utility of geological events as meaningful calibrations for molecular clocks (e.g. Buckley & Simon, 2007; Marshall *et al.*, 2016; Owen *et al.*, 2017). In addition, drought and cold tolerance (e.g. Toolson, 1987; Sanborn *et al.*, 1995) has equipped cicadas to persist through challenging environmental shifts (Buckley & Simon, 2007; Marshall *et al.*, 2009, 2012; Owen *et al.*, 2017). An aspect of cicada biology that has recently attracted attention is their interaction with heritable nutritional endosymbiotic microorganisms, which provides independent insights into the phylogenetic relationships among the cicada hosts and also offers a unique window into the genomic evolutionary processes related to symbiosis (Van Leuven *et al.*, 2014; Campbell *et al.*, 2015, 2017; Łukasik *et al.*, 2018; Matsuura *et al.*, 2018).

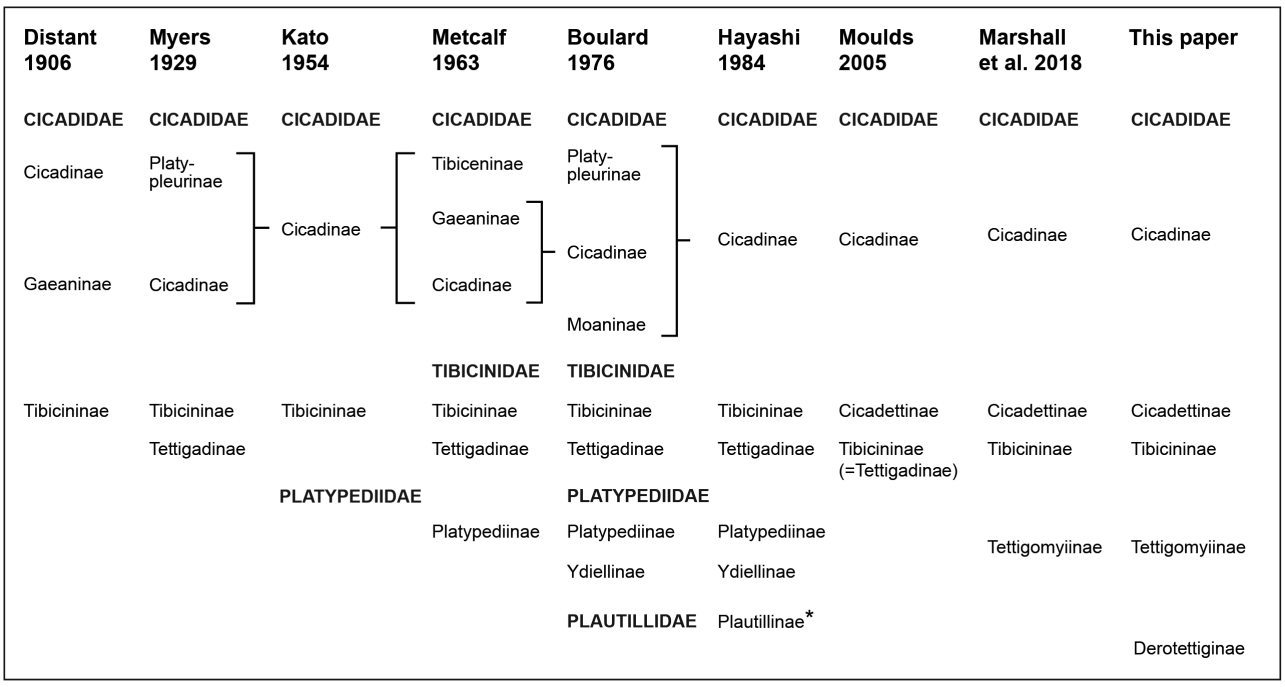

Figure 1. Historical shifts in the number of families and subfamilies in Cicadidae classification, excluding Tettigarctidae (updated from Moulds, 2005; Goemans 2016; Marshall *et al.*, 2018). **Plautilla* (Plautillinae) is strongly supported as a member of the Cicadinae (Goemans, 2016; Marshall *et al.*, 2018).

Figure 2. Provinces and ecoregions of Argentina redrawn from Pometti *et al.* (2012), with *Derotettix* localities coloured by collection year and collectors (see key). Those new to the present study are detailed in Table 1. Sanborn & Heath's (2014 records include data from five personal expeditions and examination of 11 major relevant museum collections, including three major museums in Argentina and the National History Museum, London. Two additional localities are taken from Torres (1945) but are not exact because he lists only the names of cities or villages. All records are *Derotettix mendosensis* unless noted as *Derotettix wagneri* in the key.

MATERIAL AND METHODS

Taxon sampling, song recording and analysis

From 7 to 23 December 2015, we surveyed *Derotettix* localities and documented individual cicadas with photographs (14 individuals photographed from San Juan, Rio Negro and Neuquén provinces, Argentina; Fig. 2; Table 1; Supporting Information, Figs S1–S5). We recorded songs from two populations (See Box 2). From 6 to 10 January 2018, we collected *D. mendosensis* specimens from two localities in Mendoza province, Argentina (Fig. 2; Table 1; Supporting Information, Figs S6–S8) by locating individual males from their songs and capturing them in nets or by hand. We captured females opportunistically, often near males. Specimens collected in 2018 were preserved in 95% ethanol or RNAlater and stored on wet ice for 2 weeks before laboratory storage at –20 °C. We deposited vouchers of specimens collected in 2018 in the collections of M. S. Moulds (Kuranda, Queensland, Australia) and the Department of Ecology and Evolutionary Biology, University of Connecticut Biodiversity Collections (Storrs, CT, USA). In total, we collected and exported ten *Derotettix* specimens (eight males and two females) from Mendoza province, Argentina. We took data for other cicada species used in our phylogenetic studies from Marshall *et al.* (2018) and from bycatch from an anchored hybrid enrichment study of the family Cicadidae, in progress (see 'Sample preparation, sequencing and data handling', below).

During the 2018 field season, we recorded male calling songs in the field using a digital linear pulse code modulation recorder (model PCM-D50; Sony Corp.) with an integral condenser microphone pair. Set at bit depth 16, a 96 kHz sampling rate and a low cut-off frequency of 75 Hz, this equipment recorded a frequency range of 75 Hz to 40 kHz. In the 2015 field season, we recorded songs with a device (model H4n; Zoom Corp.) set to similar specifications.

We analysed recordings in Audacity v.2.1.0 (available at www.audacity.sourceforge.net/) and visualized them with RavenLite v.2.0 (available at www.ravensoundsoftware.com). Before analysis, we used a high-pass filter set to a 1 kHz cut-off frequency and 6 dB roll-off to remove wind and other ambient noises that were not already reduced by the low cut-off frequency setting used during recording. For each recording, we calculated pulse rate manually from a 0.5 s oscillogram window. We measured the peak frequency (i.e. frequency at maximal amplitude) from three randomly selected pulses with a fast Fourier transformation (Hanning window, size 512). We performed statistical analysis using R v.3.5.2 (R Core Team, 2018).

Sample preparation, sequencing and data handling

We extracted DNA from muscle tissue of two legs of a *D. mendosensis* specimen (specimen code 18.AR.MZ.EVT.01) with a QIAGEN DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA, USA), augmenting the included protocol with an overnight incubation at 56 °C as described by Marshall *et al.* (2018). We conducted PCR with EmeraldAmp GT PCR Master Mix (Takara, Shiga Japan) for the genes *cox1*, *cox2*, EF1a and the 18S rRNA using the primers and annealing temperatures described by Marshall *et al.* (2018). The PCR products were electrophoresed on a 1% agarose gel, and excess nucleotides and primers were digested with ExoSAP-IT (USB Corp., Cleveland, OH, USA) before submission to Eurofins Genomics (Louisville, KY, USA) for Sanger sequencing

Table 1. *Derotettix mendosensis* localities new to this paper (mapped in Fig. 2).

Sample label	Date	Site name	Latitude	Longitude	Elevation (m)
18.AR.MZ.CLG	10 January 2018	Calle Lugones*	-33.513802	-69.065634	910
18.AR.MZ.EVT	6 January 2018	East of Villa Tulimaya†	-32.726388	-68.564623	600
PL767	29 December 2015	NW de Rincón de Los Sauces‡	-37.26473333	-69.07856667	664
PL769	29 December 2015	NW de Rincón de Los Sauces‡	-37.26473333	-69.07856667	664
PL754	28 December 2015	Sgto. Vidal§	-38.65226667	-68.13903333	322
PL755	28 December 2015	Sgto. Vidal§	-38.65226667	-68.13903333	322
PL756	28 December 2015	Sgto. Vidal§	-38.65226667	-68.13903333	322
PL757	28 December 2015	Sgto. Vidal§	-38.65226667	-68.13903333	322
PL758	28 December 2015	Sgto. Vidal§	-38.65226667	-68.13903333	322
PL954 (song)	23 December 2015	Ruta de Pomonaǁ	-39.665793	-65.482162	265
PL955	23 December 2015	Ruta de Pomonaǁ	-39.665793	-65.482162	265
PL752	22 December 2015	Ruta a Choele Choel¶	-39.08821667	-66.38621667	355
PL753	22 December /2015	Ruta a Choele Choel¶	-39.08821667	-66.38621667	355
PL618 (song)	7 December 2015	San Juan ruta 141**	-31.55763333	-67.43775	554
PL623	7 December 2015	San Juan ruta 141**	-31.55763333	-67.43775	554
PL624	7 December 2015	San Juan ruta 141**	-31.55763333	-67.43775	554

*Just off of Ruta Provincial 96, Mendoza, AR, corner of Calle Lugones.
†Highway 34, East of Villa Tulumaya, Mendoza, AR.
‡Side of Ruta Provincial l 6, 23 km NW from Rincón de Los Sauces, Neuquén, AR.
§Side of Ruta Nacional 151, 2 km north of Sgto. Vidal, Río Negro, AR.
ǁSide of Ruta Nacional 250, 33 km south of the village of Pomona, Río Negro, AR.
¶Side of Ruta Nacional 22 between Choele Choel & Gral Roca, Río Negro, AR.
**Road 141 between Bermejo and Marayes, San Juan, AR.

with forward and reverse primers. We visualized, quality trimmed and assembled chromatographs and confirmed accurate protein translation for relevant loci in Geneious (Biomatters Ltd, Auckland, New Zealand). We removed intronic sequences from EF1a.

For anchored hybrid enrichment, we dissected bacteriomes under a stereomicroscope from specimens preserved in ethanol and stored at −20 °C. We removed the abdominal sternites from each specimen and excised all the bacteriomes found (minus as much excess cicada tissue as possible) and placed them directly in lysis buffer for DNA extraction. We performed separate DNA extractions of bacteriomes and muscle tissue from one leg for each cicada using the QIAGEN DNeasy Blood & Tissue kit. We used the manufacturer's instructions but added an overnight incubation at 56 °C and removed RNA with RNase A (QIAGEN). We assessed the quality and quantity of DNA using the Qubit fluorometer v.2.0 (Invitrogen, Carlsbad, CA, USA) and agarose gel electrophoresis.

We trialled three different pooling methods and demonstrated that we could sequence endosymbiont (extracted from dissected cicada bacteriome tissue) and cicada DNA simultaneously using a mixture of 0.1% cicada bacteriome DNA and 99.9% cicada leg DNA. Average coverage was 146× for cicada anchored hybrid enrichment loci and 864× for endosymbiont loci. High coverage is needed to compensate for the high variance in cicada-to-endosymbiont DNA ratio (owing to variation in the size of bacteriomes among samples).

We prepared Illumina libraries from DNA extracts at the Center for Anchored Phylogenomics (www.anchoredphylogeny.com), following Lemmon *et al.*, (2012) and Prum *et al.* (2015). More specifically, we sonicated DNA using a Covaris ultrasonicator to a fragment size of 175–325 bp. We then used a Beckman-Coulter FXp liquid handling robot to add universal Illumina adapters with 8 bp indexes. After pooling, we enriched libraries using the anchored hybrid enrichment approach (Lemmon *et al.*, 2012). The targets for enrichment were developed for Paraneoptera by Dietrich *et al.* (2017), who produced a probe set containing probes representing cicadas, among other lineages. This target set was derived from 941 core loci that were determined previously to be orthologous across Diptera (Young *et al.*, 2016), Holometabola (Niehuis *et al.*, 2012), Arthropoda (Misof *et al.*, 2014) or Neuropteroidea (McKenna & Farrell, 2010; Beutel & McKenna, 2016). Dietrich *et al.* (2017) scanned for these core loci in 17 genomes and 46 transcriptomes of Paraneoptera. After the sequences obtained were aligned and filtered for taxon presence, we designed probes from 514 target loci (total target size = 151 944 bp), including ten genes (*dnaE*, *dnaK*, *fusA*, *groL*, *mnmA*, *prfA*, *rpoA*, *rpoB*, *rpoC* and *tufA*) of the obligate heritable cicada endosymbiont *Candidatus Sulcia muelleri* (hereafter, *Sulcia*) to assess whether the phylogeny of this bacterium mirrored that of the host genome. Agilent Technologies produced the probe kit, which included 55 700 probes. We captured a total of 515 anchored cicada loci, which ranged from 342

to 969 bp in length. The anchored loci themselves will be used in a future publication that includes increased taxon sampling.

To achieve the most complete possible phylogenetic dataset incorporating global cicada diversity, we supplemented existing Sanger-sequenced data for the 28S, 18S, *EF1a*, *ARD1*, *cox1* and *cox2* genes from Marshall *et al.* (2018) by mining the capture assemblies of the same or closely related individuals for loci that were previously missing and were likely to have been a part of capture bycatch owing to a high genomic copy number (18S rRNA, *cox1* and *cox2*). In addition, we supplemented this dataset with the 28S rRNA, which, although off target, was also frequently recoverable in capture assemblies.

We deduplicated both merged and unpaired reads from the capture library sequencing using clumpify in the BBMap suite (Bushnell, 2014) and trimmed them of TruSeq adaptor and low-quality (Quality Score < 20) sequences with Trimmomatic (Bolger *et al.*, 2014). We assembled the resulting trimmed reads using SPAdes v.3.12.0 (Nurk *et al.*, 2013). For host genes, we queried capture assemblies with blastn (18S and 28S rRNA) or tblastn (*cox1* and *cox2*) using *Magicicada* references on GenBank (MG953107.1 and KM000130.1) or the 28S rRNA of an unidentified cicada (JQ309936.1) as the query sequence. We aligned matching contigs back to the query sequence in Geneious v.10.1.3 and stitched them together if they consisted of two or more contigs. We processed captured loci for *Sulcia* and the 28S rRNA in a similar manner, then implemented additional processing using iterative read mapping with MIRA v.4.0.2 (Chevreux *et al.*, 1999). We then used MITObim v.1.9.1 (Hahn *et al.*, 2013), an additional read mapper that produced slightly better results, on the MIRA-corrected bait sequences, which we edited by manual trimming of apparent misassembled or duplicated segments. We aligned *Sulcia* and host 28S rRNA loci using the MAFFT v.7 E-INS-i algorithm (Katoh *et al.*, 2017), and we trimmed alignments of apparently misassembled or duplicated segments further. We constructed individual unpartitioned *Sulcia* gene trees using RAxML v.8 (Stamatakis, 2014) on the CIPRES web server (Miller *et al.*, 2010) to check and remove sequence data that showed evidence of cross-contamination based on similarity to sequence data from distantly related taxa.

The methods used to acquire and assemble the metagenome of a *D. mendosensis* specimen, PL623x1, are described by Łukasik *et al.* (2018, 2019). Briefly, we extracted DNA from the dissected bacteriome after fragmenting it using Covaris, prepped following a modified protocol by Meyer & Kircher (2010) and sequenced on the Illumina HiSeq 4000 platform. We assembled the reads using Spades v.3.7.1. We obtained the cicada *ARD1* and 28S rRNA sequence in addition to all *Sulcia* loci for *Derotettix* by querying this assembly with blastn or by using a query sequence from a relative as a seed for MITObim on the trimmed paired end reads.

Phylogenetic analysis

We aligned cicada and *Sulcia* loci using the MAFFT v.7 E-INS-i algorithm (Katoh *et al.*, 2017) and inspected and trimmed them in Geneious v.10.1.3 based on amino acid translations for protein-encoding loci. We used SequenceMatrix (Vaidya *et al.*, 2011) to concatenate loci. We created partitioning schemes based on codon positions of each protein-encoding gene and separate partitions for the two rRNA genes and the 5′ untranslated region of *ARD1* and analysed them using PartitionFinder v.1.0.1 (Lanfear *et al.*, 2012) with the greedy search algorithm and the best combination of possible partitions chosen by the Bayesian information criterion. We generated a maximum likelihood tree using this partitioning scheme with RAxML v.8 (Stamatakis, 2014) on the CIPRES web server (Miller *et al.*, 2010), with 1000 rapid bootstrap replicates. We visualized trees in FigTree v.1.4.0 (Rambaut & Drummond, 2012) and edited with GGTREE (Yu *et al.*, 2017).

Morphological cladistic analysis

For the morphological cladistic analysis, we used the same 117-character dataset as Moulds (2005). We scored *D. mendosensis* for these characters and added them to the dataset along with scores for *Tettigomyia vespiformis* Amyot & Serville, 1843 to ensure that all subfamilies were represented. See Moulds (2005) for a full description of these characters and character states. We analysed the data using the heuristic search parsimony algorithms in PAUP* v.4.0b2 (Swofford, 1998). We used the tree bisection–reconnection algorithm for tree searches and conducted 1000 random additional searches starting from random trees; we left other settings at their default values. We weighted all characters equally and treated all multistate characters as unordered. We found the most resolved trees by filtering the set of shortest trees using the Filter Trees option. We prepared the chosen tree using CLADOS v.1.2 (Nixon, 1992) with DELTRAN optimization. We dissected male genitalia needed for study and illustration from relaxed adults by cutting the intersegmental membrane holding the pygofer (often also along with sternite 8); we then cleared the genitalia in 10% KOH at room temperature for ~4–8 h, with the length of time depending on the degree of sclerotization of the genitalia. After removing the genitalia from the KOH, we washed them thoroughly in water. Using a stereomicroscope, with the genitalia submerged in a Petri dish of water, we removed excess intersegmental membrane from the pygofer hind-margin and then removed any internal undissolved muscle tissue (dark

matter). When closer examination of the aedeagus was required, we separated it from the pygofer by cutting the translucent membrane surrounding the theca and pulling the aedeagus backwards.

Descriptions of new subfamily and new tribe

The terminology for morphological features follows that of Moulds (2005, 2012). The relevance of characters in defining higher taxa follows the cladistic analysis of Moulds (2005). A discussion of the song, endosymbionts, ecology and biogeography of *Derotettix* is included in the formal description in the Results and Discussion (Box 2).

RESULTS AND DISCUSSION

Exploitation of bycatch in sequence capture data enables integration with existing datasets

The data acquired via sequence capture experiments typically allow for robust phylogenomic analyses based on hundreds of preselected loci (Bi *et al.*, 2013; Blaimer *et al.*, 2016; McCormack *et al.*, 2016). The success of enrichment of these selected loci varies depending on the phylogenetic distance of sampled species to those for which probes are designed (Bragg *et al.*, 2016; Kieran *et al.*, 2019) and many other factors, such as the amount and integrity of target DNA in individual samples, probe tiling depth and whether probes are synthesized as RNA or DNA (Gasc *et al.*, 2016). For studies in which successful captures of species within clades dating to 100–200 Mya have been performed, the ranges of on-target reads have been reported to be anywhere from ~10 to ~60% on average across the entire dataset, with rates of only up to 80% on-target reads for species from which the probes were designed (Schott *et al.*, 2017; Knyshov *et al.*, 2019). Owing to the imperfect nature of hybridization of targeted DNA, additional loci may also be recoverable from naïve assemblies of reads from capture experiments given adequate sequencing depth. In particular, we found that high-copy number genes, including those on the mitochondrion and those found as part of the rRNA operon, were frequently recoverable from non-target reads. Given that these multi-copy genes happen to be ones that were first selected as commonly used phylogenetic markers because they allowed relatively easy PCR amplification, we can integrate newly sampled taxa meaningfully with datasets collected previously that encompass much wider sampling. Such non-target bycatch data have begun to be exploited for systematic studies only in recent times (Guo *et al.*, 2012; Gasc *et al.*, 2016; Lyra *et al.*, 2017; Barrow *et al.*, 2017; Caparroz *et al.*, 2018; Matsuura *et al.*, 2018; Taucce *et al.*, 2018; Percy *et al.*, 2018; Łukasik *et al.*, 2019), but continued use of this valuable, albeit hidden, resource will help to resolve the tree of life by allowing more complete sampling in phylogenies.

Note that we were particularly successful at obtaining these data from the bycatch because of the relatively low enrichment efficiency of anchored hybrid enrichment loci (between 1 and 6% of reads map to target anchored hybrid enrichment loci), which is attributable to the large size of the cicada genome (Hanrahan & Johnston, 2011) and its diversity. Mitochondrial DNA genomes can be more difficult to obtain from bycatch for systems in which the size of the genome is small and/or the probes are designed for taxa with less variation (i.e. when > 50% of reads map to target loci). The complete mitochondrial genome of *D. mendosensis* (minus the control region) was sequenced as genomic bycatch from exon capture (Łukasik *et al.*, 2019; GenBank no. MG737807.1). Nuclear and mitochondrial metadata and gene segments used in the present study (28S, 18S, Ef1a and ARD1; *cox1* and *cox2*) can be found at GenBank numbers MN241535-MN241813). *Sulcia* gene sequences can be found at GenBank numbers MN219733-MN219984. Details of each Genbank submission by species and gene are given in Tables S1 and S2.

Multifaceted evidence for a monogeneric subfamily

Before our work, there were four Cicadidae subfamilies. Three of these, Cicadettinae (worldwide), Cicadinae (worldwide) and Tettigomyiinae (Africa + Madagascar), appear to have split from each other close together in time (Marshall *et al.*, 2018). The fourth subfamily (Tibicininae) is the sister group to the other three, as shown in our trees (Figs 3–5; Supporting Information, Figs S9–S11) and by Marshall *et al.* (2018). Our results demonstrate that four datasets [nuclear gene (Supporting Information, Fig. S9), mitochondrial genome (Łukasik *et al.*, 2019), *Sulcia* endosymbiont genes (Fig. 4) and morphological data (Fig. 5)] all strongly support the hypothesis that the genus *Derotettix* is sister to all these subfamilies. *Derotettix* is also strongly supported as sister to the rest of Cicadidae in our nuclear plus mitochondrial DNA phylogeny (Fig. 3), in the phylogeny built with 28S data alone (Supporting Information, Fig. S10) and in a tree made with all genetic data combined (Supporting Information, Fig. S11). All trees also strongly support Tibicininae as sister to the remaining three subfamilies: Tettigomyiinae, Cicadettinae and Cicadinae. Maximum likelihood bootstrap support is strong (98–100%) for the monophyly of each subfamily except Cicadinae, for which bootstrap support varied from unresolved in 28S alone to 69–89% in the other dataset combinations.

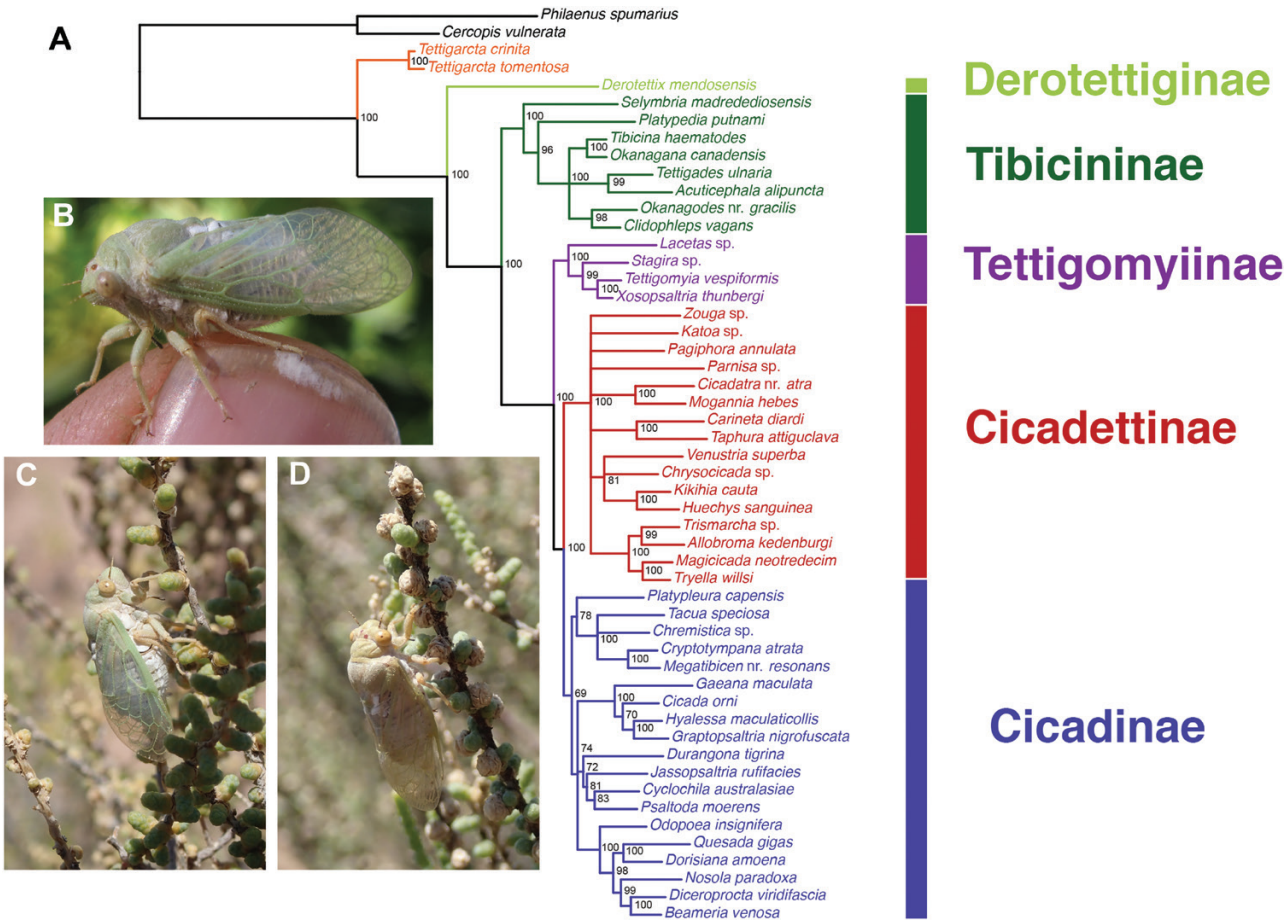

Figure 3. A, RAxML phylogeny, RNA + codon partitioned, nuclear (28S, 18S, *EF1a* and *ARD1*) plus mitochondrial DNA data. Of 8819 total characters, 2123 are parsimony informative; for the ingroup only, 1787 characters are parsimony informative. B, *Derotettix mendosensis* (PL954), Ruta de Pomona, Provincia Rio Negro, Argentina with fingertip for scale (photograph: P.Ł.). C, D, *D. mendosensis* green and yellow colour morphs, respectively, both from site 18.AR.MZ.EVT, on *Heterostachys* (see Table 1) (photographs: J.A.C.).

The nuclear gene tree (Supporting Information, Fig. S9) includes four gene segments: *EF1a*, *ARD1*, 28S and 18S. The 28S gene was not included in the study by Marshall *et al.* (2018) but proved to be informative in our phylogeny at the deeper nodes (Supporting Information, Fig. S10). This locus, consisting of a total of 4622 sites (629 informative within Cicadidae), is able to resolve the relevant relationships of *Derotettix* with respect to the rest of cicadas and the relationship of the Tibicinae as sister to the remaining three subfamilies with 100% bootstrap support but loses resolution shallower in the tree (Supporting Information, Fig. S10). Nuclear anchored hybrid enrichment genomic data analyses (C. Owen, D. Marshall, E. J. Wade, R. C. Meister, G. Goemans, K. B. R. Hill, A. R. Lemmon, E. M. Lemmon, M. Kortyna, M. S. Moulds, V. Sarkar, K. Marathe, K. Kunte, C. Simon, unpublished observations) are predicted to strengthen support for the monophyly of the subfamily Cicadinae and to resolve shallower nodes in the tree.

Auchenorrhyncha were ancestrally associated with one or more obligate bacterial endosymbionts that produced essential amino acids and vitamins and were transmitted faithfully through the female reproductive system to subsequent generations (Moran *et al.*, 2005). One of them, *Sulcia*, has been retained by the majority of Auchenorrhyncha lineages, including all cicadas characterized to date. Unlike the second ancestral endosymbiont of cicadas, *Candidatus Hodgkinia cicadicola* (hereafter, *Hodgkinia*) (McCutcheon *et al.*, 2009a, b), *Sulcia* evolves in a relatively slow manner and is easy to align across Cicadidae (Campbell *et al.*, 2015), making it useful for phylogenetic reconstructions (e.g. Matsuura *et al.*, 2018).Our maximum likelihood phylogeny of ten conserved *Sulcia* genes supports and strengthens the conclusions of the nuclear DNA, mitochondrial DNA and morphological data, but with Cicadinae, Cicadettinae and Tettigomyiinae represented as a trichotomy. The three subfamilies

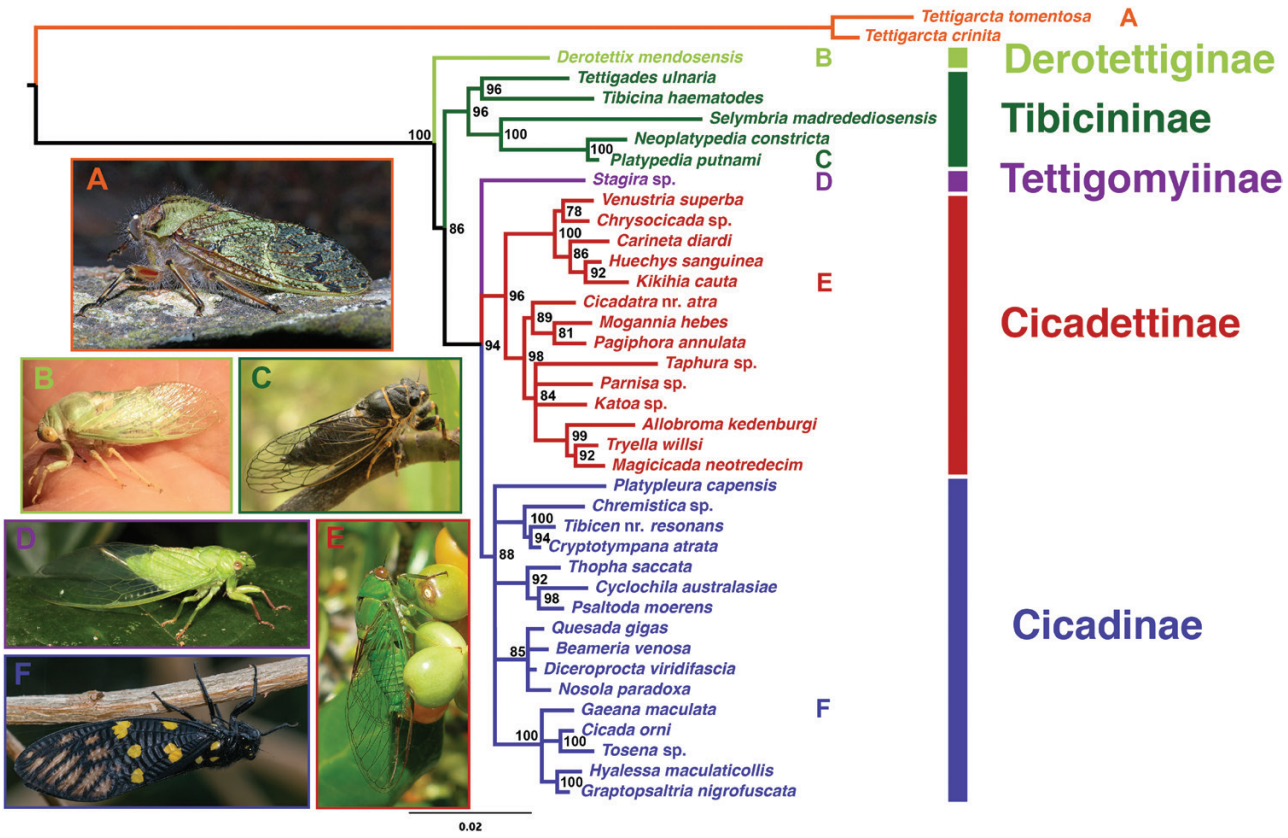

Figure 4. RAxML phylogeny, codon partitioned, *Sulcia* endosymbiont genes (*dnaE*, *dnaK*, *fusA*, *groL*, *mnmA*, *prfA*, *rpoA*, *rpoB*, *rpoC* and *tufA*). Of 22 804 sites, 1400 are parsimony informative, 899 for the ingroup. Inset photograph borders match the colour of subfamily tree branches. A, *Tettigarcta tomentosa* (Tettigarctidae), Tasmania (photograph: Simon Grove, Tasmanian Museum & Art Gallery). B, *Derotettix mendosensis* (**Derotettiginae subfam. nov.**), Argentina (photograph: C.S.). C, *Platypedia* sp. (Tibicininae), Arizona (photograph: David Marshall). D, *Stagira* sp. (Tettigomyiinae), Uganda (photograph: Nick Dean). E, *Kikihia ochrina* (Cicadettinae), New Zealand (photograph: C.S.). F, *Gaeana maculata* (Cicadinae), Hong Kong (photograph: Ray Li).

that were represented by multiple taxa were all clearly monophyletic.

Our morphological tree for 82 taxa (plus one outgroup; Fig. 5) adds *Derotettix* and *Tettigomyia* to the 117-character dataset used by Moulds (2005) (Supporting Information, Table S4) and also supports the hypothesis that *Derotettix* is sister to the other cicada subfamilies and should be placed in a new subfamily, Derotettiginae. Although clade support for Derotettiginae on the morphological tree was not strong, owing to its single non-homoplasious synapomorphy being outweighed by five homoplasious (shared) synapomorphies, the clade support for the remaining subfamilies of Cicadidae to the exclusion of Derotettiginae was strong (82% bootstrap). Despite Derotettiginae having only one non-homoplasious synapomorphy, it does have six shared attributes in unique combination, a situation not unusual for more basal nodes in large morphological analyses of groups with reasonably conservative morphology (e.g. Cicadinae has only one non-homoplasious synapomorphy and five shared attributes in unique combination; see Supporting Information, Table S4).

Hiding in plain sight

Derotettix was described in 1882 but has never been singled out as unusual. Thus, given the long history of work on higher-level cicada taxonomy (Fig. 1), the resolution of *D. mendosensis* as sister to all other species in the family Cicadidae (Łukasik *et al.*, 2019) was unexpected. The tribe Parnisini, to which *Derotettix* had been assigned, includes 23 genera, of which 13 genera are restricted to the Ethiopian biogeographical realm (four of those are endemic to Madagascar); two genera are found in both the Ethiopian and Palaearctic realms; one genus is restricted to the Palaearctic; and

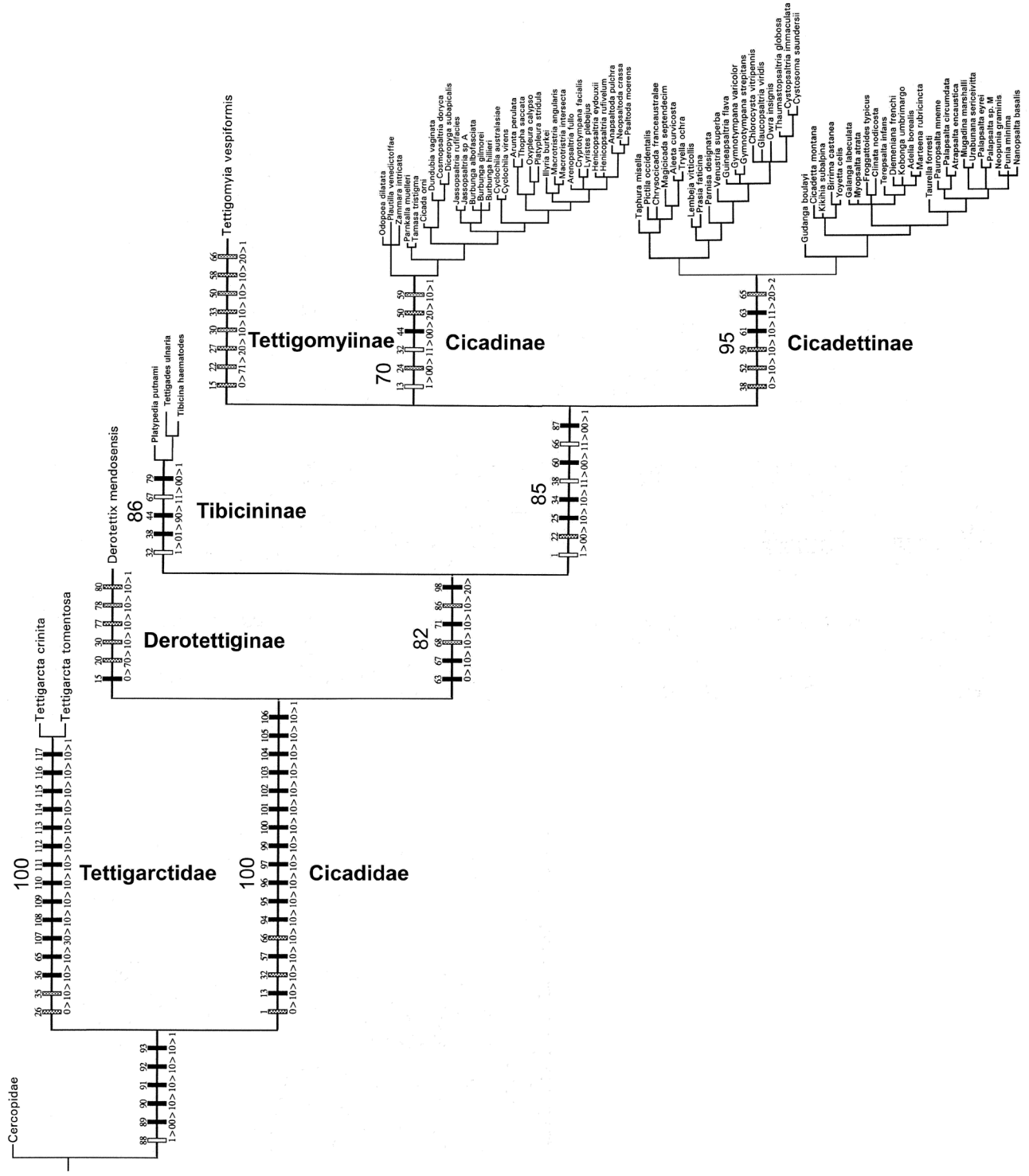

Figure 5. One of 716 most parsimonious trees (length 313, consistency index [CI] 55, rescaled consistency index [RI] 87) derived using the morphological dataset from Moulds (2005), with *Derotettix* and *Tettigomyia* included; hence, all subfamilies are represented. Differences between the 716 trees were confined to terminal taxa within Cicadinae and Cicadettinae, meaning that character transformations for more basal nodes shown on the tree were identical for all trees. Character transformations on branches are represented as follows: black bars, non-homoplasious forward change (unique); grey bars, homoplasious forward change (a shared state); white bars, reversal (whether homoplasious or not). Bootstrap values are shown for branches supporting subfamilies.

BOX 2. TAXONOMIC DESCRIPTION OF **DEROTETTIGINAE SUBFAM. NOV.** AND **DEROTETTIGINI TRIBE NOV.**

Subfamily **Derotettiginae** Moulds **subfam. nov.**

Type genus: Derotettix Berg, 1882 (type species *Derotettix mendosensis* Berg, 1882).

Included tribes: **Derotettigini** Moulds **tribe nov.**

Diagnosis: Metanotum partially exposed at dorsal midline (Supporting Information, Fig. S12). Forewing veins CuP and 1A unfused, adjacent for two-thirds of length but widely diverging in distal third. Hindwing veins RP and M unfused at their bases (Fig. 6). Male opercula rounded, reduced, enclosing tympanal cavity but not meeting. Abdominal timbal cavity lacking timbal covers. Pygofer with distal shoulder undeveloped; pygofer upper lobe absent. Claspers absent. Aedeagus restrained by tubular encapsulation below uncus, with ventrobasal pocket present; basal plate reduced on more than its basal half to form a pair of long, slender lateral arms attached to theca by sinuation (Fig. 7). The unique morphology of the male genitalia might be a feature of this subfamily rather than a feature of the tribe Derotettigini or *Derotettix*. The theca is loosely hinged with the basal plate at the extremities of the lateral projections of both structures, as in *Tettigarcta* (Tettigarctidae). The endotheca enters the somewhat flattened theca beneath a short dorsal overhang at its proximal end. At its distal end, the theca attaches to the thickened membranous vesica in an area weakly membranous, meaning that there is some flexibility between the two. At rest, the nearly straight distal half of the vesica is held within a groove along the ventral surface of the uncus.

Distinguishing features: With the following combination of features: forewing veins CuP and 1A and hindwing veins RP and M unfused (Fig. 6); aedeagus with a ventrobasal pocket present and a basal plate deeply divided basally and attached to the theca by sinuation (compare Fig. 7A–C with F–H).

Distribution: Neotropics: Argentina. Dry Chaco and Monte de Llanuras y Mesettas ecoregions.

Comments: The shape of the basal plate and its attachment to the theca by sinuation are unique among Cicadidae but are features also found in the Tettigarctidae. All other cicadas have the basal plate undivided, as illustrated by *Tibicina* Kolenati, 1857 (Fig. 7I–L), subfamily Tibicininae.

Tribe **Derotettigini** Moulds **tribe nov.**

Type genus: Derotettix Berg, 1882 (type species *Derotettix mendosensis* Berg, 1882).

Included genera: Derotettix Berg, 1882.

Diagnosis: Head including eyes wider than lateral margins of pronotum, but with supra-antennal plates much wider than distance between supra-antennal plate and eye. Postclypeus shape in transverse cross-section rounded; postclypeal ridges lacking transverse grooves towards distal ends. Pronotal collar narrow, with lateral margins confluent with adjoining sclerites and no lateral tooth. Mesonotum lacking auxiliary sound-producing structures. Forewing pterostigma absent; veins C and R+Sc close together; vein RA_1 aligned closely with subcosta (Sc) for its length; vein CuA_1 divided by crossvein so that distal portion is longest. Hindwing with anal lobe broad and vein 3A straight, very long and widely separated from wing margin. Foreleg femoral primary spine small and prostrate, lacking auxiliary spines. Hind-coxae lacking a large inner protuberance. Meracanthus broadly rounded. Male opercula not completely encapsulating meracanthus; completely covering tympanal cavity but not meeting. Male abdominal tergites with sides convex in cross-section; tergite 2 larger than tergites 3–7; epipleurites reflexed to ventral surface, without an inward V-shaped kink. Timbals extended below level of wing bases; timbal cavity with a rounded rim. Pygofer with basal lobe moderately developed; dorsal beak absent. Uncus undivided, not retractable within pygofer. Aedeagus with theca broad, almost flat but concave ventrally, lacking appendages; vesica much longer than theca, not retractable; basally with a small sclerotized plate either side; conjunctival claws and pseudoparameres absent; ventral rib of basal plate ill defined, short, fused with surface of basal plate (Figs 6, 7 A–E; Supporting Information, Figs S12–S17).

Distinguishing features: The **Derotettigini tribe nov.** differs from all other tribes in having, in combination, the foreleg femoral primary spine small and prostrate and no auxiliary spines, a male uncus that is not retractable within the pygofer, and an aedeagus that has a very broad, almost flat theca. Seven (rather than eight) apical cells in the forewing might be unique to this genus and tribe, but we cannot be certain. Likewise,

there are other unusual features of the wings that would normally be considered generic attributes but might not be relevant here at tribal rank, in particular the very elongate basal cell, thickened costal veins (C, Sc + R), the enlarged sixth apical cell in the forewings and the very wide space between hindwing vein 3A and the wing margin. The Supporting Information (Table S3), updated from Marshall *et al.* (2018) to include **Derotettiginae subfam. nov.**, compares distinguishing features of all five subfamilies of cicadas. Figure 1, also updated from Marshall *et al.* (2018), traces the historical shifts in the number of subfamilies of Cicadidae.

Genus *Derotettix* Berg, 1882
Included species: Derotettix mendosensis Berg, 1882; *Derotettix wagneri* Distant, 1905 (Supporting Information, Figs S13–S17).

Ecology: Derotettix mendosensis is found largely in patches of dry, salty soils in the Dry Chaco, Monte de Llanuras y Mesettas ecoregions of Argentina (high plains and plateaus; as defined by Pometti *et al.*, 2012), with one or two specimens located nearby in the Estepa Patagónica and Espinal ecoregions (Fig. 2). The only other species in the genus, *D. wagneri*, is known from several localities in the Dry Chaco of Santiago de Estero province, Argentina (Fig. 2; (Torres, 1945; Sanborn *et al.*, 2004; Sanborn & Heath, 2014). *Derotettix* species are found on plants in the Amaranthaceae (*Allenrolfea* and *Heterostachys*) and Chenopodiaceae (*Atriplex*, salt bush; Supporting Information, Fig S1A) typical of alkaline salty soils. *Heterostachys* is a new host record. These habitats can seemingly be degraded by human activity and still support populations of *Derotettix* (Supporting Information, Figs S2–S4, S6, S8). *Derotettix* are cryptically coloured to match their host plants (Fig. 3 insets; Supporting Information, Figs S1–S8). Other cicadas have been found on related fleshy halophytic plants. For example, in the desert southwest of North America two species of the genus *Okanagodes* are cicadas of a similar colour and found on *Atriplex*, but they belong to the subfamily Tibicininae; another tibicinine cicada, *Babras sonorivox*, is found on *Allenrolfea* in Argentina but is not pale green (Torres, 1945; Sanborn *et al.*, 2004; Sanborn & Heath, 2014). *Derotettix* has one of the highest known thermal tolerances of any cicada species (Sanborn *et al.*, 2004).

Derotettix mendosensis

Calling song: Songs were recorded from two populations in Mendoza province during the 2018 field season ($N = 6$ from site 18.AR.MZ.EVT and $N = 4$ from site 18.AR.MZ.CLG) between 12.30 and 13.30 h at temperatures that ranged from 30.6 to 36.7 °C. Male *Derotettix* produced a monotonous buzz in long bouts (~30–45 s). Males were wary and ceased singing upon disturbance but were reluctant to fly, instead relying on crypsis to avoid detection. After disturbance, calling resumed as intermittent bouts of short duration (~6–8 s). No interpopulation differences were found in pulse rate or peak frequency (Welch's two-sample *t*-tests, $P = 0.643$ and $P = 0.812$, respectively). Two singletons from San Juan and Río Negro provinces recorded during the 2015 field season were compared with the song character distributions estimated from Mendoza province. Single-comparison *t*-tests (Sokal & Rohlf, 1995: pp. 227–228) could not reject the null hypothesis that the song characters of these specimens were drawn from the same distributions. The following descriptive statistics thus include all recordings ($N = 12$ from three Argentine provinces).

Male *D. mendosensis* calling songs have a pulse rate of 210.4 ± 9.3 (range 194.8–224.7) s^{-1} (Supporting Information, Fig. S18a) and a peak frequency of 9.5 ± 0.6 (range 8.6–10.4) kHz (Supporting Information, Fig. S18). Neither pulse rate (generalized linear model, $N = 9$, $P = 0.93$) nor peak frequency ($P = 0.57$) depended on ambient temperature over the range at which our recordings were made. The fact that pulse rate was independent of temperature suggests that males thermoregulate their acoustic behaviour.

Genetic data: See GenBank numbers in Results and Discussion, above.

Endosymbionts: Derotettix mendosensis, like many but not all cicadas (Matsuura *et al.*, 2018), harbours two obligate endosymbionts: *Hodgkinia cicadicola* and *Sulcia muelleri*. The 235 kb genome of *Sulcia* is one of the smaller genomes observed for cicadas, but the family has not been characterized fully. Similar to many other cicadas (Łukasik *et al.*, 2019), the *Hodgkinia* of *Derotettix* comprise cytologically and genetically distinct but complementary lineages: one with the expected genome size of ~144 kb and high coding density; the other at much lower abundance, substantially smaller and not yet assembled fully. Our data suggest that after the split, the resulting *Hodgkinia* lineages degenerated more asymmetrically than in the previously characterized case of the *Hodgkinia* of *Tettigades undata* (Van Leuven *et al.*, 2014), but the mechanisms underlying this asymmetry are unclear.

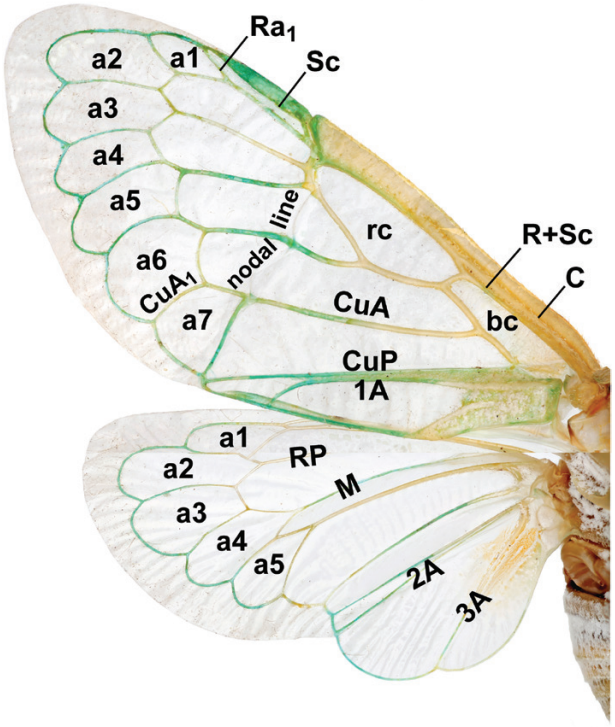

Figure 6. *Derotettix mendosensis* left fore- and hindwing, with veins labelled. Abbreviations: a, apical cells; A, anal; bc, basal cell; C, costa; CuA, cubitus anterior; CuP, cubitus posterior; M, median; R, radius; Ra, radius anterior; rc, radial cell; RP, radius posterior; Sc, subcostal. *Derotettix mendosensis* wing photograph: E.R.L.G. (using Macropod camera, Macroscopic Solutions).

finally, five genera (including *Derotettix*) are found only in the Neotropical realm.

Marshall *et al.* (2018) reviewed the tribes and subfamilies of the family Cicadidae, including four genera previously classified as Parnisini. The Neotropical genera *Parnisa* Stål, 1862 and *Calyria* Stål, 1862 were retained in this tribe, but *Quintilia* Stål, 1866 was moved into the new African subfamily Tettigomyiinae; *Arcystasia* Distant, 1882 was found to belong to Cicadettini in an earlier work (Marshall *et al.*, 2016) but not formally reassigned. Marshall *et al.* (2018) were not able to review the other 19 parnisine genera and noted that the tribe Parnisini needs further revision. Our present study does not include parnisines outside of the Neotropics; therefore, a complete evaluation of the members of this tribe awaits further sampling and future genomic studies. Marshall *et al.* (2018) questioned the make-up of tribes that, like Parnisini, have deep, seemingly global distributions (e.g. Chlorocystini, Cryptotympanini and Taphurini) and removed some taxa; future studies might remove more. However, global tribes do exist. The subfamily Cicadettinae contains two well-sampled tribes with Northern, Southern, Eastern and Western Hemisphere components: Cicadettini (Marshall *et al.*, 2016) and Lamotialnini (Marshall *et al.*, 2018). Parnisini (minus *Derotettix*) might turn out to be another widely distributed cicadettine tribe.

It is not impossible that future taxon sampling will turn up other lineages that branch deep in the cicada tree. Such candidates could come from genera in two poorly characterized tribes that have been found to be polyphyletic, i.e. Parnisini or Taphurini, or from as yet unsampled genera. For example, a new South American genus of cicada, *Gibbocicada* Ruschel, 2018 (Tibicinini, Tibicininae), was described recently from museum material and is the only member of its tribe found in the Southern Hemisphere (Ruschel, 2018).

Long branch, little cicada: palaeoclimatic and landscape changes

We propose that species extinction is a more likely cause for the lack of other species in Derotettiginae than lack of speciation over millions or tens of millions of years. This hypothesis parallels the situation we see in the Tettigarctidae, the sister lineage to modern singing cicadas. Tettigarctids were diverse throughout the Mesozoic and into the Eocene (from 250 to ~40 Mya; Moulds 2018), but today are represented by only two relatively closely related species that are cold adapted and live in remote mountainous regions of Tasmania and southern New South Wales (Kaulfuss & Moulds, 2015). The derotettigine lineage dates back to the late Cretaceous or earliest Palaeocene. We suggest that former species in this lineage went extinct as a result of landscape and climatic changes.

The Cenozoic was a time of extensive habitat modification caused by continental movements and changes in sea level and ocean currents that had a profound effect on the global distribution and diversification of plants and animals [e.g. in Australia (Byrne *et al.*, 2018), Africa (Linder & Bouchenak-Khelladi, 2015) and South America (Ortiz-Jaureguizar & Cladera, 2006)]. A general cooling and drying of the Southern Hemisphere starting in the Late Eocene/early Oligocene was triggered by many factors, including the establishment of the Antarctic Circumpolar Current and a reduction in global carbon dioxide (~41–33 Mya; (DeConto & Pollard, 2003; Speelman *et al.*, 2009). Various taxa invaded these newly arid domains around the world (e.g. Rabosky *et al.*, 2007; Kadereit *et al.*, 2012; Woodburne *et al.*, 2014; Owen *et al.*, 2017; Byrne *et al.*, 2018), with plants being aided by the rise of C_4 photosynthesis independently in many lineages (Edwards & Smith, 2010; Morando *et al.*, 2014; Zucol *et al.*, 2018).

Roig *et al.* (2009) reviewed the biogeography of Argentina and concluded that the Monte and

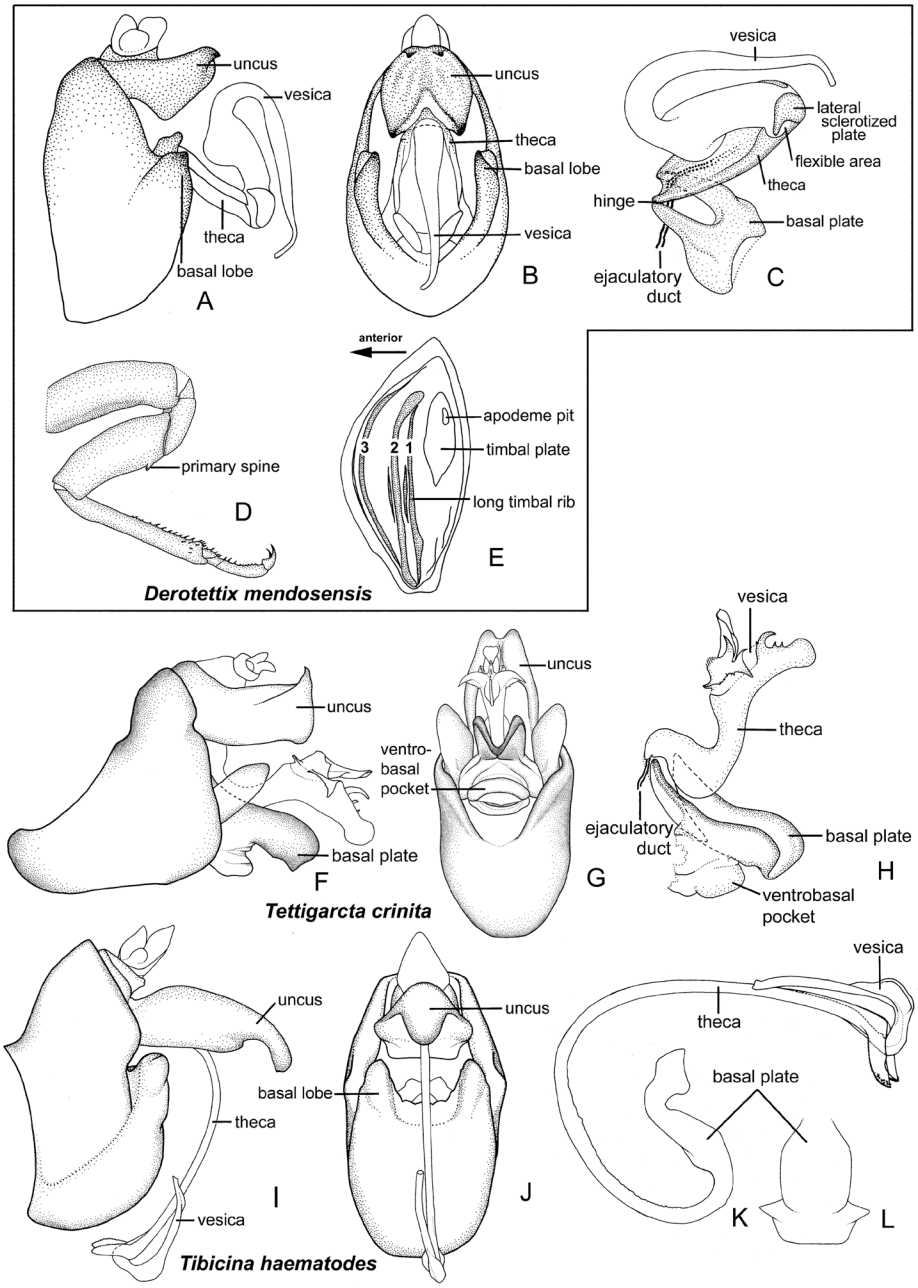

Figure 7. Distinguishing morphological features of **Derotettiginae subfam. nov.** and comparisons of genital characters of **Derotettiginae** with Tettigarctidae and Tibicininae. Clockwise from upper right: A–E, *Derotettix mendosensis* pygofer (genital capsule), lateral view (A); pygofer, ventral view (B); aedeagus (dissected) (C); front leg (D); timbal (E); F–H, *Tettigarcta crinita* Distant pygofer, lateral view (F); pygofer, ventral view (G); aedeagus (H); I–L, *Tibicina haematodes* Scopoli pygofer, lateral view (I); pygofer, ventral view (J); aedeagus (K); basal plate (L). C, H, K, views of aedeagi of the three representative species.

Chaco ecoregions were savanna at the beginning of the Cenozoic (65 Mya). Despite the wet climate of the Eocene (50–33 Mya), pollen fossils suggest that dry conditions persisted in parts of central western Argentina and increased in extent as the Andes uplifted. Retreats of extensive epicontinental seaways (Supporting Information, Fig. S19) left salt deposits in many parts of South America (Benavides, 1968), most recently in the mid Miocene, when most of the current range of both *Derotettix* species was under the Paranense Sea (Hernández *et al.*, 2005). The influence of this incursion has been seen in the

population structures of many central and northern Argentinian animal species (e.g. Delsuc *et al.*, 2012; Morando *et al.*, 2014; Brusquetti *et al.*, 2019). From the middle Miocene to the present in Argentina, rain-shadow aridity has increased owing to the rise of the Andes over the last 15 Myr, with accelerated uplift in the central Andes ~5 Mya (Farías *et al.*, 2008; Folguera *et al.*, 2011). The resulting climatic changes influenced all groups of flora and fauna (e.g. Ortiz-Jaureguizar & Cladera, 2006; Roig *et al.*, 2009; Ruzzante & Rabassa, 2011; Turchetto-Zolet *et al.*, 2013; Wallis *et al.*, 2016). In the last 2.6 Myr, the region has been affected by a series of no fewer than eight glaciation events that caused major fluctuations in the climate (Rabassa, 2008; Rabassa *et al.*, 2011; Elderfield *et al.*, 2012).

Although the derotettigine lineage stretches back >60 Myr, the current host plants of *Derotettix* are thought to have arrived much later. *Allenrolfea* and *Heterostachys* are predicted to have arrived in South America from Eurasia some time in the Miocene, 19 Mya at the earliest (Piirainen *et al.*, 2017). *Atriplex* arrived in North America in the mid Miocene (14 Mya or later) and moved into South America from there (Kadereit *et al.*, 2010). Salt tolerance, succulence and the evolution of C_4 photosynthesis might have preadapted these plant taxa to invade the steppes and deserts of the interior of South America. *Derotettix* might have arisen via a host shift in the mid Miocene after their host plants arrived (Piirainen *et al.*, 2017). Host shifts in insects are common and often lead to speciation (Forbes *et al.*, 2017). Examples are known from cicadas, including changes in gene expression likely to be associated with a shift in host plant in *Subpsaltria yangi* from an angiosperm to a gnetophyte (Hou & Wei, 2019). The ability of *Derotettix* to adapt to saline environments might have been a key innovation that facilitated their survival.

The Monte regions of Argentina currently lie in the transition between the tropical biota to the north and the Patagonian biota to the south. During our fieldwork, we observed that the cicadas of this region are a mixture of these northern (tribe Fidicinini-dominant) and southern (tribe Tettigadini-dominant) elements. The rise of cicadas in the subfamily Tibicininae, whose members in the tribe Tettigadini now dominate the temperate habitats of southern South America (Sanborn & Heath, 2014), probably also contributed to the decline of Derotettiginae. The key innovations that are lacking in Derotettiginae but present in Tibicininae are unknown but would be a fruitful area for future research.

Age of Cicadidae and a possible South American origin

The fossil record (Shcherbakov, 2009; Moulds, 2018) suggests that the modern cicadas (Cicadidae) arose during the late Cretaceous or early Palaeozoic at the latest. This would place the origin of the family Cicadidae no later than 99–60 Mya, during the time of the main Angiosperm radiation. The most comprehensive dating analyses of a major clade of cicadas conducted so far (Marshall *et al.*, 2016; Owen *et al.*, 2017) suggests that the tribe Cicadettini most probably originated around the time of the greenhouse–ice house transition at the end of the Eocene (~41–33 Mya). This tribe is contained within the subfamily Cicadettinae. The subfamily Tibicininae, known from a Palaeocene fossil 59.2–56 Myr old (Moulds 2018), is sister to Cicadettinae plus Cicadinae plus Tettigomyiinae (Figs 3–5). Thus, the Derotettiginae must have split from the rest of Cicadidae before the deposition of this tibicinine late Palaeocene fossil. Formal molecular dating studies using fossilized birth–death methods (Heath *et al.*, 2014), with much larger taxonomic and genomic sampling, are in progress.

Given that Derotettiginae is known only from South America and Tibicininae is heavily represented in South America, we hypothesize that the family Cicadidae had a South American origin. Tibicininae is largely New World, with at least two independent amphitropical Northern Hemisphere–Southern Hemisphere clade splits. Tibicininae includes an additional five (out of 23) genera that are endemic to the Palaearctic (Marshall *et al.*, 2018) and closely related to the North American tibicinines (Sueur *et al.*, 2007). Genomic sampling of additional South American and world taxa (in progress) will allow us to test the South American origin hypothesis put forth here for the first time.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website:

Table S1. Additional details for cicada genes used in this study.
Table S2. Additional details for Sulcia endosymbiont genes used in this study.
Table S3. Characters for the five subfamilies of the family Cicadidae, updated from Marshall *et al.* (2018). Autoapomorphies are highlighted in grey. Note that the Tettigomyiinae and Derotettiginae subfam. nov. each lack an autapomorphy and are diagnosable only by a combination of attributes.
Table S4. Character matrix (83 taxa × 117 characters) used in the maximum parsimony morphological character analysis. Missing data and character states not relevant to a taxon are scored as '?'.
Figure S1. A, *Derotettix mendosensis* male (PL754) on saltbush, *Atriplex* sp., Río Negro province, Argentina. B, male, dorsal view. C, male, side view. D, male, ventral view. Photographs: P.Ł. See Table 1 and Figure 2 for exact locality.

Figure S2. *Derotettix mendosensis* male (PL618), Ruta 141, San Juan province, Argentina. Clockwise from upper left: A, side view; B, dorsal view; C, side view; D, head. Photographs: P.Ł. See Table 1 and Figure 2 for exact locality.
Figure S3. *Derotettix mendosensis* (PL755 and PL756), Río Negro province, Argentina. A, female, green morph, dorsal. B, female, green morph, ventral. C, male, yellow morph. D, male, yellow morph, dorsal. E, male, yellow morph, side view. Photographs: P.Ł. See Table 1 and Figure 2 for exact locality.
Figure S4. *Derotettix mendosensis* (PL767), NW de Rincón de Los Sauces, Neuquén province, Argentina. A, male, side view. B, male, ventral view. C, male, dorsal view. Photographs: P.Ł. See Table 1 and Figure 2 for exact locality.
Figure S5. *Derotettix mendosensis* (PL954), Ruta de Pomona, Provincia Rio Negro, Argentina. A, male, dorsal view. B, male, side view. C, male, ventral view. Photographs: P.Ł. See Table 1 and Figure 2 for exact locality.
Figure S6. *Derotettix mendosensis* site (18.AR.MZ.CLG), highway 34, east of Villa Tulumaya, Mendoza province, Argentina. Three views of the habitat of *D. mendosensis*, with obvious human disturbance. Photographs: C.S. See Table 1 and Figure 2 for exact locality.
Figure S7. *Derotettix mendosensis*, east of Villa Tulumaya, Mendoza province, Argentina (site 18.AR.MZ.EVT). A, female, ventral view. B, female, side view. C, male, side view. D, male, ventral view. Photographs: C.S. See Table 1 and Figure 2 for exact locality.
Figure S8. *Derotettix mendosensis* habitat (18.AR.MZ.CLG), Calle Lugones, just off Provincial Road 96, Mendoza province, Argentina. Habitat with obvious human disturbance. Photographs: C.S. See Table 1 and Figure 2 for exact locality.
Figure S9. RAxML phylogeny for nuclear genes only, RNA + codon partitioned (28S, 18S, EF1a and ARD1). Of 6652 total characters, 1034 are parsimony informative; for the Cicadidae ingroup only, 714 are parsimony informative.
Figure S10. RAxML phylogeny, 28S gene only. Of 4622 total sites, 721 are parsimony informative, 629 within Cicadidae. Note 100% support on deepest nodes, including Cicadidae, **Derotettiginae subfam. nov.**, Tibicininae and the three remaining subfamilies as a trichotomy (Cicadinae unresolved).
Figure S11. RAxML phylogeny, all genetic data combined (nuclear NDA, mitochondrial DNA and *Sulcia*, RNA + codon partitioned). Of 31 623 total sites, 3523 are parsimony informative, 2686 within Cicadidae. All subfamilies were resolved as monophyletic.
Figure S12. Illustration of the subfamily character 'metanotum at dorsal midline' with states 'partially visible' and 'completely hidden'. Clockwise from upper left: A, Cicadettinae, *Amphipsalta zelandica* (photograph: C.S.); B, **Derotettiginae subfam. nov.**, *Derotettix mendosensis* (photograph: E.R.L.G.); C, Tibicininae, *Alarcta micromacula* (photograph: C.S.); D, Cicadinae *Neotibicen pronotalis* (photograph: David C. Marshall).
Figure S13. *Derotettix mendosensis*, 18.AR.MZ.EVT (Table 1). Colour faded by ethanol. A, dorsal view. B, head. C, lateral view. Macropod photographs: E.R.L.G.
Figure S14. *Derotettix mendosensis* male holotype, La Plata. A, dorsal view. B, ventral view. Macropod photographs: K.N.
Figure S15. *Derotettix mendosensis* female allotype, La Plata. A, dorsal view. B, ventral view. Macropod photographs: K.N.
Figure S16. *Derotettix wagneri* (= *Derotettix proseni*) male holotype, La Plata. A, dorsal view. B, ventral view. Macropod photographs: K.N.
Figure S17. A, *Derotettix wagneri* (= *Derotettix proseni*) male holotype, lateral view, La Plata. B, *Derotettix mendosensis* male holotype, lateral view, La Plata. Macropod photographs: K.N.
Figure S18. A, *Derotettix mendosensis* song structure, recording 080110-05, 35.4 °C, 18.AR.MZ.CLG. A, 0.5 s oscillogram (above). B, spectrogram.
Figure S19. (A) Paleomap reconstructions redrawn/excerpted as permitted from Scotese, C. R., 2001. Atlas of Earth History, Volume 1, Paleogeography, PALEOMAP Project, Arlington, Texas, 52 pp. Note extensive South American inland sea incursions 80 Mya and again 20 Mya.; (B) Paranense Sea transgression 13-15 Mya, with all *Derotettix* localities superimposed. Map redrawn from Hernandez et al. 2005, Figure 2. Inset below is Figure 2 from the present paper for comparison.