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The cicada genus *Tugelana* Distant, 1912 (Hemiptera, Cicadidae): phylogenetic position and conservation status

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Abstract

The cicada genus *Tugelana* Distant, 1912 is monotypic and endemic to south-eastern Africa. Material was not available for a recent molecular phylogeny of its tribe, so its precise phylogenetic placement is unestablished. Consequently, a 627 bp sequence of the cytochrome oxidase gene was obtained and its candidate relatives identified as several species of *Platypleura* Amyot & Audinet-Serville, 1843 using the BOLD Identification System and NCBI Genbank's BLAST. Bayesian inference analyses indicated that the type species, the Maputaland Orangewing Cicada *Tugelana butleri* Distant, 1912, is closely related to the Dune Koko Orangewing Cicada *Platypleura zuluensis* Villet, 1989, which has a geographical distribution that is parapatric with *T. butleri* and which has aberrant genitalia for a member of *Platypleura*. This pair of species is placed fairly deep within the African clade of *Platypleura*. We therefore formally recognized *Platypleura* Amyot & Audinet-Serville, 1843 as a senior synonym of *Tugelana* Distant, 1912, **syn. nov.**, and assign *T. butleri* Distant, 1912 to *Platypleura* as *Platypleura butleri* (Distant 1912), **comb. nov.** The species occurs on the wooded grasslands of the Maputaland coastal plateau east of Lebombo Mountains and south of Maputo Bay. Its Extent of Occurrence is about 6360 km², which would qualify it as Vulnerable under the IUCN's classification criteria for conservation status.

Keywords

Introduction

The monotypic southern African endemic genus *Tugelana* Distant, 1912 was first assigned to the tribe Hamzini Distant, 1904 (Distant 1914), which had only one other member, the Asian species *Hamza ciliaris* (Linnaeus, 1758). Based on molecular phylogenetic evidence (Price et al. 2019), Hamzini was recently synonymized with Platypleurini Schmidt, 1918, which is distributed across Asia and Africa, and contains about 30 genera. Although Platypleurini is a junior synonym of Hamzaria (Marshall et al. 2018), it has been conserved by conditional reversal of precedence (International Commission on Zoological Nomenclature 2020). *Tugelana* was not included in the phylogenetic study by Price et al. (2019), so its phylogenetic placement amongst this greater number of potential relatives is unclear.

Biogeographically, *Tugelana* is southern African (Villet 1994) and *Hamza* Distant, 1904 is distributed from Timor to Mindanao (Duffels 1991), so it seems unlikely that they are actually close relatives. Distant (1914) assigned them to the same tribe solely on the grounds that they both had tymbal covers that partially exposed their tymbal membranes (Distant 1914 plate 2, figs 28, 29; Villet 1994 fig. 4), a relatively rare character state. However, their male genitalia (Fig. 1) are not particularly similar (Duffels 1991; Villet 1994).

The wing colouration and pattern (Fig. 2; Distant 1914 plate 2, fig. 29; Armstrong and Villet 2019 figure 16), tomentum on the 7th abdominal tergite, and head shape (Distant 1914 plate 2, fig. 29; Villet 1994 fig. 1) of *Tugelana butleri* Distant, 1912 resemble those of the southern African species of *Platypleura* Amyot & Audinet-Serville, 1843 (e.g. Fig. 2). It was found that *Platypleura maritzburgensis* Distant, 1913 is a synonym of *T. butleri* (Villet 1994), which indicates phenotypic similarity between *T. butleri* and at least some species of *Platypleura*. However, the urite of the male genitalia of African species of *Platypleura* (e.g. *P. capensis* Linnaeus, 1764) (Fig. 1) is generally distinctive because the apices of the elongated medio-lateral processes are turned laterally and both the medio-lateral and lateral processes bear numerous spines (Boulard 1972). *Tugelana* has a urite with rather small lateral processes and indented medio-lateral processes (Villet 1994 figs 5, 6), conditions reminiscent of the slightly aberrant urite of *Platypleura zuluensis* Villet, 1987 (Fig. 1), which is physically similar (Fig. 2) and is phylogenetically placed quite deep within the African clade of *Platypleura* with good support from two nuclear and two mitochondrial genes (Price et al. 2019).

Since these observations create some doubt about the taxonomic distinctness of *Tugelana* and *Platypleura sensu stricto*, there is reason to seek new evidence of the relationships between these taxa. We provide molecular evidence to clarify this problem, and some notes on the biogeography and conservation status of the species.

Material and methods

A specimen of *T. butleri*, now deposited in the Albany Museum, Makhanda, was collected by Adrian Armstrong in wooded grassland hillside in the Manzengwenya
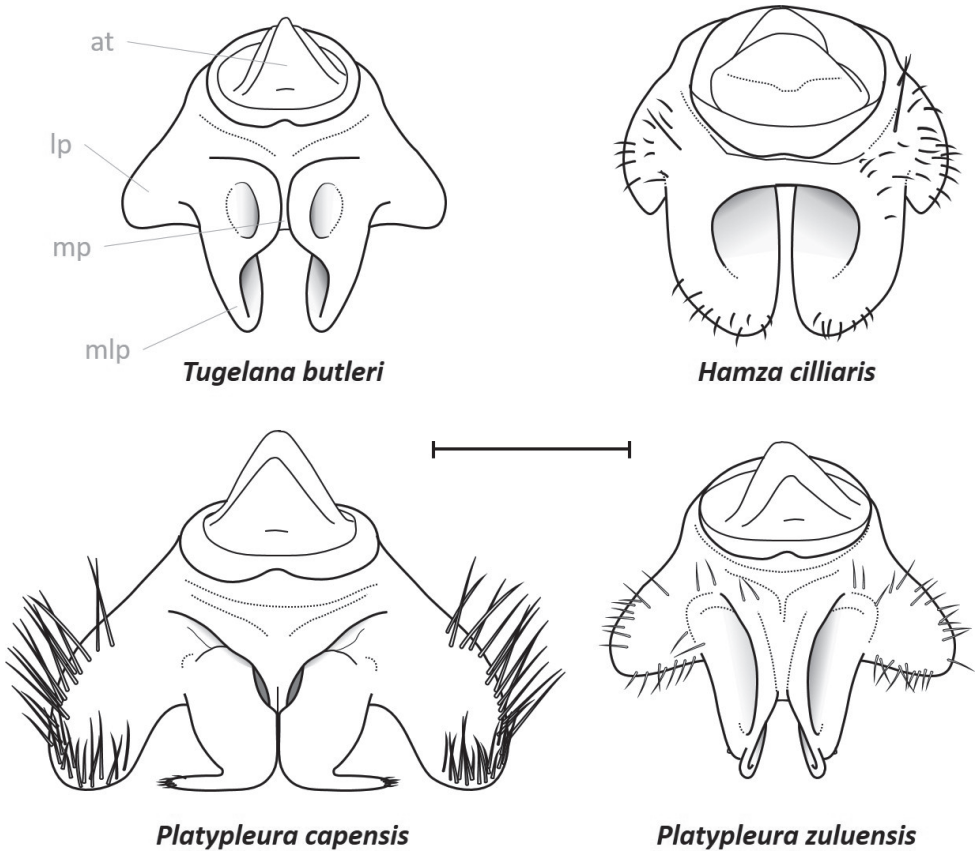
Figure 1. Urites of *Tugelana butleri*, *Hamza ciliaris*, *Platypleura capensis* and *Platypleura zuluensis*. AT = anal tube; LP = lateral process; MLP = medio-lateral process; MP = median process. Scale bar: 1 mm.

area (-27°15.17'S, 32°46.42'E) of the Isimangaliso Wetland Park, South Africa, on 26th March, 2016. Genomic DNA was extracted from the tympanal muscle of the dried, pinned specimen by a salt extraction method (Bruford et al. 1992) using lysis buffer (Buffer AL) and elution buffer (Buffer AE) from Qiagen. A section of the cytochrome oxidase I gene was amplified by polymerase chain reaction (**PCR**) using the primers LCO 1490 and HCO 2198 (Folmer et al. 1994). The 25 µl PCR mixes contained ~ 25–50 ng genomic DNA, 0.2 µM of each primer, 12.5 µl iTaq Mastermix (BioRad). The PCR cycling profile for the COI gene region was as follows: initial denaturing step at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 8 min. Sequencing was done by Macrogen (Amsterdam, Netherlands), using the forward primer, after a purification step. The resulting electropherogram trace file was checked for quality and edited using BioEdit v.7.2.5 (Hall 1999), and the resultant 627 bp-long sequence was archived on the NCBI Genbank database (Clark et al. 2016).
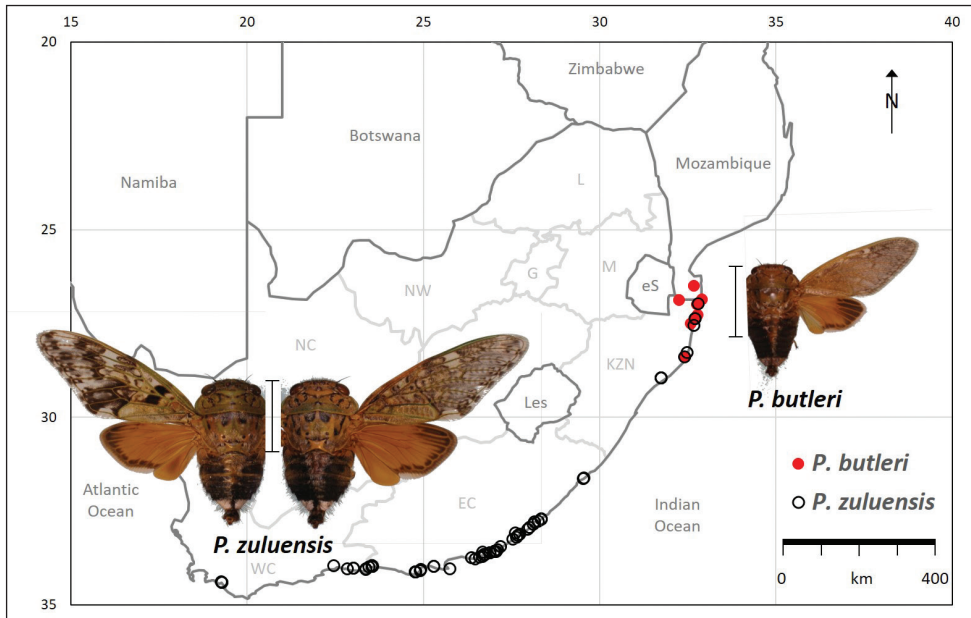
Figure 2. Geographical distribution of *P. butleri* and *P. zuluensis* within southern Africa, with insets of the two species (specimen scale lines = 10 mm). The distribution of *P. butleri* has a known standard Area of Occupancy (AOO) of 36 km² (nine localities) and an Extent of Occurrence (EOO) of 6360 km². Abbreviations: EC = Eastern Cape, eS = eSwatini, G = Gauteng, KZN = KwaZulu-Natal, L = Limpopo, Les = Lesotho, M = Mpumalanga, NC = Northern Cape, WC = Western Cape.

To get a first approximation of the relationships of *T. butleri*, the COI sequence was submitted to the on-line BOLD Identification System (IDS) for COI (https://www.boldsystems.org/index.php/IDS_OpenIdEngine) to search all records on the Barcode Of Life Database (**BOLD**) (~8,229,467 sequences at the time, including privately held data), and to NCBI Genbank's Basic Local Alignment Search Tool (**BLAST**) (Altschul et al. 1990) for comparison with holdings on GenBank and affiliated sequence databases. The BLAST results were assessed in terms of their proportional overlap, percent similarity and EXPECT-values (E-value, a measure of the probability of a random match) (Altschul et al. 1990).

The closest genus-level relatives of *T. butleri* were sought using phylogenetic analyses. Based on the results of the IDS and BLAST searches, homologous sequences of four gene regions (mitochondrial 16S, COI and COII, and nuclear EF1α) from the African species of *Platypleura* in the open-access data set of Price et al. (Suppl. material 1: Table S1) were downloaded with a spectrum of African platypleurine genera to serve as outgroups (Price et al. 2019). They were aligned using the ClustalW algorithm in MEGA X (Kumar et al. 2018), inspected manually and trimmed so that the first nucleotides in the alignment was the first codon position (for the protein coding gene regions).

Saturation of the different codon positions (each codon position separately, and codon positions 1 and 2 together) of each protein-coding region marker (COI, COII, and EF1α)

were tested using DAMBE v.7.2.152 (Xia 2018), but no saturation was found. Analyses were, therefore, run with each marker partitioned, without partitioning the codon positions. Evolutionary nucleotide substitution models best fitting the individual marker datasets (16S: GTR+Γ, COI: HKY+I+Γ, COII: GTR+I+Γ, EF1α: K80+I) were identified using the Bayesian Information Criterion (BIC) in jModeltest v.2.1.10 (Posada 2008).

Two Bayesian inference (**BI**) analyses were conducted with MrBayes v.3.2.2 (Ronquist et al. 2012) through the CIPRES Science Gateway (http://www.phylo.org), assuming uniform priors for all parameters (unless otherwise stated). The first BI analysis was done using the nucleotide substitution models for each gene partition (found using jModeltest). Two parallel runs of 20 million generations each were run for the MCMC analysis, with trees sampled every 1000 generations. The number of generations to discard as burn-in was determined using Tracer v.1.7.1 (Rambaut et al. 2018) by examining the number of generations (1) at which the standard deviation of split frequencies stabilized (at less than 0.001), (2) at which the log-likelihood tree scores reached stationarity, and (3) by ensuring that the effective sample sizes (**ESS**) of all parameters were >200. A 50% majority rule tree was constructed with the burn-in excluded (approximately 10% of total trees sampled), and nodes with ≥0.90 posterior probability were considered supported.

The second BI analysis also used the nucleotide substitution models, but used the codon model within MrBayes (Ronquist et al. 2012). This type of model basically groups the sequences into codons (3-nucleotide sets) and models the evolution of the codons, instead of the individual nucleotides. The analysis was run for 4 million generations, using codon substitution models. Trees were sampled and treated in the same way as the first Bayesian run, to produce a 50% majority rule tree.

A third phylogenetic tree was produced using maximum likelihood methods in Garli v.2.01 (Zwickl 2006). The genetic dataset was partitioned into the three gene regions and the nucleotide models obtained in jModeltest were applied to each partition. Node confidence was determined using 1000 bootstrap replicates.

Specimens were sought in museums in South Africa, England, France, Belgium, Germany and Sweden, and found in the Albany Museum, Makhanda; the Natural History Museum, London; the private collection of R. D. Steven; and the National Collection of Insects, Pretoria. Locality and habitat data were obtained from the labels of specimens and Kew's GEOCAT software (http://geocat.kew.org/editor) was used to calculate the standard Area of Occupancy (**AOO**) and Extent of Occurrence (**EOO**) of *T. butleri* (IUCN Standards and Petitions Committee 2019).

Results

A 627 bp fragment of the COI gene was obtained. The electropherogram showed no heterozygous peaks; there were no unexpected stop codons; and no single- or double-base indels appeared when it was aligned with multiple sequences drawn from ten platypleurine genera selected from the Price et al. (2019) data set. The sequence was therefore deemed to be of high quality.

Table 1. The top twenty greatest similarities found by BOLD IDS. None of these qualified as a taxonomic match by BOLD criteria.

Rank	Genus	Similarity (%)	Status
1	*Platypleura zuluensis*	95.51	Published
2	*Platypleura plumosa*	91.15	Published
3	*Platypleura capensis*	90.95	Published
4	*Platypleura capensis*	90.95	Published
5	*Platypleura capensis*	90.95	Published
6	*Platypleura capensis*	90.95	Published
7	*Platypleura capensis*	90.95	Published
8	*Platypleura capensis*	90.95	Published
9	*Platypleura capensis*	90.73	Published
10	*Platypleura capensis*	90.73	Published
11	*Platypleura capensis*	90.73	Published
12	*Platypleura capensis*	90.73	Published
13	*Platypleura capensis*	90.73	Published
14	*Platypleura capensis*	90.73	Published
15	*Platypleura capensis*	90.73	Published
16	*Platypleura capensis*	90.73	Published
17	*Platypleura capensis*	90.73	Published
18	*Platypleura capensis*	90.73	Published
19	*Platypleura capensis*	90.73	Published
20	*Platypleura capensis*	90.73	Published

The BOLD IDS reported "no match" for the sequence, but the top three hits were *P. zuluensis* (95.51% similarity), *Platypleura plumosa* Germar, 1834 (91.15%) and *P. capensis* (90.95%), which are all African platypleurine species (Table 1). The remaining hits were dominated by *P. capensis*, for which there were many samples on BOLD due to a phylogeographic study by Price et al. (2007). The top 26 hits on BLAST were *P. plumosa* (90.66–90.35% similarity) (Table 2). Other species in the top 50 included *P. capensis* (90.05%) and three undescribed species of the *P. plumosa* species group (90.20–89.89%) that were the focus of a phylogeographic study (Price et al. 2010). The *P. zuluensis* sequence lacked 186 of the 627 bases of the *T. butleri* sequences, which caused the BLAST algorithm to return a poor match between these taxa. On the basis of these results, COI gene sequences of all of the African and Asian species of *Platypleura* that were available in the Price et al. (2019) data set were included in the phylogenetic analysis to minimize the risk of taxon sampling artifacts.

The phylogenetic tree reconstructions using the three differing algorithms produced congruent topologies, and the overall topology unsurprisingly matched that found in Price et al. (2019). The Bayesian inference analyses (Fig. 3) both placed *T. butleri* as the sister taxon to *P. zuluensis* with good support. The ML analysis produced the same topology with weaker nodal support than the BI trees, however many of the deeper nodes were not supported at all, producing a polytomy nearer the base of the tree (Suppl. material 1: Figs S1 and S2). While a few of the species that made *Platypleura* polyphyletic in the Price et al. (2019) study have been reclassified into new genera or moved into existing genera, a few still remain to be reassigned; e.g. the

Table 2. The top 45 greatest similarities found by BLAST, with their assessment criteria.

Rank	Description	Query Cover	E-value	Percent Identity	Accession
1	*Platypleura plumosa* MHV0534	99%	0.0	90.66%	FJ169057
2	*Platypleura plumosa* MHV0555	99%	0.0	90.66%	FJ169070
3	*Platypleura plumosa* MHV1040	99%	0.0	90.66%	FJ169175
4	*Platypleura plumosa* MHV0329	99%	0.0	90.51%	FJ169023
5	*Platypleura plumosa* MHV0333	99%	0.0	90.51%	FJ169026
6	*Platypleura plumosa* MHV0501	99%	0.0	90.51%	FJ169052
7	*Platypleura plumosa* MHV0558	99%	0.0	90.51%	FJ169072
8	*Platypleura plumosa* MHV0567	99%	0.0	90.51%	FJ169078
9	*Platypleura plumosa* MHV1041	99%	0.0	90.51%	FJ169176
10	*Platypleura plumosa* MHV1042	99%	0.0	90.51%	FJ169177
11	*Platypleura plumosa* MHV1043	99%	0.0	90.51%	FJ169178
12	*Platypleura plumosa* MHV1045	99%	0.0	90.51%	FJ169179
13	*Platypleura plumosa* MHV1046	99%	0.0	90.51%	FJ169180
14	*Platypleura plumosa* MHV0324	99%	0.0	90.35%	FJ169021
15	*Platypleura plumosa* MHV0327	99%	0.0	90.35%	FJ169022
16	*Platypleura plumosa* MHV0422	99%	0.0	90.35%	FJ169040
17	*Platypleura plumosa* MHV0616	99%	0.0	90.35%	FJ169110
18	*Platypleura plumosa* MHV1019	99%	0.0	90.35%	FJ169166
19	*Platypleura plumosa* MHV1021	99%	0.0	90.35%	FJ169168
20	*Platypleura plumosa* MHV1039	99%	0.0	90.35%	FJ169174
21	*Platypleura plumosa* MHV0200	99%	0.0	90.20%	FJ168999
22	*Platypleura plumosa* MHV0259	99%	0.0	90.20%	FJ169010
23	*Platypleura plumosa* MHV0536	99%	0.0	90.20%	FJ169059
24	*Platypleura plumosa* MHV0537	99%	0.0	90.20%	FJ169060
25	*Platypleura plumosa* MHV0564	99%	0.0	90.20%	FJ169075
26	*Platypleura plumosa* MHV1020	99%	0.0	90.20%	FJ169167
27	*Platypleura* sp. 13 MHV1016	99%	0.0	90.20%	FJ169164
28	*Platypleura* sp. 13 MHV1018	99%	0.0	90.20%	FJ169165
29	*Platypleura* sp. 12 MHV0014	99%	0.0	90.08%	FJ168986
30	*Platypleura* sp. 12 MHV0571	99%	0.0	90.08%	FJ169080
31	*Platypleura* sp. 12 MHV0572	99%	0.0	90.08%	FJ169082
32	*Platypleura* sp. 12 MHV0573	99%	0.0	90.08%	FJ169083
33	*Platypleura* sp. 12 MHV0586	99%	0.0	90.08%	FJ169092
34	*Platypleura* sp. 12 MHV0603	99%	0.0	90.08%	FJ169102
35	*Platypleura capensis* MHV0021	99%	0.0	90.05%	FJ168988
36	*Platypleura plumosa* MHV0320	99%	0.0	90.05%	FJ169018
37	*Platypleura plumosa* MHV0421	99%	0.0	90.05%	FJ169039
38	*Platypleura plumosa* MHV0538	99%	0.0	90.05%	FJ169061
39	*Platypleura plumosa* MHV0540	99%	0.0	90.05%	FJ169063
40	*Platypleura* sp. 13 MHV1015	99%	0.0	90.05%	FJ169163
41	*Platypleura plumosa* MHV1037	99%	0.0	89.92%	FJ169173
42	*Platypleura plumosa* MHV0610	99%	0.0	89.89%	FJ169106
43	*Platypleura plumosa* MHV0614	99%	0.0	89.89%	FJ169109
44	*Platypleura* sp. 11 MHV0859	99%	0.0	89.89%	FJ169137
45	*Platypleura* sp. 11 MHV0862	99%	0.0	89.89%	FJ169139

east Asian species, *P. contracta, P. hilpa, P. kaempferi, P. kuroiwae, P. mira, P. miyakona, P. nobilis, P. arabica, P. octoguttata,* and *P. takasagona* (Villet in prep.).

Although there are several with no provenance, other specimens of *Tugelana butleri* have been collected from nine identified localities in Mozambique and South Africa.
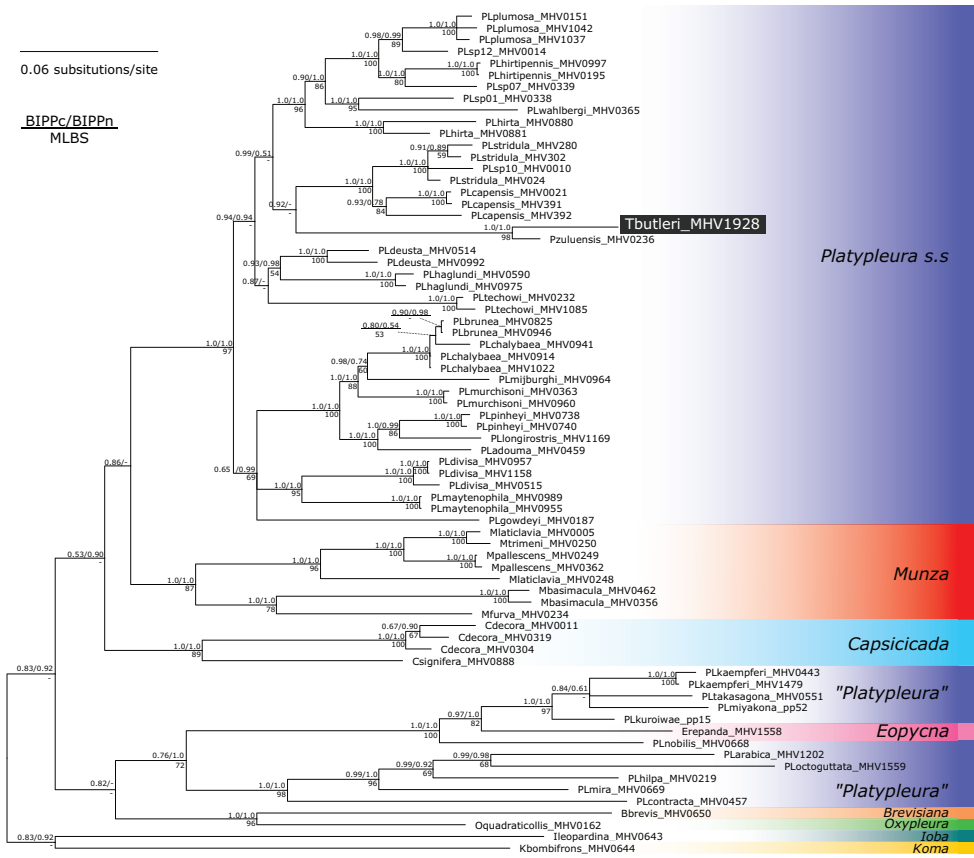
Figure 3. Bayesian Inference (BI) phylogenetic tree constructed using a codon nucleotide substitution model. *Tugelana butleri* is highlighted with a black box. Posterior probabilities (PP) from the two BI phylogenetic reconstructions are indicated above the nodes numerically (BIPPc = codon substitution model, BIPPn = nucleotide substitution model), and the bootstrap support values from the Maximum Likelihood analyses are shown in percentages below the nodes (MLBS). Nodes with BIPP > 0.95 and MLBS > 75 are considered well-supported.

Its known standard Area of Occupancy (AOO) is 36 km² (nine localities), its Extent of Occurrence (EOO) is 6360 km². The labels of two specimens record the habitat as "coastal dune vegetation" at Lake Sibaya (*leg*. R. Perissinotto & L. Clennell) and "wooded grassland hillside" in the Manzengwenya area of Isimangaliso Wetland Park (*leg*. Adrian Armstrong).

Discussion

Consistent molecular evidence was found that *T. butleri* and *P. zuluensis* are closely related. *Platypleura zuluensis* has been collected from late November to late February in coastal forests from Hermanus to at least the Mozambique border (Fig. 2); *T. butleri* has been collected from early January to late March in wooded grasslands and coastal

dune vegetation around that same border (Fig. 2), making these species parapatric in northern Zululand. Based on the molecular and biogeographical evidence, we formally propose the following synonym and new combination:

Platypleura Amyot & Audinet-Serville, 1843: 465
= *Tugelana* Distant, 1912: 646, syn. nov.
Platypleura butleri (Distant, 1912: 646), comb. nov.
= *Tugelana butleri* Distant, 1912: 646
= *Platypleura maritzburgensis* Distant, 1913: 79 (*teste* Villet 1994, 88)

The geographical distribution of *P. butleri* comb. nov. appears to be restricted to Maputaland, a wedge of wooded grassland on the coastal plateau east of the Lebombo Mountains and south of Maputo Bay, which is reflected in its vernacular name, the Maputaland Orangewing Cicada. Since it is known from only nine localities, it is not well sampled, so its Area of Occurrence may be a misleading indication of its conservation status. However, its estimated Extent of Occurrence (~6360 km²) suggests that its IUCN status may qualify as "Vulnerable" (IUCN Standards and Petitions Committee 2019).

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Supplementary material 1

Table S1, Figures S1, S2
Authors: Martin H. Villet, Shelley Edwards
Data type: docx. file
Explanation note: **Table S1.** Specimen sampling for the *Platypleura* genus tree. **Partial sequence of COI** for *Platypleura* butleri voucher number MHV1928. **Figure S1.** Maximum likelihood phylogenetic tree for the *Platypleurini*. Confidence values (MLBS) at the nodes are the bootstrap values, presented in percentages. MLBS > 75 is considered well-supported. **Figure S2.** Bayesian Inference phylogenetic tree, using nucleotide substitution models, for the *Platypleurini*. Confidence values (BIPPn) at the nodes are the posterior probabilities, presented numerically. BIPPn > 95 is considered well-supported.

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Supplementary material 2

Locality records
Authors: M.H. Villet
Data type: COL (excel table)
Explanation note: Locality data for *Platypleura butleri* (= *Tugelana butleri*).
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