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DNA Barcoding and Molecular Phylogenetics Revealed a New Cryptic Bamboo Aphid Species of the Genus *Takecallis* (Hemiptera: Aphididae)

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Abstract: *Takecallis nigroantennatus* Wieczorek sp. nov. (Hemiptera: Aphididae), associated with the cold hardy bamboo variety *Fargesia* spp. (Bambusoideae), is described and illustrated along with a key to species of the genus *Takecallis*. The results of a mitochondrial COI (DNA barcoding) and nuclear elongation factor 1 (EF1α) gene sequences, which confirm the genetic difference of the new taxon from the other congeneric species, are provided. The possible way of introduction of this species to Europe is discussed with its new locality from Belgium.

Keywords: COI; EF1α; cryptic species; *Fargesia* spp.; *Takecallis nigroantennatus*

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1. Introduction

Aphids (Hemiptera, Aphididae), due to the seasonal polymorphism and complex life cycles, are insects difficult to identify. Thus, the usability of DNA barcoding, a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene, has been used for additional diagnostic data in species identification [1–5]. Moreover, DNA barcoding enables the detection of cryptic species and the reassessment of species diversity delimitation on various taxonomic levels [6–9].

The bamboo feeding aphid genus *Takecallis* Matsumura contains seven taxa of oriental origin: *T. affinis affinis* Ghosh, *T. affinis niitakayamensis* Quednau, *T. alba* Lee, *T. arundicolens* (Clarke), *T. arundinariae* (Essig), *T. assumenta* Qiao and Zhang, *T. sasae* (Matsumura), and *T. taiwana* (Takahashi) [10]. Matsumura (1917) [11] erected *Takecallis* for *Callipterus arundicolens* Clarke (= *T. bambusae* Matsumura, 1917). The genus was revised by Higuchi (1969) [12] and later reviewed by Quednau (2003) [13]. The author synonymized *T. takahashii* Hsu with *T. arundinariae*, described *T. affinis niitakayamensis*, and provided keys to species and illustrations of all available morphs. Qiao and Zhang (2004) [14] reviewed the genus *Takecallis* from China and described *T. assumenta*. Recently, *T. alba* from Korea was distinguished based on morphological and molecular evidence [15]. Moreover, comprehensive assessment of DNA barcodes for the subfamily Calaphidinae revealed deep intraspecific divergence within the species complex of *T. arundicolens*, which indicates that there is more than one species under this name [6]. In their natural range, all known species of this genus are restricted to East Asia (China, India, Japan, Korea, Manchurian subregion, Taiwan). Three species—*T. arundicolens*, *T. arundinariae* and *T. taiwana* are now widely distributed and introduced to other continents, including Europe [16]. All of them are treated as a pest on Bambusoideae, i.e., *Arundinaria* spp., *Bambusa* spp., *Phyllostachys* spp., and *Sasa* spp. [13,17].

Aphids, as herbivorous sap-sucking insects, can cause considerable damage to crops and ornamental plants, and also as virus vectors. Therefore, it is very important to use sustainable strategies for their management. In order to use them, it is important to identify

the pest correctly, and choose the right methods of its control. An examination of aphid samples collected in 2022 from the cold hardy bamboo variety *Fargesia rufa* T.P. Yi revealed the first detection of the genus *Takecallis* from Poland. These specimens were firstly classified as belonging to the *T. arundicolens* complex [18]. In order to exclude whether the material collected in Poland is a color variant of *T. arundicolens* or an unknown cryptic species of this genus, detailed morphometric studies together with analyses of a mitochondrial COI (DNA barcoding) and nuclear elongation factor 1 (EF1α) gene sequences were undertaken, and the results are presented in this paper. In addition, the material collected from Poland was compared with aphids feeding on various species of bamboo, collected in 2023 in the Meise Botanic Garden, Belgium.

2. Materials and Methods

2.1. Specimen Collection and Identification

The aphids (adult winged viviparous females and immature alatoid nymphs) were collected directly from the host plant using a fine brush and placed into Eppendorf tubes containing 70% and 98% ethanol. Insects were slide-mounted using the method of Wieczorek [19], examined using a Nikon Ni-U light microscope equipped with a phase contrast system and photographed using a Nikon SMZ 25 stereoscopic microscope with a DS-Fi2 camera. Field photographs were taken using an iPhone 7 camera with the OlloClip Macro Pro Lens Set. The figures were prepared using Corel Draw 2021, Corel Corporation. The measurements were taken according to Ilharco and van Harten [20] and are given in millimeters.

2.2. DNA Sequencing and Phylogenetic Analysis

DNA was extracted from seven specimens (isolates 1BAN, 2BAN, 3BAN, 4BAA, 5BAN, 6BAA, 7BAN, Poland locality). Genomic DNA was isolated without modifying the protocol using the Bio-Trace DNA Purification Kit (EURx, Gdańsk, Poland). To elute the purified DNA, we applied 50 µL of an Elution Buffer onto the silica membrane. Two genes were amplified, mitochondrial COI and nuclear EF1α. To amplify a fragment of the mitochondrial cytochrome c oxidase I (COI) gene, the primer pair LCO-1490 and HCO1850 [21] was used. For the amplification of the Elongation factor 1-alpha, alpha (EF1α) primers EF2 [22] and EF3 [23] were used. Polymerase chain reaction (PCR) amplification for all DNA fragments was performed in a final volume of 20 µL containing 30 ng of DNA, 10 µL of 2× Phanta Master Mix (Vazyme Biotech, Nanjing, China), 0.4 µL of 20 µM of each primer, in a Mastercycler ep system (Eppendorf, Germany). The cycling profile for the PCR was: 95 °C for 3 min, 35 cycles of 95 °C for 15 s, Tm of oligos for 15 s, 72 °C for 1 min and a final extension period of 72 °C for 5 min.

In order to assess the quality of the amplification, the PCR products were electrophoresed in 1% agarose gel for 45 min at 85 V with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Thermo-Scientific, Waltham, MA, USA). The PCR products were purified using Clean-Up Concentrator (A & A Biotechnology, Gdańsk, Poland).

Samples were sequenced in both directions using the same primers as for the PCR reactions, except EF1α products where two or more internal primers, selected from: EF4 (5′-GAACCACCATACAGCGAAG) or EF4a (5′-GAACCACCGTACAGTGAAG), EF5 (5′-TTGATGTAACTGCTGACTTC), EF7 (5′-ATTGGAGGTATTGGAACAGT) and EF8 (5′-GGGACTGTTCCAATACCTCC) [24] were used combined with a BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) in the chain termination reaction method [25]. The sequencing reaction was carried out with the PCR product at a total volume of 20 µL, containing 2 µL of BigDye Terminator Reaction Ready Mix v. 3.1, 2 µL 5×sequencing buffer, 3.2 mol/µL of the primer solution, and 6 µL of the purified PCR product. The cycle-sequencing profile was 3 min at 94 °C followed by 30 cycles of 10 s at 96 °C, 5 s at 50 °C, and 2 min at 60 °C.

Sequencing products were precipitated using CleanDTR (CleanNa, Waddinxveen, The Netherlands), and were separated on an ABI PRISM 3130xl DNA Sequencer (Applied Biosystems, Waltham, MA, USA).

Raw chromatograms were evaluated and corrected in Geneious v10. 2.6 (https://www.geneious.com, accessed on 15 February 2023) [26]. In order to identify the numts [27,28], the mitochondrial COI and EF1 sequences were translated into amino acid sequences with Geneious v R10.2.6 using the respectively standard invertebrate mitochondrial genetic code and standard code. All of the nucleotide sequences were verified using BLAST searches of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 Febuary 2023). Protein coding data were aligned using the MAFFT versions 7.263 [29] plugin within Geneious v R10.2.6 and introns were removed from the EF1 α alignment. All sequences used in the phylogenetic analysis are presented in Table 1.

Table 1. List of species and GenBank accession numbers used in the phylogenetic analysis.

Sample ID	COI		EF1	Reference
Takecallis nigroantennatus sp. nov—isolate 1BAA	OR001758	Present Study	OR039806	Present Study
Takecallis nigroantennatus sp. nov—isolate 2BAN	OR001759	Present Study	OR039807	Present Study
Takecallis nigroantennatus sp. nov—isolate 3BAN	OR001760	Present Study	OR039808	Present Study
Takecallis nigroantennatus sp. nov—isolate 4BAA	OR001761	Present Study	OR039809	Present Study
Takecallis nigroantennatus sp. nov—isolate 5BAN	OR001762	Present Study	OR039810	Present Study
Takecallis nigroantennatus sp. nov—isolate 6BAA	OR001763	Present Study	OR039811	Present Study
Takecallis nigroantennatus sp. nov—isolate 7BAN	OR001764	Present Study	OR039812	Present Study
Takecallis taiwana	MH820965	[30]	MT039369	Direct submission
	MH820989	[30]		
	MH820958	[30]		
	MH820978	[30]		
	MH820985	[30]		
	MH820961	[30]		
	MH820975	[30]		
	MH820966	[30]		
	MH820962	[30]		
	MH820968	[30]		
	MH820980	[30]		
	MH820981	[30]		
	MH820996	[30]		
	MH820990	[30]		
	MH820973	[30]		
	MH820993	[30]		
	MH820974	[30]		
	MH820977	[30]		

Table 1. *Cont.*

Sample ID	COI		EF1	Reference
	MH820970	[30]		
	MH820987	[30]		
	MH820964	[30]		
	MH820988	[30]		
	MH820979	[30]		
	MH820963	[30]		
	MH820984	[30]		
	MH820991	[30]		
	MH820967	[30]		
	MH820982	[30]		
	MH820976	[30]		
	MH820997	[30]		
	MH820992	[30]		
	MH820969	[30]		
	MH820995	[30]		
	MH820959	[30]		
	MH820951	[30]		
Takecallis sasae	KY307057	[6]	MT262445	Direct submission
	KY307060	[6]		
	KY307059	[6]		
	KY307063	[6]		
	KY307061	[6]		
	KY307062	[6]		
	KY307058	[6]		
	KY307056	[6]		
Takecallis aroundicolens	EU701915	[1]	MT262446	Direct submission
	KY307036	[6]		
	KY307039	[6]		
	KY307037	[6]		
	KY307038	[6]		
	KY307035	[6]		
	KY307047	[6]		
	KY307049	[6]		
	KY307051	[6]		
	KY307050	[6]		
	KY307034	[6]		
	KY307048	[6]		
	KY307029	[6]		
	KY307030	[6]		
	KY307043	[6]		
	KY307031	[6]		

Table 1. *Cont.*

Sample ID	COI		EF1	Reference
	KY307053	[6]		
	KY307032	[6]		
	KY307028	[6]		
	KY307033	[6]		
	KY307046	[6]		
	KY307052	[6]		
	KY307045	[6]		
	KY307044	[6]		
	KY307040	[6]		
	KY307041	[6]		
	KY307042	[6]		
	KY307027	[6]		
	KY307022	[6]		
	KY307024	[6]		
	KY307025	[6]		
	KY307023	[6]		
	KY307026	[6]		
	KY307049	[6]		
Takecallis arundinariae	KR039454	[31]	MT262444	Direct submission
	MH820948	[30]		
	MH820950	[30]		
	MH820955	[30]		
	MH820956	[30]		
	MH820947	[30]		
	MH820954	[30]		
	MH820957	[30]		
	MH820952	[30]		
	KR579007	[32]		
	MH820951	[30]		
	JX205217	Direct submission		
	KR034713	[31]		
	MH820953	[30]		
	KR034488	[31]		
	KY307054	[6]		
	KF639650	Direct submission		
	KY307055	[6]		
	KR043148	[31]		
	GU135641	[33]		
Euceraphis lineata	KR033551	[31]		
Calaphis magnoliae	KY306818	[6]	MT262369	[9]
Glyphinaphis bambusae	-		MN167448	Direct submission

Models for Bayesian (BI) and maximum likelihood (ML) analyses were calculated in MrModeltest [34] using the Akaike information criterion (AIC). BI analyses were performed in MrBayes v3.2.6 [35] with four independent runs, each having three heated and one cold chain. Analyses were run for 2 million generations with trees sampled every 1000 generations. The first 25% of each run was discarded as burn-in. Convergence among the runs was assessed using Tracer [36], and examination of the potential scale reduction factor (PSRF) values and average standard deviation of the split frequencies in the MrBayes output was performed. The ML analyses were performed using IQ-TREE v.1.6.8 [37] with the ultrafast bootstrap approximation for branch support with 1000 replicates [38]. All trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 20 February 2023). Trees were edited and annotated in Corel Draw 17.1.0.572, 2014 Corel Corporation. Pairwise distances were calculated using MEGA X with Kimura-2 parameter model [39].

2.3. Species Delimitation

Three DNA-based species delimitation methods, Poisson tree processes (bPTP) model with Bayesian implementation [40], Automatic Barcode Gap Discovery (ABDG) [41], and Assemble Species by Automatic Partitioning (ASAP) [42] were used to confirm independently evolved linage in the Takecallis genus. Poisson tree process modeling is a method intended for delimitating species in single-locus molecular phylogenies. ABDG and ASAP use pairwise distances to group intra-sequences into potential species based on detecting gaps in the variation between supposed intra- and interspecific groups (barcode thresholds). bPTP analysis was performed with PTP web server (https://species.h-its.org, accessed on 12 June 2023) using the IQ-TREE result from the previous analysis as an input. Automatic Barcode Gap Discovery (ABDG) was performed using ABDG web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.htm, accessed on 12 June 2023) with default parameters using the alignment of sequences as an input file. Assemble Species by Automatic Partitioning (ASAP) was performed using the ASAP web server (https://bioinfo.mnhn.fr/abi/public/asap/, accessed on 12 June 2023) with alignment of sequences as an input file.

3. Results

3.1. Molecular Phylogeny

Phylogenetic inferences were obtained for COI and EF1α genes using Bayesian inference (BI) and Maximum likelihood (ML).

For the COI, the jModelTest estimated GTR + G + I as the best fitting model. ML and BI analysis resulted in phylograms with the same topologies. The ESS values reported for BI analysis were >200, and the observed PRSF was 1.0. The phylogenies wereresolved and the main splits were supported by high bootstrap values and posterior probabilities. *Calaphis magnoliae* Essig and Kuwana and *Euceraphis lineata* Baker were used to root the topologies. Phylogram shows two main clades, first formed by two sister subclades; one with species belonging to the *T. taiwana*, and the second by species belonging to the *T. sasae*. The second clade is formed by two subclades. The first subclade is formed by the *T. nigroantennatus* sp. nov., described in the present paper (Figure 1). The second subclade consists of two groups, first formed by species belonging to the *T. arundinariae*. The second group is formed by two sister phylogenetic lines formed by species belonging to the *T. arundicolens*.

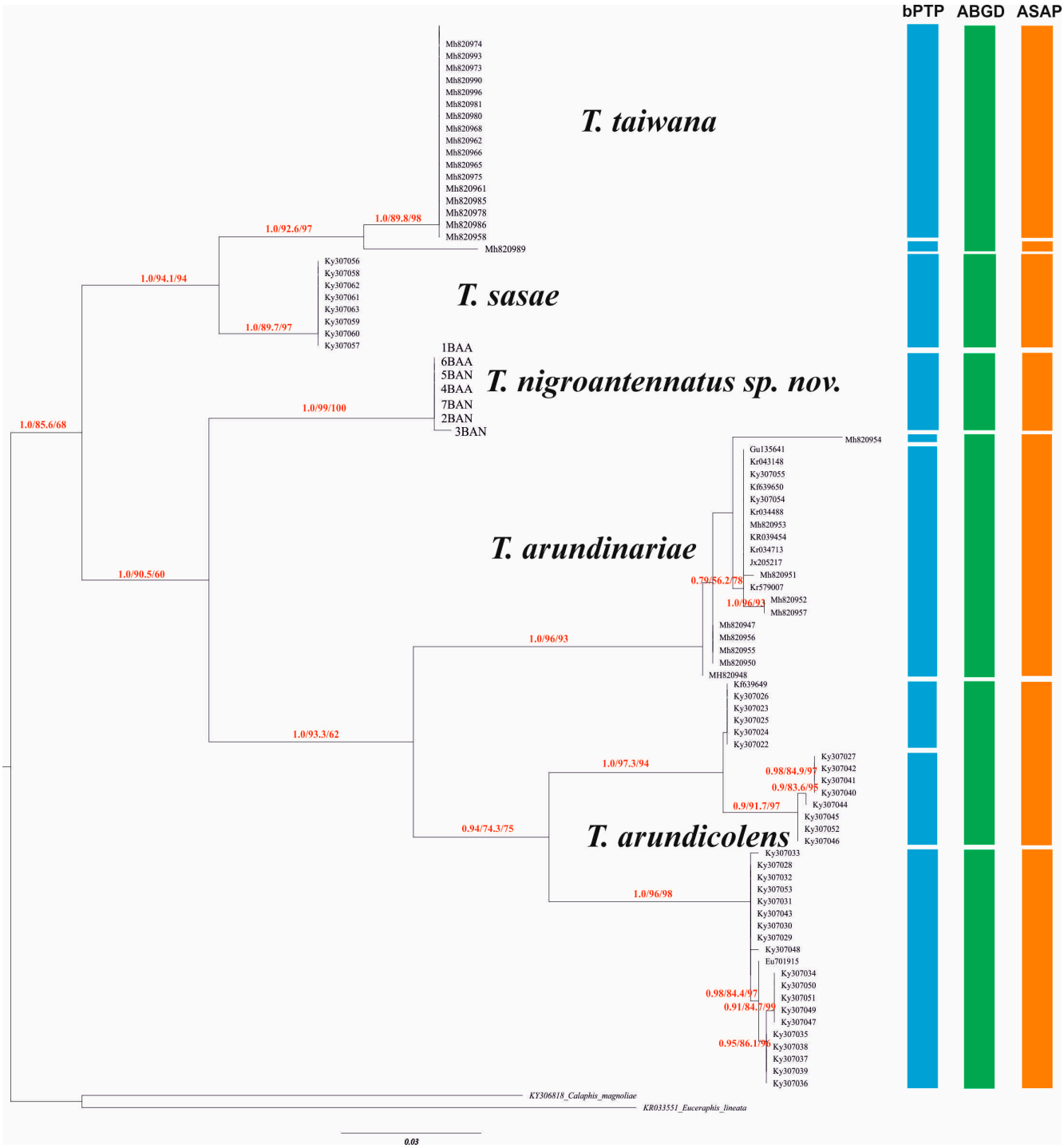

Figure 1. Phylogenetic tree showing the phylogenetic position of *Takecallis nigroantennatus* sp. nov. among *Takecallis* species, inferred from maximum likelihood (ML) and Bayesian Interference (BI) analysis based on cytochrome oxidase I (COI) gene sequences with results of species delimitations (bPTP, ABGD and ASAP). The first number on the branch is posterior probabilities from MrBayes; the second, SH-aLRT support (%); and third, ultrafast bootstrap support (%) from IQ-TREE. Scale bar: expected substitutions per site.

For the EF1α, the jModelTest estimated GTR + I as the best fitting model. ML and BI analysis resulted in trees with the same topologies. The ESS values reported for BI analysis were >200, and the observed PRSF was 1.0. The phylogenies were resolved and the main splits were supported by high bootstrap values and posterior probabilities. *Calaphis*

magnoliae and *Glyphinaphis bambusae* van der Goot were used to root the topologies. The phylogram shows two main clades. First is formed by two subclades, one represented by *T. sasae* and the second by *T. taiwana*. The second clade is formed by two subclades; one formed by two phylogenetic lines, *T. arundicolens* and *T. arundinariae*. The second subclade is formed by the *T. nigroantennatus* sp. nov., described in the present paper (Figure 2).

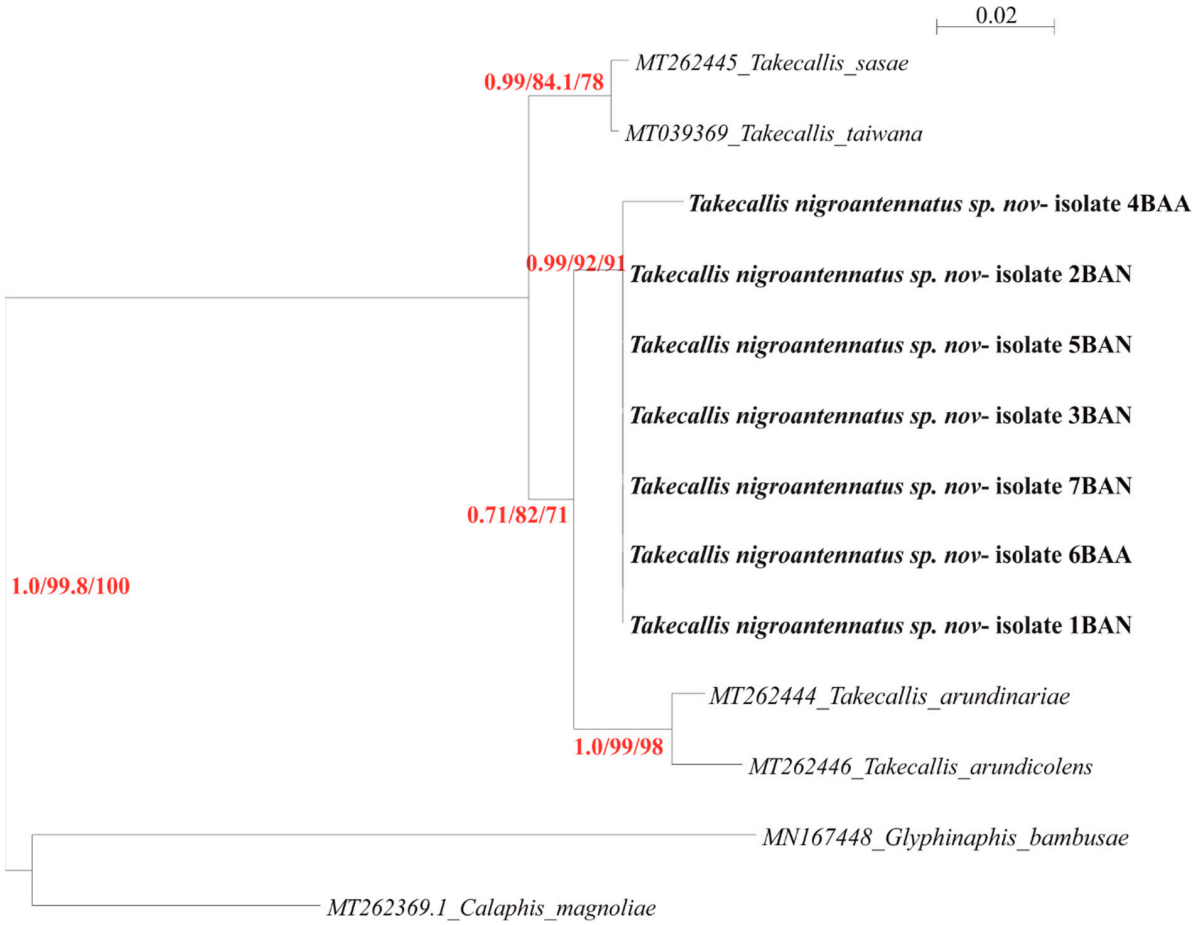

Figure 2. Phylogenetic tree showing the phylogenetic position of *Takecallis nigroantennatus* sp. nov. among *Takecallis* species, inferred from maximum likelihood (ML) and Bayesian Interference (BI) analysis based on elongation factor 1 (EF1α) gene sequences. The first number on the branch is posterior probabilities from MrBayes; the second, SH-aLRT support (%); and third, ultrafast bootstrap support (%) from IQ-TREE. Scale bar: expected substitutions per site.

p-distance between investigated specimens and group of species of *T. taiwana*, *T. sasae*, *T. arundicolens* and *T. arundinariae* was 9.3%, 7.8%, 10.2%, and 9.3%, respectively.

3.2. Species Delimitation

The different species delimitation methods (bPTP, ABDG and ASAP) resulted in different numbers of molecular operational taxonomic units (MOTUs) in the analyzed representative of the *Takecallis* genus (Figure 1). ASAP analysis identified 5–21 hypothetical species. The first ASAP-score (1.0) was chosen, which provides the best-fit scenario at the threshold distance of 2.78% (JC69) with five hypothetical species. The pairwise distance gap approach (ABDG) with default settings (X = 0.5) suggested six species with a barcode gap distance of 4.9%. The bPTP showed nine hypothetical species. All performed analyses indicated that *Takecallis nigroantennatus* sp. nov may be a species.

3.3. Taxonomy

Family Aphididae Latreille, 1802

Subfamily Calaphidinae Oestlund, 1919
Tribe Therioaphidini Börner, 1944
Genus *Takecallis* Mastumura, 1917
Species *Takecallis nigroantennatus* Wieczorek sp. nov.

Materials examined: 28 adult viviparous females and 14 alatoid nymphs (collected in the type locality, Poland); specimens were mounted on microscope slides (20 adult viviparous females + 6 alatoid nymphs) and processed for DNA sequencing (8 + 8). Additional fresh material for comparative morphological study was collected by the first author on 4 May 2023 in the Meise Botanic Garden, Belgium: 10 adult viviparous females of *T. arundicolens* from *Pseudosasa japonica* (Siebold and Zucc. ex Steud.) Makino ex Nakai; 10 adult viviparous females of *T. arundinariae* from *Sasa megalophylla* Makino and Uchida; and five adult viviparous females of *T. nigroantennatus* sp. nov. from *Fargesia nitida* x *murielae*.

Type of locality and host plant: 54°27′52.19″ N, 17°02′01.55″ ES; Słupsk, Poland; coll. from *Fargesia rufa* T.P.Yi on 9 July, 1 August, and 3 September 2022 by Karina Wieczorek.

Type of repository: Holotype (Słupsk, Poland, *Fargesia rufa* 1 August 2022, leg. K. Wieczorek, slide DZUS 1/8.22_7) and three paratypes (slides DZUS 9/7.22_8, DZUS 1/8.22_9, DZUS 3/10.22_10 same data as holotype) were deposited in the Entomology Collection of the University of Silesia in Katowice, Poland (DZUS).

Etymology: The species name refers to the antennae black, except for the antennal segments I–II, and the very base of segment III, clearly visible in live, fresh fixed, and slide-mounted adult viviparous females.

3.3.1. Differential Diagnosis

T. nigroantennatus sp. nov. is morphologically close to *T. affinis niitakayamensis*. However, this species can be distinguished from the latter by the following characteristics: body length 1.40–1.95 mm, antennae 0.95–1.14 times body length, antennal segment I with 4 setae, URS with 4 accessory setae, cauda with 8–11 setae, each lobe of anal plate with 5–9 setae, spinal abdominal setae barely visible. On the contrary, body length 2.45 mm, antennae 1.3 times body length, antennal segment I with 5–7 setae, URS with 3 accessory setae, cauda with 18 setae, each lobe of anal plate with 15 setae, spinal abdominal setae on low elevations are characteristic for *T. affinis niitakayamensis*.

3.3.2. Species Description

Winged viviparous female (Table 2).

Table 2. Measurements of winged viviparous females of *T. nigroantennatus* sp. nov.

No.	Body	Antenna	Antennal Segments				Ultimate Rostral Segment	Second Segment of Hind Tarsus
			III	IV	V	VIbase + VI Processus Terminalis		
1 Holotype	1.79	1.88	0.64	0.39	0.34	0.21 + 0.20	0.07	0.10
2	1.42	1.62	0.52	0.35	0.30	0.19 + 0.20	0.07	0.11
3	1.40	1.57	0.47	0.35	0.29	0.19 + 0.19	0.07	0.12
4	1.75	1.89	0.59	0.41	0.35	0.22 + 0.21	0.08	0.12
5	1.74	1.87	0.64	0.45	0.40	0.21 + 0.22	0.07	0.10
6	1.70	1.87	0.62	0.39	0.33	0.20 + 0.21	0.08	0.10
7	1.74	1.85	0.63	0.56	0.33	0.22 + 0.21	0.08	0.11
8	1.95	1.87	0.64	0.45	0.38	0.22 + 0.22	0.07	0.10
9	1.74	1.90	0.63	0.43	0.36	0.25 + 0.20	0.07	0.09
10	1.86	1.89	0.63	0.48	0.33	0.20 + 0.21	0.08	0.11

Color in life: uniformly yellow, with antennae black, except for the antennal segments I–II, and the very base of segment III, which are pale (Figure 3A). This same coloration is clearly visible in the fresh fixed specimen (Figure 3B).

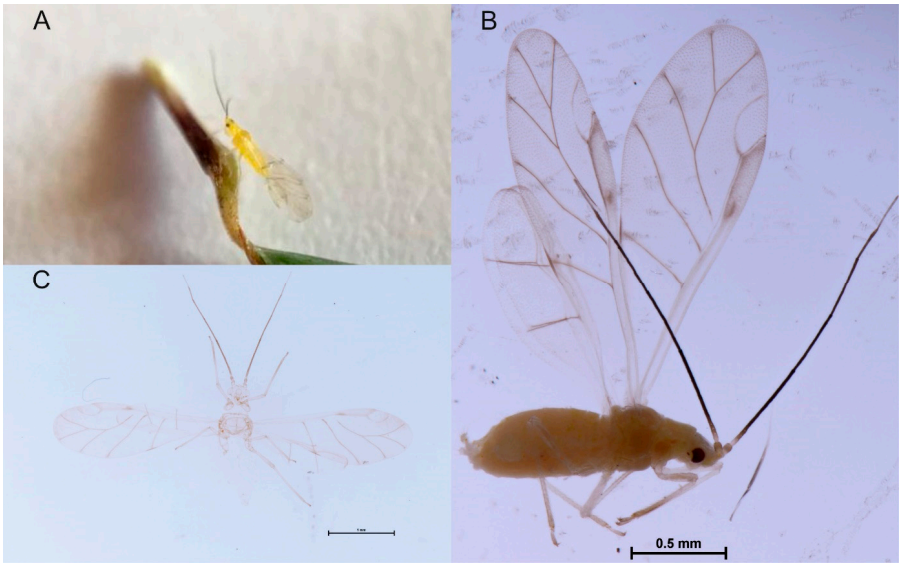

Figure 3. *Takecallis nigroantennatus* sp. nov. winged viviparous female. (**A**)—life; (**B**)—fresh fixed; (**C**)—slide-mounted specimen (holotype DZUS 1/8.22_7).

Pigmentation of cleared specimens on slide: uniformly pale with tarsi dusky and antennae black, except for the antennal segments I–II and the very base of segment III which are concolorous with head. Wing veins dusky (Figure 3C).

Morphometric characters: Body oval. Head with three pairs of anterior and two pairs of posterior short and pointed discal setae about 0.01–0.04 mm, median protrusion on frons developed, epicranial suture and antennal tubercle developed, head dorsum without tubercles (Figure 4A). Antennae 6-segmented, 0.95–1.14 × body length. Antennal segment III, the longest; antennal segment IV slightly longer than antennal segment V; antennal segment V always shorter than antennal segment VI; processus terminalis 0.95–1.05 × base; other antennal ratios: VI:III 0.70–0.86, V:III 0.51–0.56, IV:III 0.49–0.57. Antennal segment III with 3–6 transversely elliptical ciliated secondary rhinaria in a row arranged rather near the base of the segment (Figure 4A); antennal segment IV without secondary rhinaria; base of antennal segment VI with 3 accessory rhinaria. Antennal segments IV–VI and partly antennal segment III imbricated. Antennal segments: I with 4 setae; II with 3 setae; III with 13–16 setae; IV with 5–8 setae; V with 3–5 setae; base with a single seta; processus terminalis with 5 apical setae. Antennal setae pointed, fine and very short, longest seta on antennal segment III 0.30 × basal articular diameter of this segment. Clypeus with large nose-like processus. Rostrum very short, reaching to fore coxae, ultimate rostral segment short, blunted, 0.07–0.08 mm long with 4 accessory setae, 0.33–0.40 × base, 0.61–0.70 × second segment of the hind tarsus. Thorax smooth, without tubercles. Tibiae setose, with numerous stout, pointed setae and rastral organ on its distal part (Figure 4B). First tarsal segment with 4 setae. The forewings typical with a normal venation; the radius strongly curved, the media with three branches. The hind wings have two oblique veins. Pterostigma and wing veins slightly pigmented. Dorsal abdominal tergites I–VII with a pair of spinal and marginal seta on low elevations; spinal setae barely visible. Siphunculus short, cylindrical, with 0.02–0.04 mm of single marginal seta at its base (Figure 4C). Cauda knobbed with 8–11 setae (Figure 4D). Anal plate bilobed, each lobe with 5–9 setae (Figure 4E).

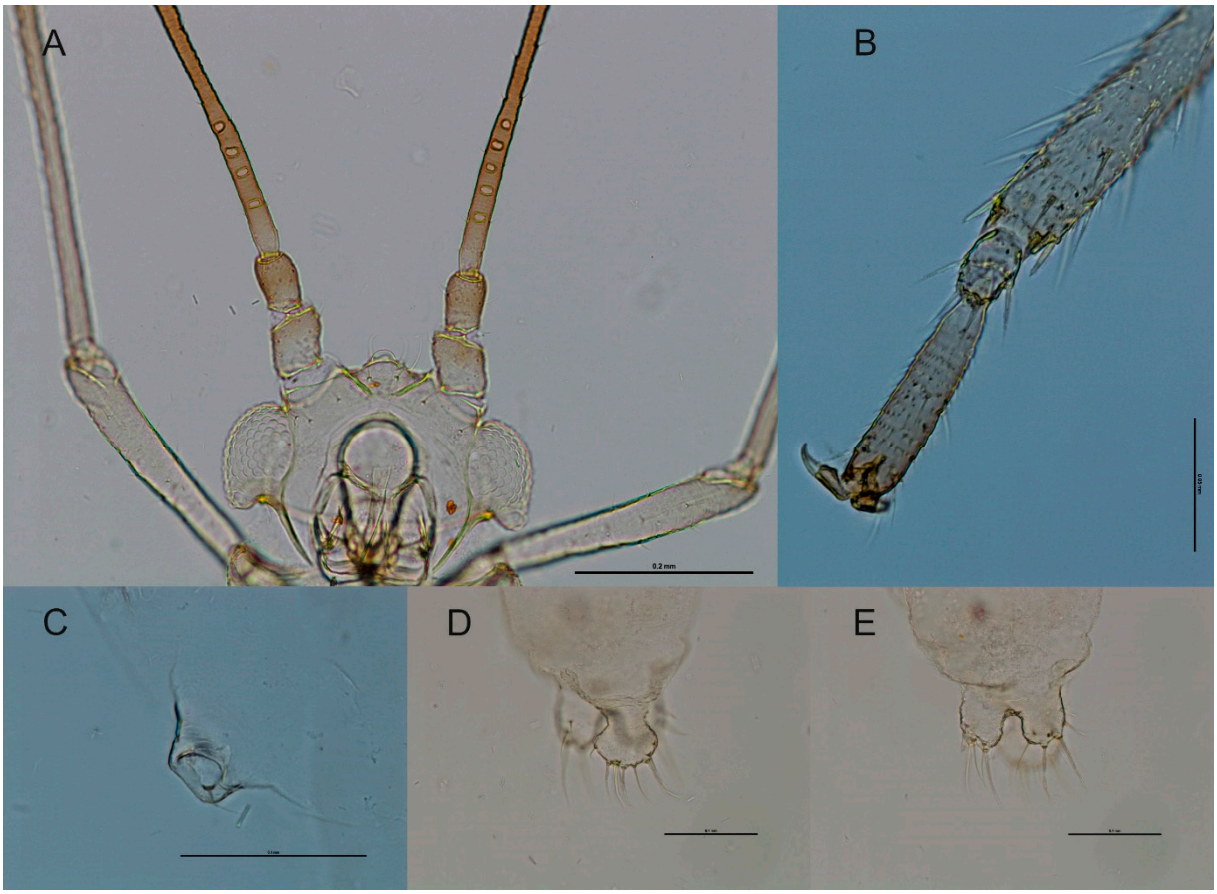

Figure 4. *Takecallis nigroantennatus* sp. nov. winged viviparous female. (**A**)—head and antennal segments I–III; (**B**)—distal part of hind tibia with rastral organ and tarsus; (**C**)—siphunculus with single marginal seta at its base; (**D**)—cauda; (**E**)—anal plate.

3.3.3. Key to Species of the Genus *Takecallis*

1. Antennae longer than body.....2
   – Antennae shorter than body.....8
2. Ultimate rostral segment without any accessory setae. Abdominal tergites 1–7 with paired dark circular spots around spinal setae.....*T. assumenta*
   – Ultimate rostral segment with 3–4 accessory setae. Abdominal tergites 1–7 with or without dark spots.....3
3. Antennal segment I with (4-) 5–7 setae. Antennal segments III–VI dark, except for basal part of antennal segment III. Antennal segment III with 4–15 secondary rhinaria.....4
   – Antennal segment I usually with 4, rarely with 3 or 5, setae. Antennal segments III–VI or IV–VI base usually pale basally and dark apically. Antennal segment III with 3–9 secondary rhinaria.....6
4. Antennal segment III with 10–15 secondary rhinaria, marginal seta on abdominal tergite VI not positioned on base of siphunculus.....*T. affinis* s. str.
   – Antennal segment III with 4–7 (often 5) secondary rhinaria, marginal seta on abdominal tergite VI positioned on base of siphunculus.....5
5. Antennae 1.3 times body length, antennal segment I with 5–7 setae, cauda with 18 setae, each lobe of anal plate with 15 setae, spinal abdominal setae on low elevations.....*T. affinis* ssp. *niitakayamensis*

– Antennae 0.95–1.14 times body length, antennal segment I with 4 setae, cauda with 8–11 setae, each lobe of anal plate with 5–9 setae, spinal abdominal setae barely visible..... *T. nigroantennatus* sp. nov.

6. Thorax and abdomen usually without dark dorsal markings, never with paired elongate dark abdominal patches. Cauda slightly dusky or blackish.....7
   – Thorax with variably-developed longitudinal dark stripes, and abdomen with a pair of elongate dark patches on each tergite. Cauda pale or dusky.....*T. arundinariae*
7. Antennae 3.36–4.00 mm, secondary rhinaria densely concentrated on very short dark
   – Section of proximal third of antennal segment III.....*T. alba*
   – Antennae 2.36–2.51 mm, secondary rhinaria spread over longer dark section oc cupying most of proximal third of antennal segment III.....*T. arundicolens*
8. Secondary rhinaria confined to basal third of antennal segment III. Each abdominal tergite bearing only 2 spinal setae .....*T. taiwana*
   – Secondary rhinaria extending about half of length of antennal segment III. Each abdominal tergite bearing at least 4 setae besides the marginal ones.....*T. sasae*

4. Discussion

In the present study, *T. nigroantennatus* sp. nov. was identified as separate from the *T. arundicolens* complex by either morphology or molecular analysis. This species (Figure 5A) is uniformly yellow, with antennae black, except for the antennal segments I–II, and the very base of segment III, which are pale. Three to six secondary rhinaria of antennal segment III are arranged rather near base of the segment. The body length is 1.4–1.9 mm. *T. arundicolens* (Figure 5B) is characterized by a lack of dorsal abdominal markings, the black cauda and variegated antennae with 5–10 elliptical secondary rhinaria, distributed on the proximal quarter of the III antennal segment. The body length is 1.8–2.8 mm. The third similar species introduced to Europe and occupying the same trophic niche, i.e., the underside of mature leaves of different bamboos species is *T. arundinariae* (Figure 5C). However, winged females of this species are whitish, pale yellow or greyish-yellow, with longitudinal dark stripes on the thorax, paired black elongate spots on abdominal tergite 1–7, variegated antennae with 5–10 elliptical secondary rhinaria, and a pale cauda. The body length is 1.7–2.6 mm.

It can, therefore, be assumed that the *Takecallis* forms with black antennae discovered in the United Kingdom [16,43,44] belong to the newly described species *T. nigroantennatus* sp. nov., and are not a color variant of *T. arundicolens*. This is also supported by the same host plant—both *T. nigroantennatus* sp. nov. and 'black antenna form' from the United Kingdom were collected from *Fargesia* bamboo. At the same time, no other species belonging to the genus *Takecallis* have so far been reported from *Fargesia* spp. Thus, the discovery of a new species may be related to the recent introduction of frost-resistant varieties of bamboo (i.e., *Fargesia* spp.), frequently found in European garden centers. Until now, the species has probably been confused with *T. arundicolens* or overlooked, and its distribution is wider, as evidenced by the finding of winged adult females and immatures of this species on *Fargesia nitida* x *murielae* in the Meise Botanic Garden in Belgium.

Relatively obscure insects that are moved in commerce can become better known in areas where they are non-native. This has happened in the case of *Cinara curvipes* Path or *Appendiseta robiniae* Gillette. Their bionomics are better known in Europe [45,46] than in North America. As all species of the genus *Takecallis* (Figure 6A–D) introduced to Europe are treated as pests, knowledge of their biology and distribution as well as correct identification is needed, especially due to the unique ornamental and aesthetic values of bamboo. Chemical control is still the most important measure to combat populations of aphids. However, it currently is replaced by the plant-pest-biological control agent

interactions as a more sustainable pest management method. Thus, the tritrophic bamboo-aphid-parasitoid interactions should be the cost-effective aphid management measures and significant part of bamboo protections, in particular, in their plantations, botanic gardens, arboretums, parks, or public and private gardens.

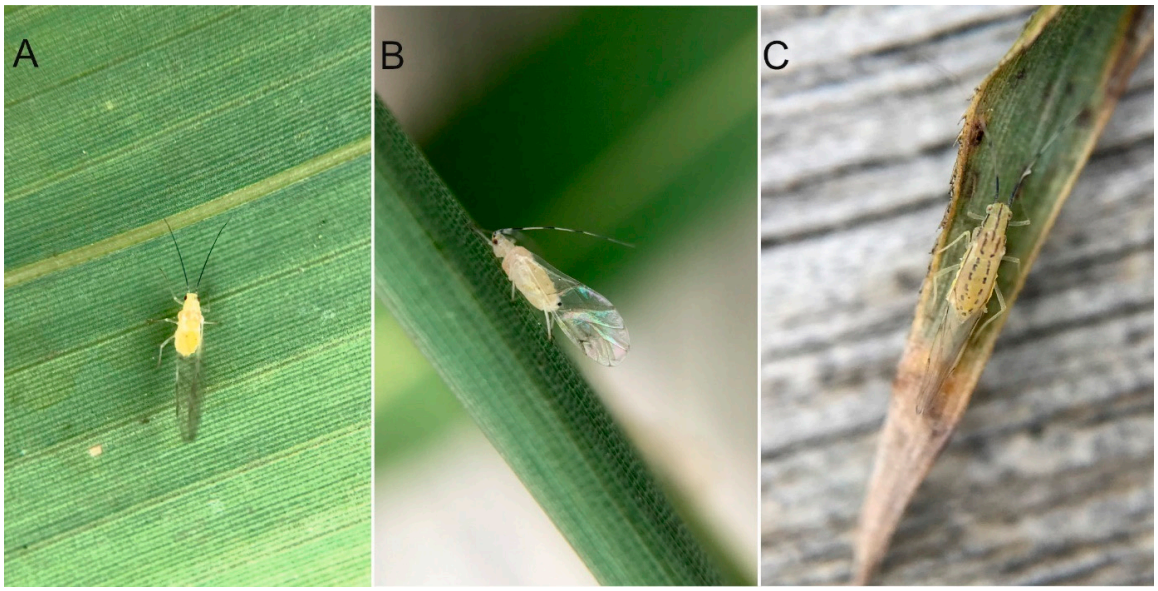

Figure 5. Comparison of yellow-colored winged viviparous female of the genus *Takecallis* introduced to Europe, feeding on the underside of mature leaves of bamboo. Live specimens. (**A**)—*T. nigroantennatus* sp. nov.; (**B**)—*T. arundicolens*; (**C**)—*T. arundinariae*.

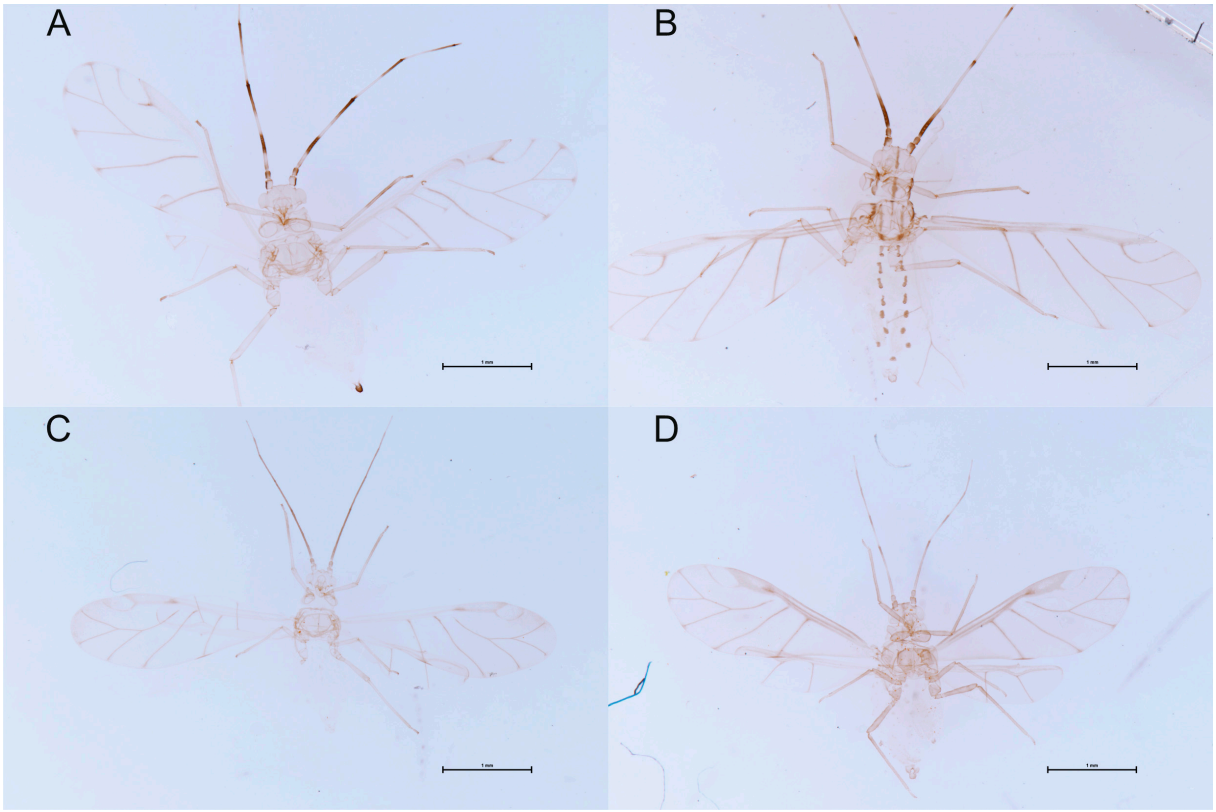

Figure 6. Comparison of winged viviparous female of the genus *Takecallis* introduced to Europe. Slide-mounted specimens. (**A**)—*T. arundicolens*; (**B**)—*T. arundinariae*; (**C**)—*T. nigroantennatus* sp. nov.; (**D**)—*T. taiwana*.

DNA barcoding provides a significant tool to aid in the determination of species boundaries and the discovery of new taxa, evolutionary biology, and conservation. Although sequence variation in traditional DNA barcodes is sometimes insufficient for species-level discrimination in many large clades, the advances in computational and sequencing technology are changing the concept of DNA barcodes, from just a few loci to large, genome-scale sequences from organelles [47]. In particular, with the development of the next-generation sequencing (NGS) technologies, increasing numbers of insect complete mitogenomes (mitochondrial DNA sequence, mtDNA) have been extensively used in species identification for insects [48]. Therefore, in the integrative taxonomy, the use of mtDNA is also promoted as an effective tool in a broad taxonomic toolkit.

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