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Taxonomic Revision of Tribe Aleurocanthini Takahashi 1954 stat. rev. Using Consortium Gene Analysis (Mito-Nuclear-Primary Endosymbiont) with the First Evidence for Mitochondrial Recombination in Whitefly (Hemiptera: Aleyrodidae)

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Abstract: The discovery of the *Aleurocanthus* cf. *Aleurocanthus spiniferus* (Tea spiny whitefly *spiniferus* morphotype; previously found in Tokyo) for the first time in Shizuoka Prefecture raised the possibility that this species had invaded Honshu Island, Japan. Unlike the allied species, *Aleurocanthus spiniferus* and *A. camelliae*, which have been intercepted from China to Japan, the origin of the current species remains unclear. Despite the status of this species as a minor pest on the ornamental plant, *Eurya japonica,* the cryptic diversity among the black spiny whitefly is fascinating to be elucidated, specifically how the primary endosymbiont of whiteflies, *Portiera aleyrodidarum*, coevolved and contributed to the classification of whiteflies. The current study examines the taxonomic status of five species of whiteflies, i.e., *A. spiniferus* (Quaintance), *Aleurocanthus* aff. *A. camelliae, Aleurocanthus* cf. *A. spiniferus*, *A. camelliae* Kanmiya and Kasai, *Aleurotrachelus camelliae* Kuwana, and *A. ishigakiensis* Takahashi. Using consortium molecular typing targeting mitochondrial DNA (COI and 16S of mitoribosome), the nuclear gene (ITS1), and the ribosomal gene of *Portiera,* the phylogenetic clustering analysis has been conducted and revealed that the genus *Aleurotrachelus* sensu lato was clustered together with Aleurocanthini Takahashi, 1954 **stat. rev.** and reinstated *Crenidorsum ishigakiensis* **comb. nov.** due to crescent-shaped scallops being clearly defined. The current study also unveiled several putative species in the *A. spiniferus* species complex, molecularly. In addition, the recombination event was not detected in *Portiera* but has been detected in the mtCOI genes of the *A. spiniferus* cryptic species and the *A. woglumi* sequences deposited in the NCBI database. The mitochondrial recombination gives an insight into the speciation process among this species complex.

Keywords: *Aleurocanthus* cf. *A. spiniferus*; *A. camelliae*; Aleurocanthini; mtCOI recombination; *Portiera*; endosymbiont

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1. Introduction

Cryptic speciation is a biological process that generates cryptic species, two or more taxa combined into a single species, which has not yet resulted in the appearance of discernible morphological characters, and they are genetically more similar than more typical, easily distinguishable species [1]. Such cryptic species are evolutionarily early forms [2,3] or perhaps the retention of a highly conserved morphology due to a stabilizing selection in homogenous habitats [4,5] which is often characterized by a poor dispersal ability [6,7]. Despite the fact that morphological-based species delimitation is a widely

accepted framework in taxonomy, cryptic species eventually emerged as a significant biological issue, particularly concerning how to incorporate the idea of speciation into the taxonomy. The general lineage species concept (GLSC) assumed that speciation is fundamentally a process rather than an event [8,9], thus, "species" should be determined using the speciation evidence that is available from the particular species concept that is being studied. For instance, based on the phylogenetic species concept, "species" from such a cryptic diversity can be described solely by the monophyletic DNA sign as their speciation evidence [9–13]. At this point, the examination of the cryptic species required molecular typing to designate the putative distinct species among populations [14]. In the Aleyrodidae, some species seem to consist of a lot of cryptic diversity with more complex definitions such as *Bemisia tabaci* and *A. camelliae* Kanmiya and Kasai [15,16]. *B. tabaci* has long been considered a complex species and termed with several "biotypes" to designate whitefly populations. However, it was then considered a cryptic species complex, involving at least 39 morphologically indistinguishable species [15,17,18]. A similar case was recorded in *A. spiniferus* which consists of at least seven genetically distinct populations, including *A. spiniferus*, *A. camelliae*, and *A. woglumi* morphospecies [16,19]. These species were also categorized as the invasive pests of tea and citrus, necessitating an accurate identification for their detection and dispersal control.

The cryptic species problem in whitefly perhaps is also an indication of inadequate taxonomy [20] since it has long been suggested that the morphological characteristics of puparium appear to be non-genera specific and conflict with tribal circumscriptions [21]. Such a case is seen in the genus *Aleurotrachelus* Quaintance and Baker which is a polyphyletic group and shares similar traits with *Cohicaleyrodes* Bink-Moenen and *Crenidorsum* Russell [22,23]. Such a conflict is also found at the tribal level. Specifically, the tribe Aleurocanthini, which was initially proposed by Takahashi [24], consists of two allied genera, *Aleurocanthus* and *Aleurotrachelus*. Following the initial description, Miyatake [25] placed two more genera, *Pentaleyrodes* and *Mixaleyrodes*. Then, in 1990, David [26] revised the definition of the tribe Aleurocanthini as a group with many prominent spines on the dorsum. This definition resulted in the replacement of the genus *Aleurotrachelus* from the tribe Aleurocantini to the tribe Aleyrodini. Per his description, the whitefly was reclassified into at least twelve tribes existing in India, but this classification failed to accommodate *Mixaleyrodes indicus* David and Selvakumaran, which later was revised into the genus *Cohicaleyrodes* [27]. Therefore, a molecular confirmation is needed to provide a better assessment of the tribal definition of Aleurocanthini, including the *Aleurotrachelus* sensu lato (s.l.) species.

Mitochondrial COI (mtCOI) is the common genetic marker used to determine cryptic species in whiteflies [28,29]. However, when closely related species have previously undergone a hybrid introgression with one another, mitochondrial DNA markers cannot be utilized to identify the species [30–35]. Moreover, sometimes a recombination happens in the mtDNA of insects [36–38] even though it is commonly accepted that unlike plants, fungi, and protists [39], the mtDNA recombination is considered to be absent in animals [40]. Despite the fact that a number of studies in recent years have questioned whether the absence of a recombination holds throughout the animal kingdom, all widely used phylogenetic reconstruction methods currently operate under the presumption that all mtDNA genes have undergone the same evolutionary process [38]. On the other hand, a recombination will lead to various evolutionary histories for the particular parts of a given sequence, which ultimately causes an issue in the mtDNA-based identification. Therefore, integrative molecular typing, such as the combination of nuclear gene typing with a reproductive isolation assay [15] or primary endosymbiont characterization [41,42] in the cryptic species instead of the mt-COI as the sole approach, becomes important to understand the cryptic speciation itself and for species delimitation.

Whitefly harbor the obligatory primary endosymbiont, *Portiera aleyrodidarum* (hereinafter referred to as *Portiera*) to provide essential nutrients [43–46] since plant sap is rich in carbohydrates and deficient in amino acids and other nitrogenous compounds [47,48].

The strict association between *Portiera* and whitefly was eventually proved by the co-cladogenesis phenomenon, the branching or diversification of lineages over time that results in congruent clades between *Portiera* and whitefly [49]. The lineage reconstruction of *Portiera* has been suggested to be much better than the mtCOI tree to represent the subfamily to the tribal level of whitefly [42,44,50–52].

Therefore, the current study aims to examine the cryptic diversity of *A. spiniferus* s.l. using the consortium molecular typing targeting mitochondrial DNA (COI and 16S), the nuclear gene (ITS1), and 16Sr RNA of *Portiera*. In addition, the tribal taxonomy of genera *Aleurocanthus* and *Aleurotrachelus* s.l. were tested using this framework.

2. Materials and Methods

2.1. Sample Collections and Morphological Identification

The five species of whiteflies belong to Tribes Aleurocanthini Takahashi, i.e., *Aleurocanthus spiniferus* (Quaintance), *Aleurocanthus* cf. *A. spiniferus*, *A. camelliae* Kanmiya and Kasai, *Aleurotrachelus camelliae* (Kuwana), and *A. ishigakiensis* Takahashi were collected from Shizuoka, Tokyo, Shiga, and Kagoshima Prefectures. *A. spiniferus* (Quaintance) was collected from Citrus plants in the Citrus Research Station, the Institute of Fruit Tree, and the Tea Science of National Agriculture and Food Research Organization (NARO), Shizuoka City (35°03′16.6″ N 138°31′20.7″ E); *Aleurocanthus* cf. *A. spiniferus* was collected from *Eurya japonica* in Otta City, Tokyo (35°35′24.9″ N 139°39′56.3″ E), Ito City- Shizuoka (10.VII.2022), and Shizuoka City, Shizuoka (34°57′52.9″ N 138°25′34.7″ E); *A. camelliae* was collected from *Camellia sinensis* in Kagoshima (7.III.2022), *A. camelliae* (34°57′46.6″ N 138°25′48.9″ E) and Shiga (35°11′51.2″ N 135°55′24.1″ E); and *A. ishigakiensis* was collected from *Hedera rhombea* in Shizuoka City (34°57′46.6″ N 138°25′48.9″ E).

The puparium samples were slide-mounted after being bleached using hydrogen peroxide (H_2O_2) overnight. The species' descriptions of *Aleurocanthus* spp. [53–56], *A. camelliae* Kuwana [57], and *A. ishigakiensis* (Takahashi) [58] have been applied for the morphological identification of the puparium whiteflies collected. In addition, the key to the genus of *Aleurotrachelus*, *Cohicaleyrodes*, and *Crenidorsum* [23] was also applied to a reappraisal of the genera placement of *Aleurotrachelus* spp. All slides mounted were deposited in insect collections of the Applied Entomology Laboratory, Shizuoka University (SU)

2.2. DNA Extraction and Polymerase Chain Reaction (PCR)

The genomic DNA of each individual was extracted using a slightly modified Hot-Shot method, as described in the previous study [16]. The amplification reaction was performed in a total volume of 20 μL of KOD FX (TOYOBO, Osaka, Japan) containing 10 μL of 2 X PCR buffer for KOD FX, 4 μL of 2mM dNTPs, 0.8 μL of each primer, 1 μL of DNA template, 3 μL of distilled water, and 0.4 μL of KOD FX. The thermocycler protocol was: pre-denaturation at 95 °C (2 min) followed by 37 cycles of 95 °C of denaturation (30 s), annealing temperature (Table 1) for 50 s, extension at 68 °C (1 min), and a subsequent final extension step at 68 °C (5 min). The PCR products were visualized on agarose gel after electrophoresis.

Table 1. Universal and specific primer sets.

No.	Primer Name	Sequence (5′→3′)	Annealing (°C)/Size (bp)	Gene Target	Ref.
		A. Primary endosymbionts			
1	Por16S-431F Por16S-1020R	CAGAAGAAGCACCGGCTAAC ATTTCACACACGAGCTAAC	55/590	16S rRNA (*Portiera*)	This study
		B. Mitochondrial DNA of whitefly			
2	16Sar 16Sbr	CGCCTGTTTAACAAAAACAT CCGGTCTGAACTCAGATCACGT	53/212	rrnL = 16S rRNA	[59]

3	TSW-F TSW-R	ATTTCACACTTAATTAGGAGTGA CTGCACGAAATACAACAAATG	53/680	COI	[19,60]
4	OSW-F OSW-R	GTGTCCCATTTAATTAGTAGAGA GAGCCATAATAAAAGACTCCATC	53/680	COI	[19,60]
5	LCO1490 HCO2190	GGTCAACAAAATCATAAAAGATATTGG TAAAACTTCAGGGTGACCAAAAAATCA	52/700	COI	[61]
		C. Nuclear gene (ITS1)			
6	TW81 5.8R	GTTTCCGTAGGTGAACCTGC ATCCGCGAGCCGAGTGATCC	54/450	ITS 1 of rDNA	[62]

2.3. DNA Sequencing and Phylogenetic Analysis

The amplified fragments of the representative samples were directly sequenced by a commercial Sanger sequencing service (Fasmac; Atsugi, Japan). The sequences obtained were aligned with ClustalW in MegaX [63], and a phylogenetic analysis was conducted using neighbor-joining (NJ) [64] and maximum likelihood (ML) methods [65] in a 1000 bootstrap replication.

2.4. Recombination Detection and Genetic Diversity

RDP5 [66] was used to assess the detection of putative recombinants in multiple sequence alignments from mtCOI (COI-2) downloaded from the database (Table S1). RDP [67], GENECONV [68], BootsScan [69], MaxChi [70], ChiMaera [71], SiScan [72], Phylpro [73], LARD [74], and 3Seq [75] were the nine techniques used in the analysis. The program's default search parameters were applied, and a p-value ≤ 0.05 was considered to be acceptable. The genetic diversity parameters of the *A. camelliae* species complex sequences, such as the number of segregating sites [76], the number of haplotypes (h), haplotype diversity (Hd) [77], and nucleotide diversity (π/bp) [77], were estimated using DNASP version 6 [78]. Using this software, the neutrality test was conducted using Tajima's D [79] and Fu and Li's D* and F tests [80].

3. Results

3.1. Morphological Identification

An asterisk (*) indicates a new host plant or distribution record and an obelisk (†) indicates the host plant which was sampled.

Aleurocanthus* cf. *A. spiniferus (Figure 1A,B).

Diagnosis: Having 11 submarginal spines (Figure 1A) and inhabit tea-related plants (*Eu. Japonica*, Pentaphylaceae) (Figure 1B), has about more than 200 marginal teeth and the sub-median abdominal spines are not in line; 2nd and 4th placed distal (blue arrows, Figure 1A) and 3rd and 5th proximal (yellow arrows, Figure 1A). The pattern of the transverse molting suture differs from *A. spiniferus* in appearance (Figure S1).

Material examined: 12 puparia on 10 slides, Japan, Shizuoka City, Shizuoka University, E. Andrianto, deposited in SU.

Distribution: Japan (Ito City, Shizuoka and Ota City, Tokyo).

Host plant: Pentaphylacacea: †*Eurya japonica.*

Remarks: In Japan, the tea spiny whitefly (TSW, *A. camelliae*) has a separated ecological character from the citrus spiny whitefly (OSW), *A. spiniferus*, which is only found in tea-related plants (*Theaceae* sensu lato, including *Pentaphylaceae*) [19]. Additionally, there is a distinct morphologically on a number of marginal teeth and the arrangement of submedian abdominal spines number 2nd to 5th. However, these characteristics failed to separate the current species from *A. spiniferus*.

Figure 1. Tea spiny whitefly *A. spiniferus* morphospecies. (**A**) Slide-mounted puparia, (**B**) high infestation on *Euryae japonica* leaves in Ito City, Shizuoka.

***Aleurotrachelus camelliae* (Kuwana)** [53] (Figure 2A,B).
Syn. Aleyrodes camelliae Kuwana [57].

Diagnosis: Shiny black puparium with an almost octagonal form and a thin layer of clear wax covering it. The anterior margin of the cephalothorax is pointed and broadest at the position of the transverse molting suture. On the thoracic, a smooth region surrounds the thoracic segments. The vasiform orifice is subcordate, the posterior outer margin is somewhat split, the inner margin is without teeth, and the operculum is similarly formed and filling orifice.

Materials examined: 3 puparia on 3 slides, Japan, Shizuoka City, Shizuoka University, E. Andrianto, deposited in SU.

Distribution: Japan, China, Hong Kong.

Host plants: Theaceae: †*Camellia japonica*, *Camellia sinensis*, *Camellia sinensis* var. *sinensis*.

Remarks: The living image resembles the late instar of *Aleuroplatus* species [81,82] but is different by having a pair of cuticular longitudinal folds or longitudinally pigmented areas in the slide mounted (Figure 2B).

***Crenidorsum ishigakiensis* (Takahashi) comb. nov.** (Figure 2C,D)
Syn. Trialeurodes ishigakiensis Takahashi [58].
Aleurotrachelus ishigakiensis (Takahashi) [83].

Diagnosis: White to yellowish. Slightly indented at the hind end, with no distinct ridge on the cephalothorax. The dorsum, with numerous small circular pores, a little concaved (crescent-shaped scallops). A pair of cephalic setae and metathoracic setae. The thoracic tracheal folds are distinct. The caudal furrow is wanting. The vasiform orifice has a huge subcordate which is notched, it is not on an elevated area, it is rounded on the rear edge, and it is as long as it's wide. The operculum fills more than half the orifice.

Material examined: 5 puparia on 5 slides, Japan, Shizuoka City, Shizuoka University, E. Andrianto, deposited in SU.

Distribution: Japan, Korea.

Host plants: Araliaceae: *Gilibertia trifida*, †*Hedera rhombea*; Cornaceae: *Cornus* sp.; Daphniphyllaceae: *Daphniphyllum macropodum,* *†*Daphniphyllum teijsmannii*; Euphorbiaceae: *Sapium japonicum*; Fabaceae: *Pueraria hirsuta*. Lauraceae: *Lindera obtusiloba*; Moraceae: *Ficus erecta*, *Morus alba*, *Morus bombycis*; Pittosporaceae: *Pittosporum tobira*, *Pittosporum tobira* 'variegata' Pentaphylacacea: †*Eurya japonica*; Umbelliferae: *Heracleum lanatum*.

Remarks: Transfer to the genus *Crenidorsum* based on the crescent-shaped appearance of the sub-median/subdorsal folds.

Aleurocanthus* aff. *A. camelliae (Figure 2E–I).

Diagnosis: The crenulation or number of marginal teeth is less than 200 or 7 to 9 per 100 μm (*A. camelliae* characters), the sub median abdominal spines are not in line; 2nd and 4th placed distal and 3rd and 5th proximal (*A. spiniferus*). The pattern of the transverse molting suture is close to *A. camelliae* and differs from *A. spiniferus* in appearance (Figure S1).

Material examined: 5 puparia on 5 slides (three♀ and two♂), Japan, Shizuoka City, Shizuoka University, E. Andrianto, deposited in SU.

Distribution: *Japan (Shizuoka City).

Host plant: Pentaphylacacea: *†*Eurya emarginata.*

Remarks: One of the essential characteristics which distinguishes *A. camelliae* from *A. spiniferus* is the number of marginal teeth. In 1928, whitefly inhabiting an unidentified plant was designated as *A. spiniferus* var. *intermedia* by Silvestri [55] due to the characteristics of having fewer of the number of marginal teeth (8 or 9 per 100 μm) than a description of Quaintance [84] on the syntypes *A. spiniferus* on *Citrus* sp. and *Rosa* sp., (Garolt (=Garut), Java, Indonesia) (12 per 100 μm). The arrangement of the sub-median abdominal spines perhaps varies on whitefly inhabiting *Eu. emarginata,* but the number of submarginal teeth tends to be *A. camelliae.*

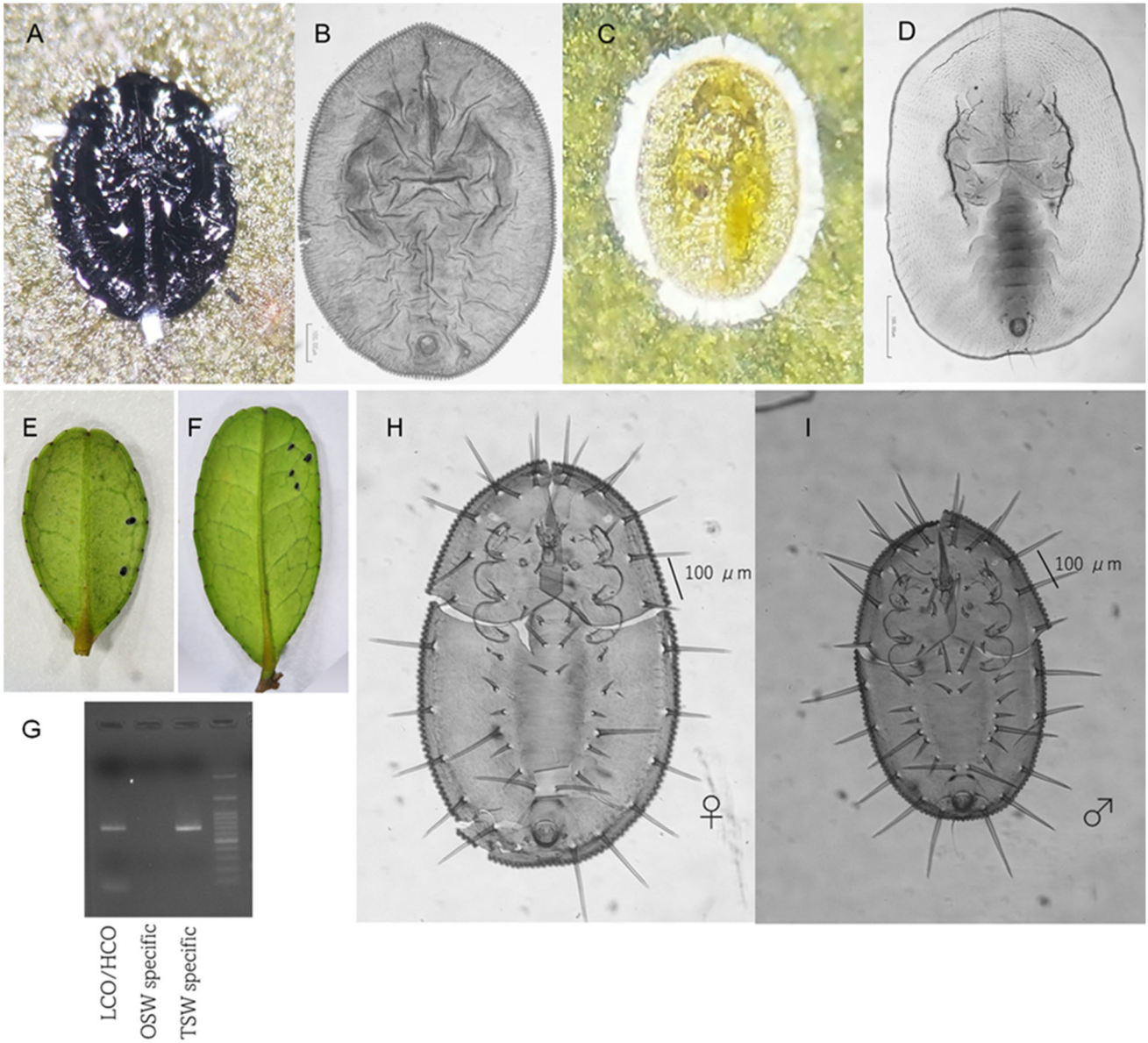

Figure 2. The Aleurocanthini samples examined. (**A**,**B**) *Aleurotrachelus camelliae* Kuwana, (**A**) habitus; (**B**) puparium slide-mounted); (**C**,**D**) *Crenidorsum ishigakiensis* **comb. nov.,** (**C**) habitus; (**B**) puparium slide-mounted; (**E**,**F**) habitus of whitefly on *Eu. emarginata* leaves, (**E**) nymphs for molecular identification, (**F**) exuviae for slides-mounted, (**G**) PCR visualization of general and specific mtCOI primers [60,61] (**H**,**I**) dimorphism of *Aleurocanthus* aff. *A. camelliae*, (**H**) female puparium slide-mounted, (**I**) male puparium slide-mounted.

3.2. Nuclear Gene Analysis in A. spiniferus Species Complex

The tea spiny whitefly collected from Ito City-Shizuoka was genetically identical to *Aleurocanthus* cf. *A. spiniferus* from Tokyo (Figure 3). This whitefly species was separated from *A. camelliae* and *A. spiniferus*. Interestingly, the ITS1 gene analysis was clustering *A. spiniferus* into the same clade with *A. camelliae* both "*spiniferus*" (*Aleurocanthus* aff. *A. camelliae* on *Eu. emarginata*) and "*camelliae*" (*A. camelliae* on *Eu. japonica*) morphotypes. The two morphotypes (the arrangement of sub-median abdominal spines) are confirmed as *A. camelliae* haplogroup B1 based on the mtCOI analysis (Figure S2).

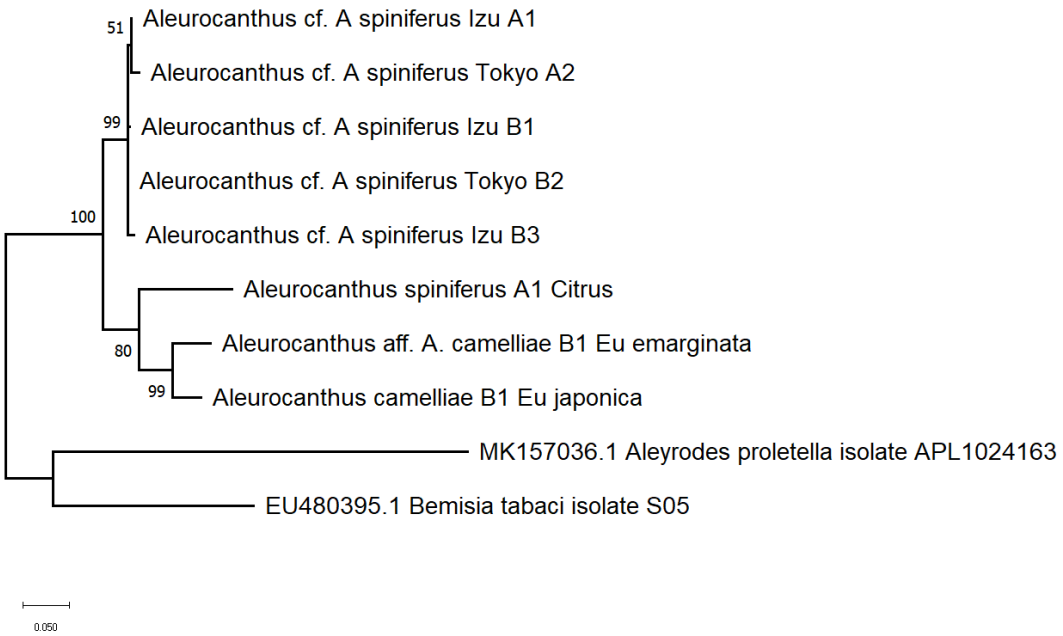

Figure 3. The ML phylogenetic tree of *A. camelliae* species complex is based on the ITS1 gene constructed using the Tamura and Nei model [65]. The whiteflies *Aleyrodes proletella* (MK157036.1) and *Bemisia tabaci* (EU480395.1) were assigned as out-group.

3.3. Estimation of Genetic Diversity of A. spiniferus Species Complex

Eighty-seven sequences retrieved from the database (Table S1) were analyzed to estimate the current genetic diversity of the *A. spiniferus* cryptic species. The genetic diversity of *A. camelliae* is lower than *A. spiniferus* (Table 2). There are five haplotypes (h) with a low haplotype diversity (Hd: 0.3), while *A. spiniferus* consist of 12 haplotypes (including *A. woglumi*) and is considered to have a moderate-to-high diversity of haplotypes (Hd: 0. 72). The diversity of the nucleotides among *A. spiniferus* is more than two times higher (π: 0.03793) than that in the *A. camelliae* group (π: 0.01463).

Table 2. Haplotype diversity of the *Aleurocanthus spiniferus* species complex was estimated from 450 bp COI-2 sequences retrieved from the NCBI database. *A. spiniferus* species complex includes *A. camelliae, A. spiniferus,* and *A. woglumi* sequences.

Gene	Sample Pool	N	S	h	Molecular Diversity Indices			Neutrality Tests	
					Hd	π	k	Tajima's D (*p*)	Fu and Li's F (*p*)
COI-2	*A. camelliae*	28	63	5	0.27	0.01463	7.94	−1.99361(<0.05) *	−1.53563(>0.10) ^{ns}
	A. spiniferus	59[a]	173	12	0.72	0.03793	20.59	−1.94440(<0.05) *	−3.01549(<0.05) *

N, number of sequences; S, number of segregating or polymorphic sites; h, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; k, mean number of nucleotide differences. [a] Include the *A. woglumi* sequence (ID JX281760); ^{ns} $p > 0.10$, * $p < 0.05$, level of significance of Tajima's D and Fu * Li's F tests.

There are at least 17 haplotypes in total (Table 2; Figure 4) consisting of 3 morphospecies, i.e., *A. spiniferus* (haplotypes 1,10, and 11; Figure 4), *A. camelliae* (haplotypes 6, 14–17; Figure 4), and *A. woglumi* (haplotype 12; Figure 4) which have been described and two putative species, i.e., **putative species 1**: *Aleurocanthus* cf. *A. spiniferus* (haplotype 13) and **putative species 2** or *A. spiniferus* haplogroup A2 (haplotypes 2–5, 7–9). The high number of median vectors (mv) observed in *A. camelliae*, i.e., mv2–mv4, and mv8–mv10, suggested the high number of unexamined haplotypes or those which are extinct associated with *A.*

camelliae morphotypes. The *Aleurocanthus* aff. *A. camelliae* inhabit *Eurya emarginata* (Figure 2E–I) is a new haplotype associated with the *A. camelliae* B1 cluster (Figure S2).

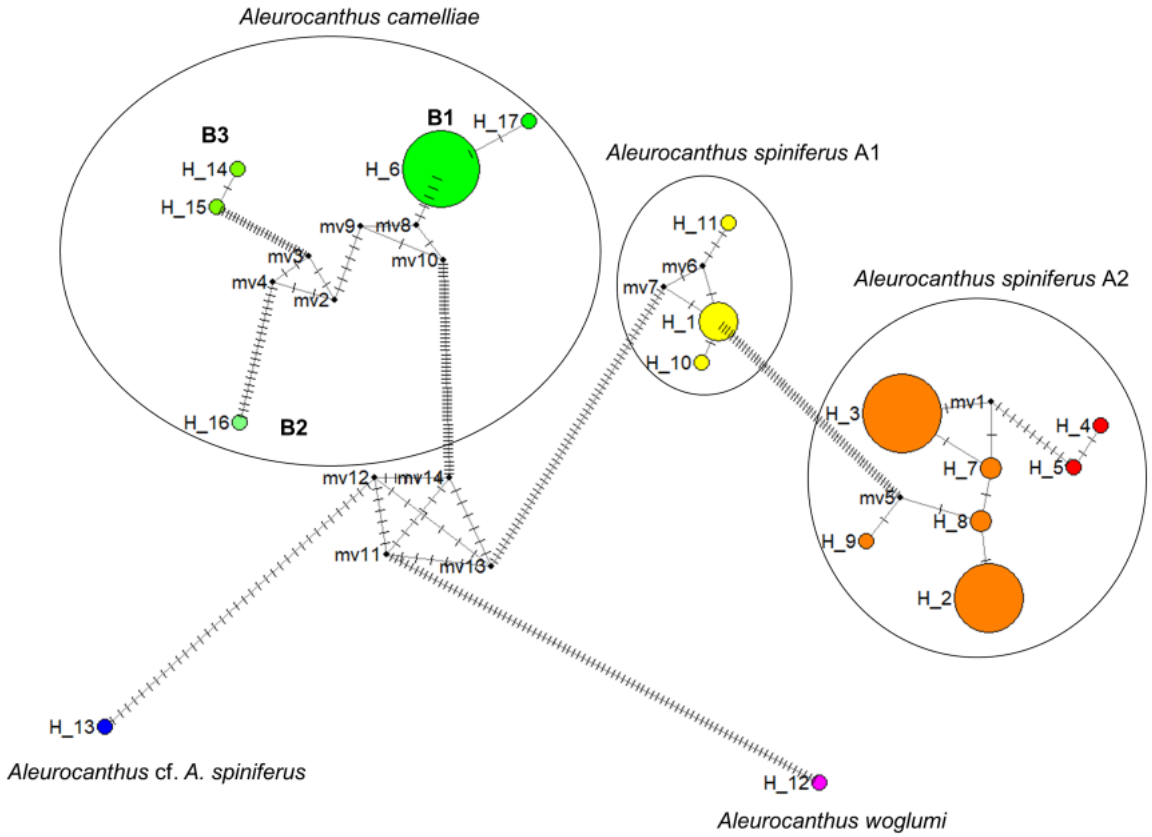

Figure 4. Haplotype network diagram based on mtCOI (COI-2) sequences of *Aleurocanthus spiniferus* species complex. Green nodes are *A. camelliae* haplotypes; yellow and orange nodes are *A. spiniferus* haplogroup A1 and A2, respectively; blue node is *Aleurocanthus* cf. *A. spiniferus*; purple node is *A. woglumi*; red nodes are *A. spiniferus* A2 recombinant haplotypes. Black nodes are median vectors. Striped lines indicate the number of nucleotide mutations. The size of nodes indicates the number of sequences clustered in haplotypes.

3.4. Mitochondrial Recombination in A. spiniferus Species Complex

The recombination events have been detected on the mtCOI of *A. spiniferus* Species Complex; first, the sequences of *A. spiniferus* A2 Greece haplotypes (IDs MH700446 and MH700445) (Haplotypes 4 and 5, Figure 4) and second, *A. woglumi* (ID JX281760). The putative recombinant was confirmed by at least four analysis methods. About 23 nucleotides (Figure 5A,B) are inserted into the first recombinant event with *A. spiniferus* A2 (AB786718.1 AS) as the predicted major parent (similarity 99.5%). A. *woglumi* sequence (ID JX281760) suggested being recombinant with about 294 nucleotides (Figure 5C) inserted from a minor parent, *Aleurocanthus* cf. *A. spiniferus* (OP323057.1 ASC) (similarity 86.8%).

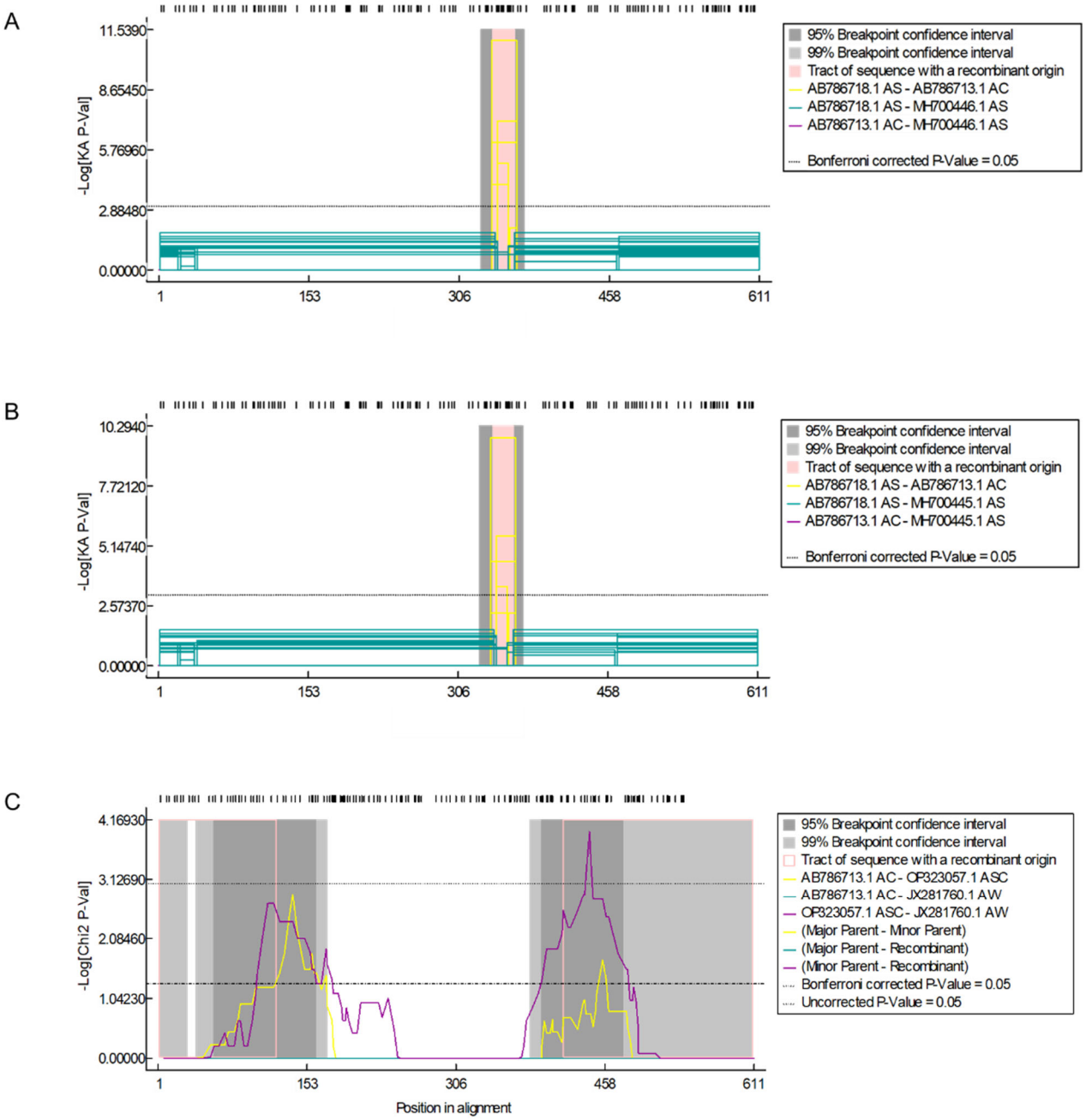

Figure 5. Recombination hotspots analysis. (**A**,**B**) Recombinant event 1 GENECONV output of sequences ID MH700446.1 AS- MH700445.1 AS. (**C**) Recombination event 2 MaxChi output of the sequence ID JX2817601. AW (possibly sequence ID OP323057.1 ASC is the real recombinant). The pink or pink border region denotes the presence of the recombination event, with the overlapping peaks on the plot serving as the recombination's start and end breakpoints. The 99% and 95% confidence intervals for the breakpoint prediction are represented by the grey zone at the ends.

3.5. Molecular Placement of Tribe Aleurocanthini Takahashi

Based on the phylogenetic clustering analysis of the 16S mitoribosome and 16S r RNA of Portiera, the putative genera members of the tribe Aleurocanthini were *Aleuroplatus, Crenidorsum, Tetraleurodes, Aleurotrachelus, Aleurocanthus* (Figure 6), *Aleurothrixus*, and *Acaudaleyrodes* (Figure 7) with a moderate bootstrap support of 50% in the ML analysis. The genus *Aleurotrachelus* was separated from the genus *Aleyrodes* (Figures 6 and 7), confirming that *Aleurotrachelus* should not be placed as the same tribe as *Aleyrodes* (tribe Aleyrodini Sampson). The clustering among those genera then failed to support the

existence of the tribe Tetraleurodini David. Since a lack of an Aleurolobus specimen, the ML cladogram of the 16S mitoribosome cannot confirm whether the *Bemisia* genus should be placed as a member of the tribe Aleurolobini Takahashi or Bemisini David. On the other hand, the Aleurolobus clustered together with Bemisia as the same tribe of Aleurolobini in the *Portiera*-based phylogenetic tree (Figure 7). The only *Singhiella simplex* was clustered with the tribe Aleurolobini (Figure 7), and the 16S mitoribosome-based cladogram placed it in the genus of *Bemisia* clades (Figure 6). Because of inadequate support, the current tree (Figure 6) cannot justify tribes such as Aleyrodini Sampson, Siphoninini Sampson, Dialeurodini Sampson/Trialeurodini Russell, and Aleurochitonini Sampson, which are identified by their typical genus. On the other hand, the genus *Apobemisia* was clustered with *Pealius*, suggesting that this genus has an affinity to *Pealius* instead of Bemisia.

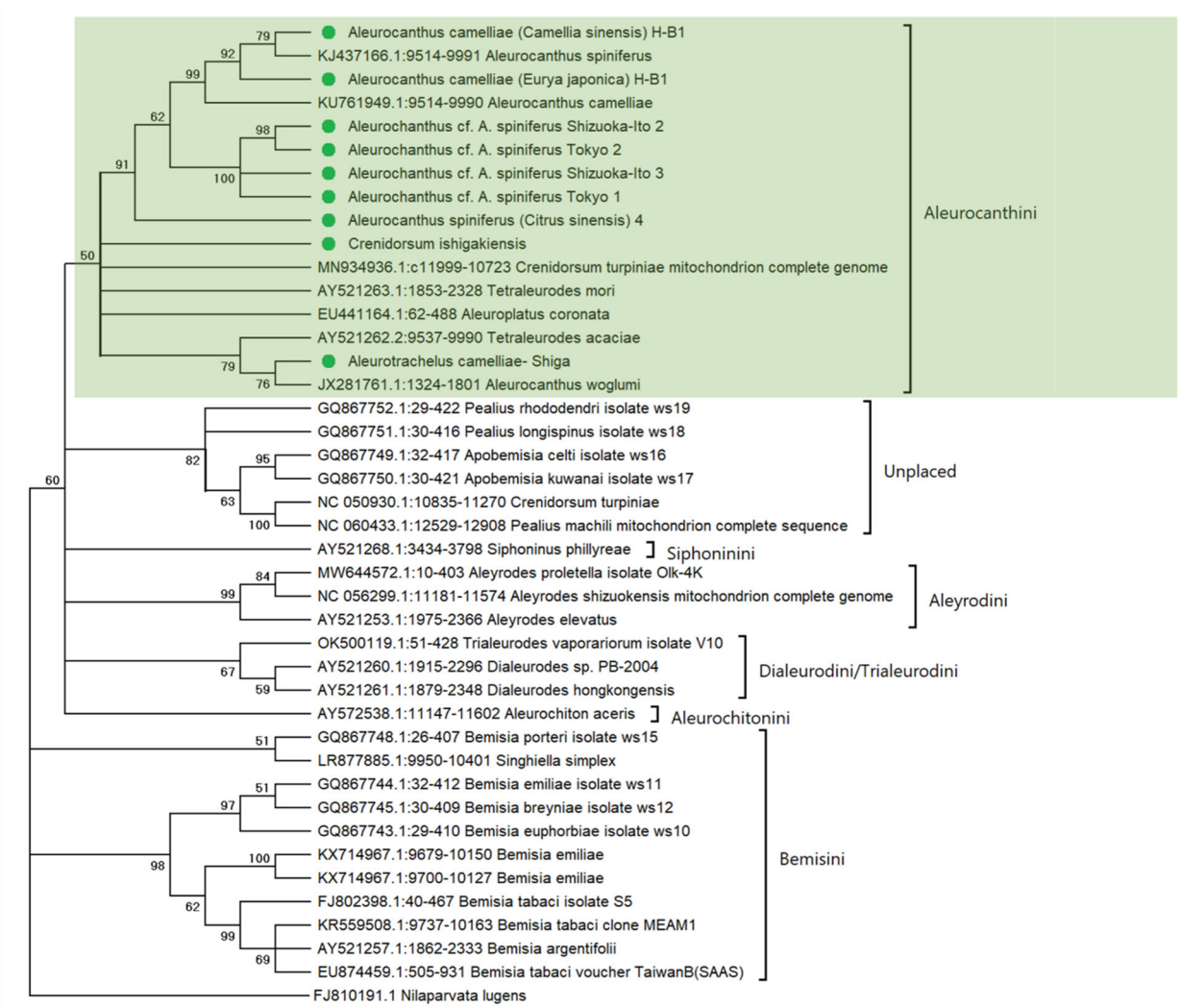

Figure 6. Cladogram of 16S mitoribosome constructed using Jukes–Cantor model [85] with partial deletion option. Forty-two sequences retrieved from the database were included in the analysis. The sequence of *Nilaparvata lugens* (No. FJ810191) was placed as an out-group. The green circles are the samples examined. Alignment length 416 bp. Note: identical sequences of *Crenidorsum turpiniae* (NC_050930.1) with *Pealius machili* (NC_060433.1) suggested they were contaminated or misidentified.

The phylogenetic tree reconstruction on these single gene analyses (Figures 6 and 7) failed to illustrate the clustering genera at least in the tribe Aleurocanthini. In the phylogenetic reconstruction of *Aleurocanthus woglumi*, for instance, both the mitoribosome (ID JX281761) and *Portiera* (ID JX281794) are consistently paraphyletic within *Aleurocanthus*

species. The same pattern was found in the sequences *Tetraleurodes mori* (ID AY521262) and *Tetraleurodes acaciae* (ID AY521262) on the mitoribosome phylogenetic tree (Figure 6). The *Portiera* strains of Aleurotrachelus spp. were separated into at least three clusters (Figure 7), i.e., *Crenidorsum (=Aleurotrachelus) ishigakiensis, Aleurothrixus (=Aleurotrachelus) trachoides* (ID LR738504), and *Aleurotrachelus camelliae*. However, interestingly, all molecular typing using ITS1, the 16S of mitoribosome, and the 16SrRNA of *Portiera* reconstructed the close association of *A. spiniferus* to *A. camelliae* instead of *Aleurocanthus* cf. *A. spiniferus* (Figures 3, 6 and 7).

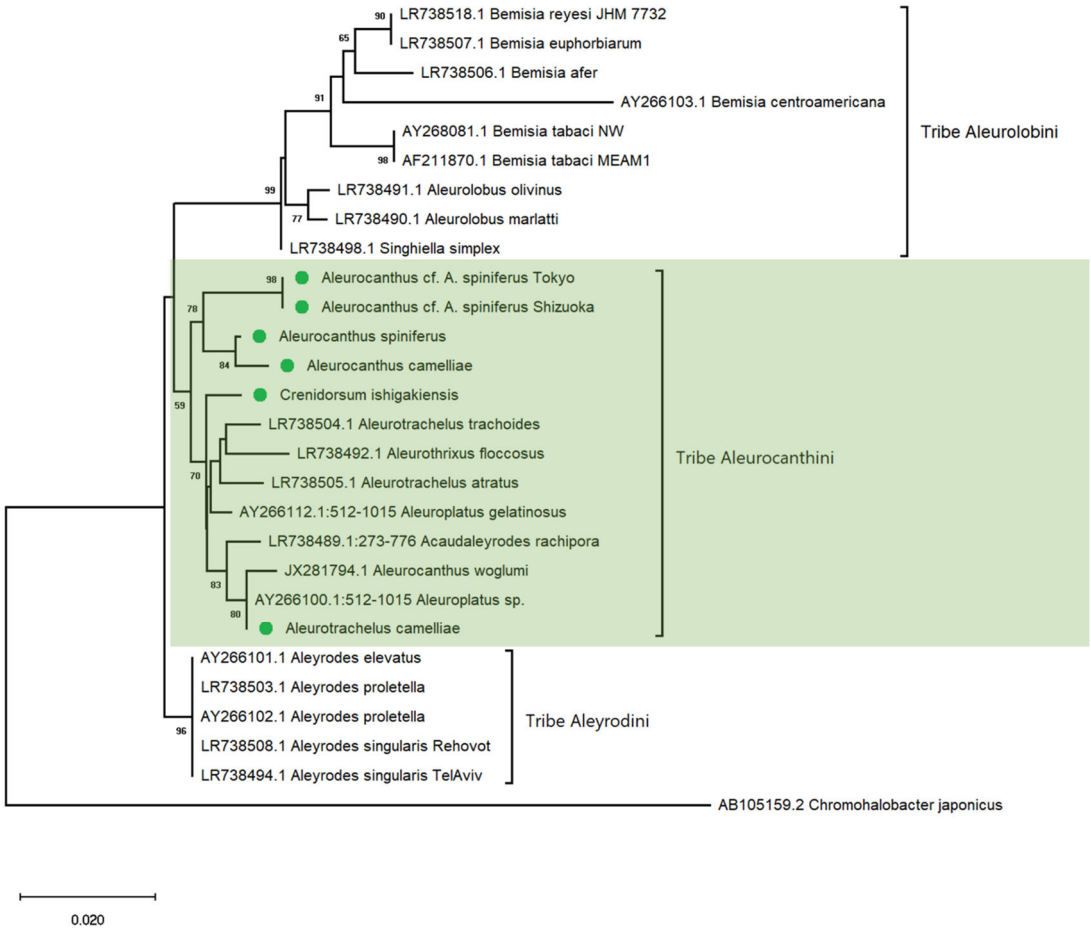

Figure 7. *Portiera* strain clustering of 16Sr RNA by NJ phylogenetic tree constructed using LogDet (Tamura–Kumar) method [86]. The sequence of *Chromohalobacter japonicus* (No. AB105159) was placed as an out-group. The green circles are the samples examined. Alignment length 504 bp.

4. Discussion

The basic issue of whitefly taxonomy is the placement of species in tribes and genera [21]. Some species were incorrectly placed into genera, which subsequently caused a more complicated reappraisal of the tribes. For instance, the genus *Apobemisia* was proposed by Takahashi from the type-species *Bemisia kuwanai* Takahashi and included *Pealius celti* Takahashi [24]. Then, these species became the only two members of the *Apobemisia* genus, *A. kuwanai* (Takahashi) and *A. celti* (Takahashi). The genus was designated as a member of the tribe Aleurolobini, which was subsequently associated with the genera *Aleurolobus*, *Bemisia*, *Asterobemisia*, *Parabemisia*, *Metabemisia*, *Acanthobemisia*, and *Heterobemisia* [25]. However, instead of being allied into the genus *Bemisia* or even a separated genus, these species were perfectly grouped into the genus *Pealius* molecularly (Figure 6). Thus, it is suggested that the genus *Apobemisia* is actually congeneric with *Pealius* **syn. nov.**

Therefore, *Pealius kuwanai* (Takahashi) comb. nov., and *Pealius celti* Takahashi **rev. comb.** were eventually also proposed here. The genus *Aleurocanthus* as well as *Aleurotrachelus* is another example of a very complex genus [23,87]. They were suggested to be allied with some distinct genera [23,87]. Some species of *Aleurotrachelus* have been transferred to the allied genera, such as *Cohicaleyrodes* and *Crenidorsum*. The morphological characteristics, such as the coloration and crescent-shaped scallops of *Aleurotrachelus ishigakiensis,* suggested that this species should be transferred into the genus *Crenidorsum*. The molecular analysis then supported the separation of the *Aleurotrachelus* s.l. and *Crenidorsum ishigakiensis* **comb. nov.** (Figures 6 and 7; Figure S2).

The *Aleurocanthus* and *Aleurotrachelus* genera are assigned to the tribe Aleurocanthini Takahashi [24] which has characteristics such as a seventh abdominal segment that is approximately the same length as the sixth or just slightly shorter; a vasiform orifice that is rounded rather than elongated, and which is occasionally elevated; a lingula that is hidden under the operculum; the absence of a caudal furrow; and tracheal pores or clefts. Then, it was redescribed by David [26] by pointing out characteristics such as having prominent dorsum spines that eventually separated the *Aleurotrachelus* genus from Aleurochantini. The tribal classification of whitefly remains debatable morphologically [21]. Moreover, it also lacks molecular support due to the limitation of mtCOI to reconstructing the subfamily [88]. The latest definition of David on the tribe Aleurocanthini, however, seems to have no support molecularly both in the mitochondrial gene and *Portiera* coevolution frameworks (Figure 6,7), as well as on the tribe Bemisini and tribe Tetraleurodini [26]. Instead of being separated from the genus *Aleurolobus* and associated with the genus *Pealius*, the genus *Bemisia* is clustered together with *Aleurolobus* and separated from the genus *Pealius* based on the current analysis (Figure 6,7). The current finding was supported by the previous analysis [50] of the tribe Aleurolobini Takahashi. However, the definition of this tribe morphologically remains in conflict with the genus *Singhiella,* which has an affinity to the genus *Dialeurodes* in the tribe Dialeurodini instead of Aleurolobini [89]. According to previous studies on *Portiera* and COI gene analysis [50,88], *Massilieurodes fici* and *Singhiella simplex* were also clustered with *Bemisia* and *Aleurolobus*.

As the preliminary analysis (Table S2), the genera that were clustered into the tribe Aleurocanthini molecularly (*Acaudaleyrodes, Aleurocanthus, Aleuroplatus, Aleurothrixus, Aleurotrachelus, Crenidorsum,* and *Tetraleurodes;* see Figures 6 and 7*)* shares similar morphological characteristics such as the margin of the pupal case being distinctly toothed, the vasiform orifice is elevated with an oval or subcordate shape, the caudal furrow is absent, the seventh abdominal segment is not significantly reduced medially, abdominal rachis is present, and the antenna is in the mesial position to the prothoracic legs. However, the current morphological comparison (Table S2) in some tribes such as Bemisini, Aleurolobini, and Zaphanerini remains unclear to confirm their clusters. Therefore, a comprehensive review of the tribal classification of whiteflies using a larger number of specimens based on morpho-molecular analysis is particularly needed.

The cryptic diversity in the genus *Aleurocanthus* has been recognized as a consequence of the few distinctive features in the genetically diverse population. Initially, *A. camelliae* cryptic species complex suggested having three morphospecies, including "*spiniferus*", "*camelliae*", and "*woglumi*" [16,87,90], however, this seems to be overestimated. *A. woglumi* can be distinguished by having only ten and rarely eleven of the number of submarginal spines [91,92]. The molecular analysis found (Figure 6,7) that *A. woglumi* (ten submarginal spines) and *A. spiniferus* s.l. (eleven submarginal spines) are paraphyletic. It is perhaps an indication that they are distinct clusters. Therefore, the status of *A. woglumi* should not be considered as a member of the *A. camelliae* cryptic species complex due to its lack of molecular support. Even though, excluding the *A. woglumi*, this complex group is containing two putative species (*A. spiniferus* haplogroup A2 and *Aleurocanthus* cf. *A. spiniferus*) and several unexamined haplotypes (Table 2; Figure 4). At this point, the morphological discrimination of those putative species would be beneficial to estimate their cryptic diversity. Moreover, the current study also revealed evidence that "*camelliae*"

morphospecies has morphological variations in the sub-median abdominal spine arrangement (Figure 2H,I). This whitefly was then assigned as *Aleurocanthus* aff. A. *camelliae*. Therefore, the combination characters, such as the number of marginal teeth and arrangement of sub-median abdominal spines [87], were insufficient to identify *A. camelliae*.

The mitochondrial recombination that was detected on *A. spiniferus* and *A. woglumi* or A*leurocanthus* cf. *A.spiniferus* (Table 3, Figure 5) brings the discussion into one of the most controversial phenomena, at least for its evolutionary implications and the applicability of mtDNA as a phylogenetic marker [93]. For a long time, mtDNA has been considered nonrecombinant [40], but recently it was detected in animals including insects [36,37]. DNA polymorphisms (π: >1%, Table 2), and the extensive exchange of the mobile elements in mtDNA (Table 3, Figure 5) of the *A. spiniferus* s.l. point to several hypotheses. First, the *Wolbachia*-insect horizontal gene transfer (HGT). Mitochondria are known to derive from *Rickettsia*-like bacteria (alpha-proteobacteria), which was the endosymbiont of the primordial eukaryotes [94]. *Wolbachia* is an alphaproteobacterial symbiont that has the ability to transfer the mobile element into the host chromosomes [95–97] and affects the mitochondrial diversity of hosts. However, the possibility of *Wolbachia* transferring a fragment of mtDNA to another mtDNA is still unclear. The second hypothesis pertains to the doubly uniparental inheritance (DUI). The mtDNA recombination was a peculiarity of DUI [98] because recombination events are difficult to detect in a mainly homoplasmic mtDNA system. However, it was considerably easier to detect under DUI due to the significant degree of sequence divergence between the male and female genomes [93]. Due to the lack of sex information data of the sequences examined, the existence of DUI cannot be confirmed yet and needs a further examination. On the other hand, a hybrid speciation [99,100] is also possible to occur in this case, even though the probability of this mechanism remains debatable [100–102]. Therefore, a confirmation of the reliable mechanism of the mitochondrial recombination of the *A. spiniferus* species complex is quite important for further study.

Table 3. Intragenic recombination in mtCOI (COI-2) by using nine different methods implemented in RDP5 software.

Analysis	Result		
Event number (main method) [a]	1 (GENECONV)	2 * (MaxChi)	2 * (MaxChi)
Putative recombinant [b]	MH700446.1 AS	JX281760.1 AW	OP323057.1 ASC
#seq. With the same event	MH700445.1 AS	-	-
Major parent (% similarity) [c]	AB786718.1 AS (99.5)	Unknown (AB536794.1 AC)	AB536794.1 AC (84.4)
Minor parent (% similarity) [d]	Unknown (AB786713.1 AC)	OP323057.1 ASC (86.8)	JX281760.1 AW
Methods (Av. *P*-val):			
1. RDP	-	2.623×10^{-02}	2.623×10^{-02}
2. GENECONV	2.144×10^{-09}	-	-
3. BootScan	-		
4. MaxChi	9.234×10^{-04}	2.603×10^{-02}	2.603×10^{-02}
5. Chimaera	1.251×10^{-02}	8.185×10^{-03}	8.185×10^{-03}
6. SiScan	-	-	-
7. 3Seq	8.720×10^{-06}	1.138×10^{-02}	1.138×10^{-02}
8. LARD	-	-	-
9. Phylpro	-	-	-
Start breakpoint	340	416	453
End breakpoint	363	122	113

[a] Recombination events detected by more than two analysis methods. [b] Putative recombinant: strains suspected to experienced recombination. [c] Major parent: parent contributing the larger fraction of the putative recombinant sequence. [d] Minor parent: parent contributing the smaller fraction of the putative recombinant sequence. * The actual recombinant perhaps their minor parent.

5. Conclusions

It has long been suggested that the whitefly's tribal classification is erroneous because the current morphological characteristics of the puparium do not appear to be genus-specific. The molecular approach is then required to re-examine the tribal classification of whiteflies. The co-cladogenesis as the implication of coevolution among *Portiera* and the mitochondrial gene has been applied to confirm the tribe Aleurocanthini Takahashi, which consists of at least the genera *Acaudaleyrodes*, *Aleurocanthus, Aleuroplatus, Aleurothrixus, Aleurotrachelus, Crenidorsum,* and *Tetraleurodes,* that eventually revises the latest tribal definition proposed by David in 1990. In addition, the polymorphism and mitochondrial recombination detected in the *A. spiniferus* species complex imply the complicated speciation process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15010080/s1, Figure S1: Variation of transverse molting suture of *Aleurocanthus* species; Figure S2: The ML phylogenetic tree of Tribe Aleurocanthini based on the mtCOI. Table S1. MtCOI sequences from the GenBank database analyzed. Table S2. Morphological comparison of the putative tribal characters of whiteflies

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