



## Introduction of a non-native lineage is linked to the recent black cocoa ant, *Dolichoderus thoracicus* (Smith, 1860), outbreaks in Taiwan

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(Manuscript received 22 March 2022; Accepted 28 April 2022; Online published 4 May 2022)

**ABSTRACT:** The black cocoa ant, *Dolichoderus thoracicus* (Smith), is widely distributed across the Indomalayan region including Taiwan. Until the recent localized outbreaks in central Taiwan, this ant has never been considered as a pest. The current study tested if an introduction of non-native lineage(s) potentially contributes to the ant's sudden outbreaks in Taiwan by analyzing phylogenetic relationships of *D. thoracicus* in Taiwan and Southeast Asia. Our results showed that all ants were grouped into two major mitochondrial clades (Clade I and II) separated by a p-distance of 0.0414. Clade I is widespread in Taiwan and represented by six haplotypes that are genetically similar to the haplotypes from the Philippines, indicating that these ants are likely a result of range expansion of the Philippine lineage(s). In contrast, ants from central Taiwan harbor a single Clade II haplotype that is predominantly found in the samples collected from the Indochina region. Coupled with the low genetic diversity, Clade II *D. thoracicus* in Taiwan was most likely introduced from the Indochina region. The possibility is further supported by the fact that most *D. thoracicus* intercepted at the Taiwanese borders had an Indochina origin. Overall, this study suggests that a non-native lineage is responsible for the recent outbreaks of this ant in Taiwan, highlighting a potential research avenue for future research on the association between the "pest" trait and non-native genetic variant in invaded area.

**KEY WORDS:** Biogeography, biological invasions, Dolichoderinae, Formicidae, Hymenoptera, Indomalayan region, insect pest.

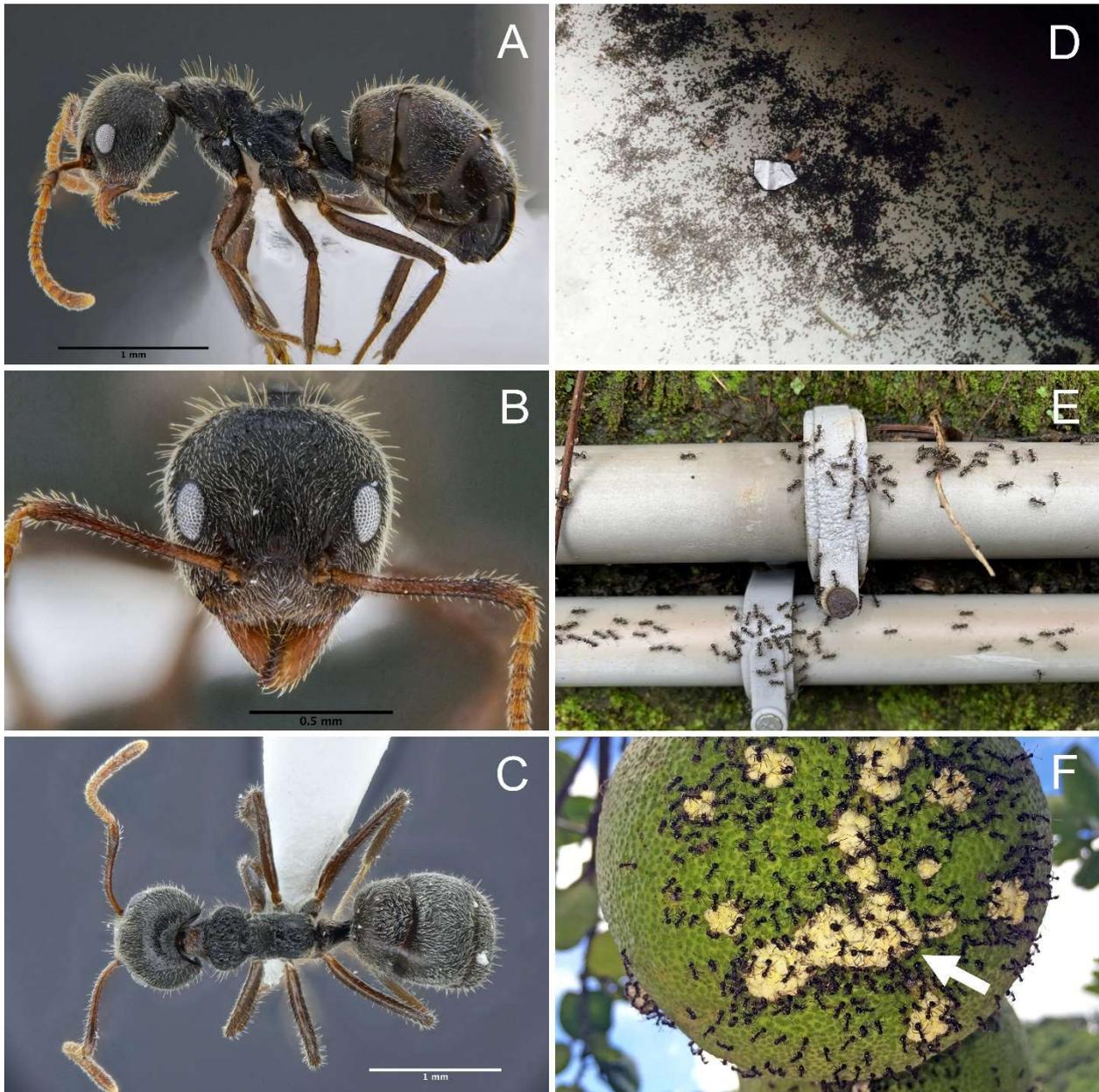
### INTRODUCTION

Globalization has been a major driver of long-distance dispersal of numerous exotic species and significantly facilitated species introductions into new habitats (Meyerson and Mooney, 2007; Hulme, 2009). Among these species, ants are an emerging global invasive group due to their highly adaptive nature and close associations with human activities such as international trade networks (Suarez *et al.*, 2001; Fournier *et al.*, 2012; Bertelsmeier *et al.*, 2017, 2018). Invasion history of alien ant species could be properly inferred using genetic markers in conjunction with phylogenetic/population genetic analyses. For example, although many invasive ants are known to be geographically widespread, reconstruction of their invasion history among geographical populations has been demonstrated to be achievable with appropriate genetic markers and analyses (Tsutsui *et al.*, 2001; Corin *et al.*, 2007; Mikheyev, 2008; Ascunce *et al.*, 2011; Tseng *et al.*, 2019).

The *Dolichoderus* is a diverse ant genus occurring in various terrestrial ecosystems throughout the world except the continent of African (Dill *et al.*, 2002; Shattuck and Marsden, 2013). In Southeast Asia, *Dolichoderus* can be separated into five species groups based on

morphological characteristics by Dill *et al.* (2002). *Dolichoderus thoracicus* (smith) species group is the most taxonomically complicated among the five groups as evident by a total of 11 valid species and 19 subspecies to date and that some of which are morphologically similar (Dill *et al.*, 2002; Janicki *et al.*, 2016; Guénard *et al.*, 2017; Barabag and Jaitrong, 2022; Bolton, 2022). *Dolichoderus thoracicus* is an arboreal ant widely distributed in the Indomalayan region including Taiwan (Dill *et al.*, 2002; Terayama, 2009; Janicki *et al.*, 2016; Guénard *et al.*, 2017). The type specimen of this species was described from Indonesia (Sulawesi), with six subspecies subsequently described from Borneo, Cambodia, Laos and Sumatra. However, the *D. thoracicus* complex (including subspecies) seems to consist of several unidentified cryptic species, and species delimitation solely based on morphology remains extremely challenging (Dill *et al.*, 2002).

To date, only a single species, *D. thoracicus*, has been recorded in the genus *Dolichoderus* in Taiwan island (Terayama, 2009). The first record of *D. thoracicus* in Taiwan is reported by Forel as *D. bituberculata* (described from Luzon, Philippines, now a junior synonym of *D. thoracicus* by Donisthorpe in 1932) more than a century ago (see Forel, 1912). Coupled with the widespread distribution across the East and Southeast Asian regions, it



**Fig. 1.** *Dolichoderus thoracicus*. **A.** The lateral view. **B.** The full-face view. **C.** The dorsal view (Dt104-TWN, collected in Xihu Township, Miaoli County, Taiwan). **D.** Corpses of *D. thoracicus* piling up on the floor in a residential house after chemical treatment using aerosol pesticide in Xihu Township, Miaoli County, Taiwan. **E.** Worker ants moving in lines on a water pipe. Water pipes often serve as a common conduit for the ant's invasion into residential areas in Xihu Township, Miaoli County, Taiwan. **F.** Worker ants tending scale insects (arrow indicated) on grapefruits in an orchard in Xihu Township, Miaoli County, Taiwan.

is thus conceivable that *D. thoracicus* is most likely a native species in Taiwan. Almost three decades after the first record of this ant, Sonan (1939) reported indoor infestation and food contamination by a large number of *D. thoracicus* in eastern Taiwan (Taitung County). However, in recent years (from the year of 2012 onwards) the outbreaking cases of this ant are increasing in Taiwan (Chang and Lin, 2015; Lin *et al.*, 2017; Huang, 2018; Lai, 2019) where extremely high densities were discovered in orchards, farms, and residential houses (Fig. 1).

Several potential hypotheses have been proposed to explain such sudden yet localized outbreaks of ants. For example, thriving populations of *D. thoracicus* may be attributed to an unrestricted access to honeydew-producing insects on fruits/crops in orchards or farms, as this has been commonly observed in many other invasive ants (Helms and Vinson, 2008; Gaigher *et al.*, 2011; Wittman *et al.*, 2018). An additional possibility is that these outbreaking *D. thoracicus* may have originated from other geographical populations. At least two lines of

**Table 1.** Primers used in this study

Primers (Sequence 5' - 3')	Length	Reference
Cytochrome <i>c</i> oxidase subunit I ( <i>COI</i> )		
LCO1490 GGTCAACAATCATAAAGATATTGG	658 bp	Folmer <i>et al.</i> (1994)
HCO2198 TAAACTTCAGGGTGACCAAAAAATCA		
Wingless ( <i>Wg</i> )		
578F TGCACNGTGAARACYTGCTGGATGCG	409 bp	Ward and Downie (2005) Abouheif and Wray (2002)
1032R ACYTGCAGCACCARTGGAA		
Elongation factor 1-alpha ( <i>EF1α</i> )		
F1-1424 GCGCCKGCGGCTCTCACCACCGAGG	359 bp	Schultz and Brady (2008)
F1-1829 GGAAGGCCTCGACGCACATMGG		
Long-wavelength rhodopsin ( <i>LR</i> )		
LR143F GACAAAGTKCCACCRGARATGCT	588 bp	Ward and Downie (2005)
LR639ER YTTACGRTTCCATCCRAACA		

evidence provide strong support to the scenario involving the introduction of a non-native population. First, *D. thoracicus* has been frequently intercepted at the Taiwanese borders in association with bamboo, fruits and live plants from China and Southeast Asia (Lee *et al.*, 2020), implying a high level of ongoing propagule pressure of this ant to Taiwan. Although propagule pressure is a complex measure of introduction effort, high propagule pressure can be generally translated into invasion success (Jeschke and Starzer, 2018). Second, a number of studies showed that sudden outbreaks of numerous existent pests can be linked to additional introduction of geographically isolated/genetically differentiated lineages (Shi *et al.*, 2018; Corrêa *et al.*, 2019). As these additional introductions tend to be relatively challenging to track solely by historical or observational data, inclusion of genetic analyses thus holds the key to reconstruct such a complex invasion scenario.

To test if introduction(s) of non-native *D. thoracicus* has occurred in Taiwan, genetic variations in ants among multiple locations in Taiwan and other parts of Southeast Asia were assessed and compared. Our results strongly suggest the occurrence of two major phylogenetic clades of *D. thoracicus* across Southeast Asia. The same phylogenetic pattern (e.g., two major phylogenetic clades) was also observed in Taiwan, with one of the two clades most likely being introduced. Virtually all outbreaking *D. thoracicus* populations share a single, presumably “introduced” mitochondrial haplotype, providing promising support of the association between the “pest” trait and non-native genetic variant. These findings are consistent to our prediction that the recent pest status of this ant in Taiwan may have been driven by the introduction of a non-native lineage possibly from Southeast Asia.

## MATERIALS AND METHODS

### Sample collection

Specimens of *D. thoracicus* were collected from various locations in Taiwan and several Southeast Asian

countries including Laos, Cambodia, Thailand, Malaysia, and the Philippines. Given the complicated taxonomic status of *D. thoracicus* and other similar species in this group in Southeast Asia, all samples in this study were carefully inspected by both morphological characteristics and molecular information to ensure no cryptic species was included (see Results and Fig. 2 for more details). Samples from a total of 63 locations were confirmed to be valid (48 from Taiwan, 14 from Southeast Asia, as well as one intercepted in a cargo container originating from China at the Taiwanese border, whose sample code was denoted as “Dt38-CHN” in the subsequent analyses). Profile information and GPS coordinates for all *Dolichoderus* samples used in this study were summarized in Supplement (Table S1). All ant samples were preserved in 95% ethanol and kept at 4°C until processed.

### DNA sequencing

DNA was extracted from a single individual of *D. thoracicus* per location ( $N = 63$ ) and an individual of *Dolichoderus* sp. (cf. *affinis*, see Fig. S1 in Supplement). DNA was isolated from the whole ant body except gaster using the Gentra Puregene Tissue Kit (Qiagen, Venlo, Netherlands) following the manufacturer’s protocol. Partial sequences of a mitochondrial gene (cytochrome *c* oxidase subunit I, *COI*) were amplified by polymerase chain reaction (PCR) from all samples ( $N = 64$  including an outgroup). Three nuclear genes (*Wingless*, *Wg*; *Elongation factor 1-alpha*, *EF1α*; *Long-wavelength rhodopsin*, *LR*) were also amplified from a subset of the samples ( $N = 21$  including an outgroup; see Results and Fig. 2 for more details). PCR amplification was performed using Taq 2x Master Mix Red (Ampliqon, Odense, Denmark) with the respective pairs of degenerate primers listed in Table 1. PCR reactions for *COI* involved an initial denaturation at 94°C for 1 min, then 35 cycles of 94°C for 1 min, 48°C annealing for 1 min, and 72°C for 1 min, with final extension at 72°C for 10 min. The PCR cycling parameters for *Wg*, *EF1α*, and *LR* followed Clouse *et al.* (2015) with slight modification and were detailed in Supplement (Table S2).



### Genetic analysis

Partial sequences of mitochondrial *COI* gene and three nuclear loci (*Wg*, *EF1a*, and *LR*) were obtained from individuals of *D. thoracicus* (mitochondrial *COI* gene,  $N = 63$ ; nuclear *Wg*, *EF1a*, and *LR* genes,  $N = 20$ ) and an individual of *Dolichoderus* sp. (cf. *affinis*, see Fig. S1 in Supplement) as outgroup. Sequences of *COI*, *Wg*, *EF1a*, and *LR* were edited and aligned using Sequencher v. 4.1.4 software (Gene Codes Co., Ann Arbor, Michigan, USA) and MEGA 7 (Kumar *et al.*, 2016). All generated sequences were deposited in GenBank (Accession numbers available in Supplement Table S1). Haplotype diversity (Hd), and nucleotide diversity ( $\pi$ ) of mitochondrial *COI* gene were calculated using DnaSP v6.12 (Rozas *et al.*, 2017). The mitochondrial and concatenated nuclear maximum-likelihood (ML) phylogenetic trees were reconstructed using IQ-TREE (Nguyen *et al.*, 2015; Chernomor *et al.*, 2016) on the web interface (Trifinopoulos *et al.*, 2016). Substitution models were determined using ModelFinder (Kalyaanamoorthy *et al.*, 2017) (the mitochondrial ML tree: TN+F+I; the nuclear ML tree: F81+F and JC, more details available in Table S3 and S4). Branch support was assessed by the ultrafast bootstrap approximation algorithm (UFBoot, Hoang *et al.*, 2018) and the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT, Guindon *et al.*, 2010), both with 1,000 replications. The phylogenetic tree was visualized using FigTree version 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>). Haplotype network (TCS network) was reconstructed and visualized using PopART 1.7 (Leigh and Bryant, 2015). To evaluate whether the two genetic clades (see Results for more details) detected in this study should be regarded as a single or separate species, we measured the potential species boundary by calculating the interspecific and intraspecific genetic divergence in the genus *Dolichoderus* based on all *Dolichoderus* sequences available in GenBank and Barcode of Life Data Systems (BOLD) databases. Note that sequences ( $N = 2$ ) named as “*D. thoracicus*” in the two databases were excluded from the analysis due to the unverified taxonomic status of these samples. The genetic divergence was estimated based on uncorrected p-distance using the R package SPIDER v. 1.5.0 (Brown *et al.*, 2012). We first calculated the minimum interspecific distance to the nearest neighbor and maximum intraspecific distance for each sequence, and then calculated the mean maximum intraspecific genetic divergence and minimum interspecific divergence for each *Dolichoderus* species. The classifications of *D. bispinosus*, *D. sibiricus* and *D. taschenbergi* from the two databases seem questionable because the maximum intraspecific distances for the three species are larger than the minimum interspecific distances (Table S5). We calculated the average of minimum interspecific divergence and maximum intraspecific distance for *Dolichoderus* after removing the three species to prevent the potential bias deriving incorrect classifications.

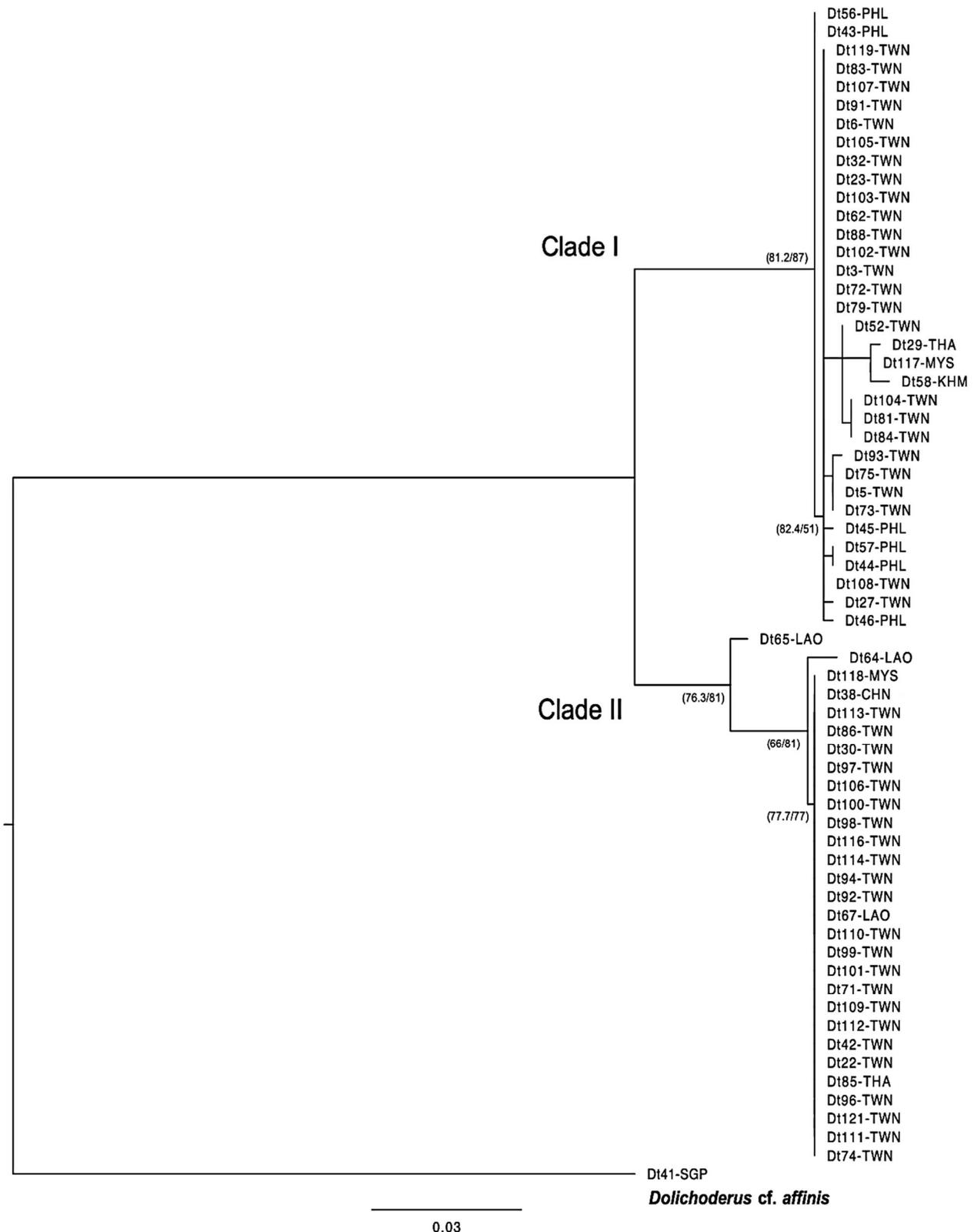
### RESULTS

Partial sequences of *COI* gene (590 bp) from 63 individuals of *D. thoracicus* were successfully amplified and sequenced together with *Dolichoderus* sp. (cf. *affinis*) as an outgroup in this study. The phylogenetic analysis of *D. thoracicus* indicated the presence of two distinct clades (Clade I and II) (Fig. 2). Samples clustered in Clade I were mainly distributed in the Philippines and Taiwan, whereas those in Clade II were mainly distributed in the Indochina and Taiwan (Fig. 2). However, three samples (Dt29, 58, and 117) belonging to Clade I were collected from the Indochina region, including Cambodia (Phnom Penh), Malaysia (Johor), and Thailand (Chiang Mai), respectively (Fig. 2). In the nuclear ML tree, no geographic pattern was observed (Fig. 3). Three Taiwanese samples (Dt73, 81, and 121) were genetically similar but distributed in geographically distant locations (Taitung, Chiayi, and Nantou) (Fig. 3). Nucleotide variations were only found at three positions in the concatenated nuclear sequences (*Wg*, *EF1a* and *LR*, 1,224 bp in total), even though these samples harbor the majority of the discovered mitochondrial *COI* haplotypes (14/16) and were collected from geographically distant locations (Fig. 3).

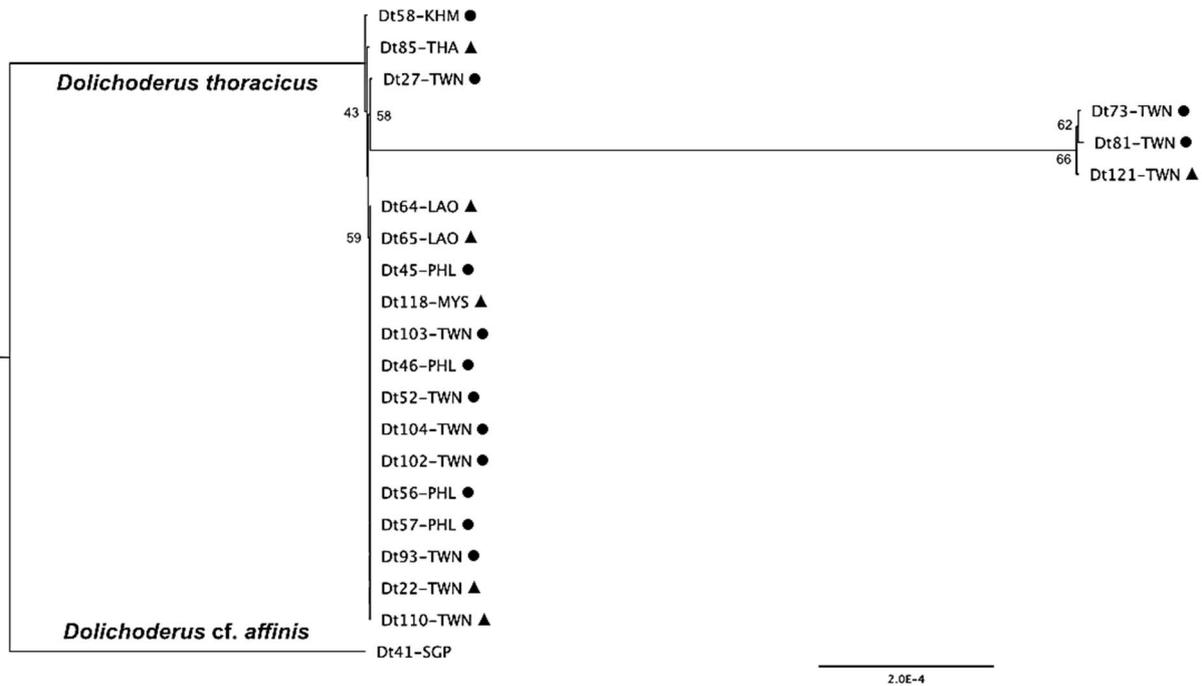
The average of the mean interspecific distance across *Dolichoderus* spp. is 0.137 (95% CI: 0.112–0.162), while the average of the mean intraspecific distance is 0.083 (95% CI: 0–0.092) (Table 2). The p-distance between Clades I and II was 0.0414, which appeared to be lower than the interspecific minimum average genetic distance in the genus *Dolichoderus* (Table 2). Given the fact that the genetic distance among the two *D. thoracicus* clades is smaller than the average of interspecific distance for *Dolichoderus* spp., we conclude that the two clades could be reasonably accepted as intraspecific genetic variation within the same species.

The result of TCS haplotype network revealed a total of 16 *COI* haplotypes that were also separated into two groups (Fig. 4). The grouping pattern agreed perfectly with that in the phylogenetic tree (Fig. 2, Clade I and II). In Clade I, four haplotypes (Hap\_9-11 and 13) were only found in the Philippines, whereas six haplotypes (Hap\_3-5, 12, 15 and 16) were only found in Taiwan. Three haplotypes (Hap\_2, 8, and 14) in Clade I, however, were found in the Indochina and distantly related to other haplotypes in Clade I (Fig. 4). In Clade II, while Hap\_1 was found to be widespread across our sampled locations in Laos, Thailand, Malaysia, China (intercepted at Taiwanese border) and Taiwan, there were two other haplotypes (Hap\_6 and 7) found in Laos (Fig. 4).

In Taiwan, seven mitochondrial haplotypes of *D. thoracicus* were identified with six belonging to Clade I but only one belonging to Clade II (Fig. 4 and Table 3). In Clade I ( $N = 25$ ), the haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) are  $0.580 \pm 0.107$  and  $0.00207 \pm$



**Fig. 2.** The maximum-likelihood tree of 63 *Dolichoderus thoracicus* samples based on the partial sequence of *COI* gene (590 bp) with *Dolichoderus cf. affinis* as outgroup. Bootstrap support values (%) of the UFBoot (left) and the SH-aLRT (right) are shown at major nodes. Country codes: CHN, China; KHM, Cambodia; LAO, Laos; MYS, Malaysia; PHL, Philippines; SGP, Singapore; THA, Thailand; TWN, Taiwan. Note that the sample denoted as “China” is an individual ant worker intercepted at the Taiwanese border.



**Fig. 3.** The maximum-likelihood tree of 20 *Dolichoderus thoracicus* samples based on the concatenated sequences of three nuclear loci (*Wg*, *EF1 $\alpha$*  and *LR*, 1,224 bp in total) with *Dolichoderus cf. affinis* as outgroup. Bootstrap support values (%) of the UFBoot support are shown at major nodes. Black circles: mitochondrial Clade I; black triangles: mitochondrial Clade II. Country codes: KHM, Cambodia; LAO, Laos; MYS, Malaysia; PHL, Philippines; SGP, Singapore; THA, Thailand; TWN, Taiwan.

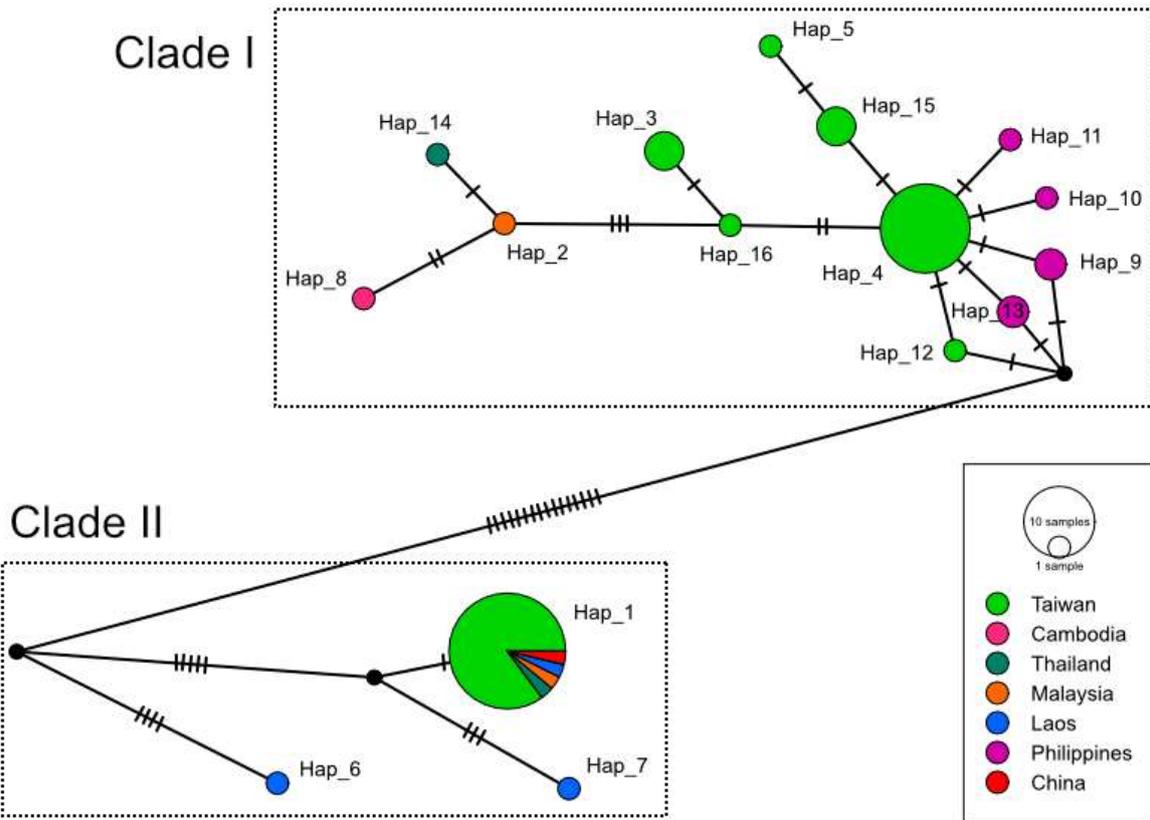
**Table 2.** Inter- and intraspecific genetic divergence (p-distance) of multiple species in the genus *Dolichoderus*.

Species	Number of minimum sequences	Mean inter-specific distance	Mean maximum intra-specific distance
<i>D. attelaboides</i>	2	0.197	0.093
<i>D. germaini</i>	2	0.091	0
<i>D. imitator</i>	2	0.198	0.145
<i>D. lutosus</i>	3	0.17	0.111
<i>D. mariae</i>	20	0.101	0.013
<i>D. quadripunctatus</i>	12	0.033	0.004
<i>D. rugocapitus</i>	146	0.104	0.004
<i>D. validus</i>	2	0.176	0.003
<i>D. voraginosus</i>	1	0.091	NA
<i>D. bidens</i>	1	0.16	NA
<i>D. debilis</i>	1	0.059	NA
<i>D. decollatus</i>	1	0.193	NA
<i>D. diversus</i>	1	0.147	NA
<i>D. feae</i>	1	0.181	NA
<i>D. flatidorsus</i>	1	0.032	NA
<i>D. ghilianii</i>	1	0.177	NA
<i>D. incisus</i>	1	0.105	NA
<i>D. inermis</i>	1	0.182	NA
<i>D. lamitus</i>	1	0.184	NA
<i>D. pilosus</i>	1	0.159	NA
<i>D. quadridenticulatus</i>	1	0.154	NA
<i>D. rugosus</i>	1	0.227	NA
<i>D. taprobanae</i>	1	0.032	NA
Average		0.137	0.047
95% CI		(0.112-0.162)	(0-0.096)

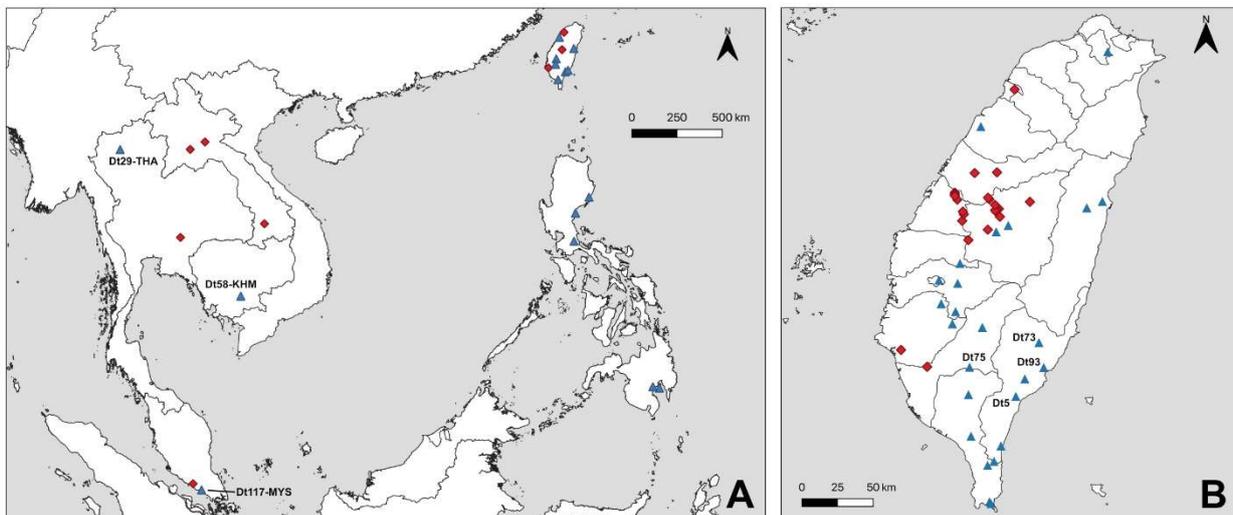
0.00051, respectively (Table 3). Since only a single Clade II haplotype was found, both diversity indices were zero ( $N = 23$ , Table 3). The geographic pattern of haplotype distribution differed between the two clades in Taiwan. Individuals collected in central Taiwan shared a single Clade II haplotype (Hap\_1, Fig. 4 and 5B). Although Clade I haplotypes are widespread in Taiwan, shared or genetically similar haplotypes tend to occur in geographically adjacent or nearby sampled locations, indicating potential geographic structure of haplotype distribution (Fig. 4). For example, most samples collected in eastern Taiwan (Dt5, 73, 75 and 93) harbor the haplotype that are genetically similar (Fig. 2 and 5B).

## DISCUSSION

This study reveals the presence of two mitochondrial clades (Clade I and II) in *D. thoracicus* from Taiwan. The divergence between the two clades appeared to be lower than the interspecific minimum average genetic distance in the genus *Dolichoderus* (Table 2), suggesting the divergence could be reasonably accepted as intraspecific genetic variation. The ants belonging to Clade I are widespread in Taiwan, while those in Clade II are mainly found in central Taiwan (Fig. 5B). The presence of Clade II haplotype (presumably originating from the Indochina) with a localized distribution pattern suggests that Clade II *D. thoracicus* in Taiwan are most likely a result of a recent introduction from the Indochina. Several lines of



**Fig. 4.** TCS haplotype network of *Dolichoderus thoracicus* based on the partial sequence of *COI* gene. Each bar on the branch corresponds to a single nucleotide substitution. Circle area is proportional to the number of individuals carrying a given haplotype. Note that the sample denoted as “China” was an individual ant worker intercepted at the Taiwanese border.



**Fig. 5.** The distribution of the two mitochondrial clades of *Dolichoderus thoracicus* in Southeast Asia (A) and Taiwan (B), with blue triangles denoting Clade I and red diamonds denoting Clade II.

evidence provide additional support to this notion: 1) *D. thoracicus* has been among the top most frequently intercepted ant species at the Taiwanese border during the last decade, and most of which were found to be associated with cargo containers originating from ports in the Indochina region (Lee *et al.*, 2020); 2) all *D.*

*thoracicus* in central Taiwan share one single Clade II haplotype (Hap\_1), suggesting an extremely low level of genetic diversity, potentially indicative of genetic bottleneck; 3) one of the intercepted *D. thoracicus* samples originating from China (code: Dt38-CHN; Lee *et al.* (2020)) was included in this study, and this individual

**Table 3.** Genetic diversity indices between two mitochondrial *COI* evolutionary clades of *Dolichoderus thoracicus* in Taiwan.

Group	Sample size	Number of Haplotype (h)	Haplotype Diversity (Hd) $\pm$ S.E.	Nucleotide Diversity ( $\pi$ ) $\pm$ S.E.
Taiwan clade I	25	6	0.580 $\pm$ 0.107	0.00207 $\pm$ 0.00051
Taiwan clade II	23	1	0	0

ant shares the identical haplotype (Hap\_1) with the ants in central Taiwan. All these data, coupled with the fact that outbreaks of this ant are mainly reported in central Taiwan, allow us to link the current pest status of *D. thoracicus* to recent introduction of a non-native lineage of this ant most likely from the Indochina.

While intrinsic mechanisms responsible for an extraordinarily high population density of this putative introduced lineage of *D. thoracicus* remains unclear, we argue that interplays with environmental factors cannot be ruled out. One potential explanation is that, like many arboreal ant species, these *D. thoracicus* populations often establish a symbiotic relationship with native and exotic honeydew-producing insects such as aphids or mealybugs to gain access to honeydew as their major food resources (Fig. 1F). This possibility, despite being untested, may be analogous to the outbreak of invasive yellow crazy ant (*Anoplolepis gracilipes*) on Christmas Island. A larger colony size, higher reproduction rates and higher level of aggression of yellow crazy ant have been considered to be attributable to a greater level of carbohydrate resource supplied by several introduced honeydew-producing scale insect species (Wittman *et al.*, 2018). Efforts in identifying honeydew-producing insects frequently tended by *D. thoracicus* in the field and also assessing population dynamics of this ant with/without these “partner” insects would help understand mechanisms underlying recent *D. thoracicus* outbreaks in Taiwan.

## ACKNOWLEDGMENTS

We would like to thank Dr. Hui-Yun Tseng and Ting-Shuo Wang (National Taiwan University), Leocris Batucan Jr. (National Taiwan Normal University), Yen-Jui Chen (Taiwan's Forest Conservation and Management Administration), Ping-Chih Lin, Han-Yu Lin, Yi-Tsen Lin, Po-Cheng Hsu, and Dr. Ching-Chen Lee (National Changhua University of Education) for their help with field sampling. We also thank to Ming-Hsun Chou and Dr. Jen-Pan Huang (Academia Sinica) for their constructive comments on earlier versions of the manuscript. We thank Dr. Chuan-Kai Ho (National Taiwan University) and two anonymous reviewers for their constructive comments on our manuscript. This study was supported by grant from the Ministry of Science and Technology (MOST 108-2622-B-018-002-CC2).

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