

Identification of Vietnamese *Coptotermes* pest species based on the sequencing of two regions of 16S rRNA gene

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Abstract

Coptotermes, found in urban areas, is regarded as the most abundant building termite pest genus, widely distributed in Vietnam. The objectives of this study were to classify the *Coptotermes* found in certain provinces in Vietnam and assess the feasibility proposed PCR method by Szalanski *et al.*, 2004 for identification of *Coptotermes* species. The proposed PCR method distinguishes species by the presence or absence of DNA fragments amplified with universal (LR-J-13007, LR-N-13398) and *Coptotermes formosanus* Shiraki specific (FST-F, FST-R) primer pairs. For this purpose, we collected six *Coptotermes* samples from five localities in Ha Noi (Van Quan-Ha Dong, Thai Ha-Dong Da, Vong Thi-Tay Ho, Tan Linh-Ba Vi, Bui Xuong Trach-Dong Da) and from Van Lam-Hung Yen province, Vietnam and employed the proposed simple PCR-based diagnosis for *Coptotermes* species (Szalanski *et al.*, 2004). We amplified the universal 430-bp region from DNA templates of all six individual termite samples and the 151-bp regions of mtDNA 16S rRNA from DNA templates extracted from Tan Linh and Van Quan termites. Appearance of the species specific DNA fragments suggested that two *Coptotermes* samples can be classified as *C. formosanus*. However, by comparing the nucleotide sequences of the 430 bps and the 126 bps (eliminating the primer sequences from 151-bp region) with the corresponding sequences from *Coptotermes* species from GenBank, we found all six samples belonged to *Coptotermes gestroi* (Wasmann). Thus, in this case, PCR products were obtained employing primers with mismatches to the mtDNA 16S rRNA gene of *C. gestroi*. Therefore, the nucleotide sequencing is necessary for the identification of *Coptotermes* species including *C. formosanus* or *C. gestroi* based on the 430-bp and 151-bp regions of mtDNA 16S rRNA gene.

Key words: *Coptotermes gestroi*, *Coptotermes formosanus*, taxonomy, mtDNA 16S rRNA.

Introduction

Coptotermes (Rhinotermitidae Coptotermitinae) is one of the most important genera of wood-destroying pests believed to have originated in Asia (Austin *et al.*, 2004a). In Vietnam, there are 141 termite species classified into four families: Kalotermitidae, Termopsidae, Rhinotermitidae, and Termitidae (Trinh *et al.*, 2010). *Coptotermes formosanus* Shiraki and *Coptotermes ceylonicus* (Holmgren) are widely distributed in all the parts of Vietnam. *Coptotermes gestroi* (Wasmann), *Coptotermes emersoni* Ahmad, and *Coptotermes travians* (Haviland) are frequently found in Ha Noi, and *Coptotermes curvignathus* Holmgren has been identified in the Southern Vietnam (Nguyen *et al.*, 2007; Trinh *et al.*, 2010).

For a long time, termite classification has been based on morphological and/or morphometrical character sets of soldier or worker and alate (or winged) termites (Scheffrahn *et al.*, 1990). Morphometry is an important tool of taxonomy, and identification of different species especially for the soldier cast by analyzing the head-capsule is a very helpful. However, this methodology has showed restriction and caused misidentification and taxonomic confusion between some species of *Coptotermes* such as *C. travians*, *Coptotermes havilandi* Holmgren, *C. gestroi* (Kirton and Brown, 2003). During the past decade, the application of molecular techniques

for termite classification/identification as well as phylogenetic analysis has provided a reliable method for identifying many termite species (Clément *et al.*, 2001; Szalanski *et al.*, 2003; Szalanski *et al.*, 2006; Austin *et al.*, 2004b). The 16S rRNA gene has been proven to be an accurate, reliable, and repeatable mtDNA marker for termite identification (Tripodi *et al.*, 2006). In the 16S rRNA gene, amplifying the 430-bp region by universal primers LR-J-13007, LR-N-13398 (Simon *et al.*, 1994) is an widely applied method for the identification of species of lower and higher termites, in particular, it is useful for the identification of species belonging to *Coptotermes* and *Reticulitermes* (Austin *et al.*, 2004b; 2012; Szalanski *et al.*, 2004; 2006; Wang *et al.*, 2009; Ghesini *et al.*, 2011b; Cheng *et al.*, 2011). In 2004, Szalanski *et al.* (2004) designed a pair of specific primers to amplify a 151-bp region of *C. formosanus* 16S gene based on alignment data of the gene region from 12 *Coptotermes* species including *C. gestroi*. Based on their findings, Szalanski *et al.* (2004) introduced a convenient and quick PCR method for identification of *C. formosanus*. The method involves running independently two PCR reactions for amplifying the 430-bp region by primers FST-F, FSF-R and 151-bp region of mtDNA 16S rRNA gene by the *C. formosanus* specific primers. If PCR products of both regions are visualized on agarose gel, the DNA template can be traced to be originated from *C. formosanus* (Szalanski *et al.*, 2004).

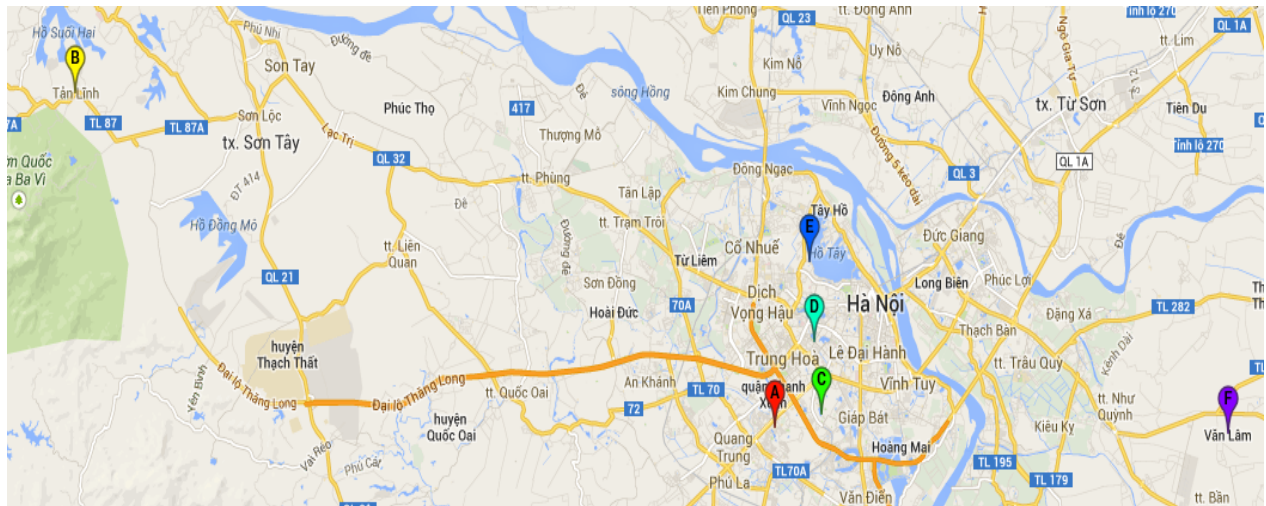


Figure 1. Geographical distribution of the six termite samples collected for this study. A: Van Quan-Ha Dong; B: Tan Linh-Ba Vi; C: Bui Xuong Trach-Dong Da; D: Thai Ha-Dong Da; E: Vong Thi-Tay Ho of Ha Noi; F: Van Lam-Hung Yen, Vietnam.

We undertook this study with an aim to classify *Coptotermes* species collected in Ha Noi (five places) and Hung Yen (one region), Vietnam. We also attempted to assess the feasibility of the PCR method introduced by Szalanski *et al.* (2004) for the identification of *C. formosanus* in Vietnam.

Materials and methods

Termite collection and dissection

The *Coptotermes* termites used in this study were harvested by visual observation of morphological characters. The termites were collected during March to June in 2012 from five dry wood-based nests found in Van Quan-Ha Dong (A, figure 1), Tan Linh-Ba Vi (B, figure 1), Bui Xuong Trach-Dong Da (C, figure 1), Thai Ha-Dong Da (D, figure 1), Vong Thi-Tay Ho (E, figure 1) of Ha Noi and one dry wood-based nest in Van Lam-Hung Yen province (F, figure 1). Six termite samples were separately maintained in laboratory at room temperature on a pine wood diet.

The healthy worker termites were rinsed in 75% ethanol and washed with distilled water to remove surface contaminants. The total DNA of individual termites sample was extracted separately as follows. The 0.15 g of termite heads were scratched in 1 ml lysis buffer (10% sucrose, 1% CTAB, 1.5 M NaCl, 100 mM Tris-HCl pH 8, 100 mM EDTA pH 8) with 0.34 g of glass bead (diameter = 0.6 mm) by shaking at 250 rpm for 30 min at room temperature. The samples were supplemented with 3.5 µl protease K (20 mg/ml) and incubated for 60 min at 50 °C with vigorous shaking at 250 rpm. The specimens were homogenized by grinding and then supplemented with 133.5 µl of 20% (w/v) SDS. The specimens were then incubated for 20 min at 60 °C, at 250 rpm. The total DNA from supernatant was obtained by QIAquick Spin kit (QIAGEN) and further purified by the troughing method (Harnpicharnchai *et al.*, 2007).

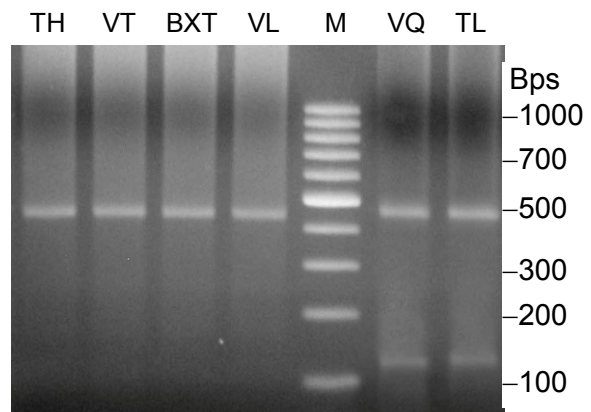


Figure 2. The mixed PCR products of 430-bp and 151-bp amplified with corresponding primers LR-J-13007, LR-N-13398, and primers FST-F, FST-R from DNA templates extracted from *Coptotermes* termites collected in Thai Ha-Dong Da (TH), Vong Thi-Tay Ho (VT), Bui Xuong Trach-Dong Da (BXT), Van Lam-Hung Yen (VL), Van Quan-Ha Dong (VQ) and Tan Linh-Ba Vi (TL) on agarose gel. M: Marker (Fermentas).

PCR condition

The following PCR primers were used to amplify the 430-bp region of the mtDNA 16S rRNA gene - LR-J-13007: 5'-TTACGCTGTTATCCCTAA-3', LR-N-13398: 5'-CGCCTGTTTATCAAAAACAT-3' (Simon *et al.*, 1994). For amplifying the 151-bp region of 16S rRNA gene the following *C. formosanus* specific PCR primers were applied - FST-F: 5'-TAAACAACAAACAACAACAAC-3', FST-R: 5'-ATGGCTTGACGAGGCACAA-3' (Szalanski *et al.*, 2004). The PCR reaction was conducted in a 25 µl reaction mixture containing 2.5 µl Thermo Pol Buffer 10X (Biolabs), 1 µl 25 mM MgSO₄, 1 µl 2 mM dNTP, 2 µl each primer (10 pmol), 8.6 ng DNA template, and 0.25 µl Vent DNA polymerase (2 U/µl, Biolabs).

PCR was carried out in 40 cycles after initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing at 54 °C (for the primer pair LR-J-13007 and LR-N-13398) or 57 °C (for the primer pair FST-F and FST-R) for 45 s, and elongation at 72 °C for 45 s. The PCR products were purified by QIAquick Gel Extraction kit (QIAGEN) followed by direct sequencing using a ABI3100 sequencer (AB Applied Biosystems, USA). The nucleotide sequences of six 430-bp PCR products (amplification of the 430-bp regions of the 16S rRNA genes extracted from every termite samples) were deposited in GenBank (Accession No KF703756-KF703761). The nucleotide sequences of all PCR products were subjected to GeneDoc for alignment analysis.

Results and discussion

The 430-bp regions of the 16S rRNA gene were amplified from six *Coptotermes* isolates collected from six places by universal primers LR-J-13007, LR-N-13398 (Accession No KF703756-KF703761). However, the 151-bp region of the 16S rRNA gene was only amplified from the DNA samples extracted from worker termites collected from Tan Linh and Van Quan using the primer pair FST-F, FST-R. In this study, by mixing PCR products of 430-bp and 151-bp regions (figure 2) as described by Szalanski *et al.* (2004), we may infer that *Coptotermes* from Tan Linh and Van Quan belong to *C. formosanus*.

Table 1. The homology of 430-bp nucleotide sequences of the 16S rRNA gene from six *Coptotermes* termite isolates collected in Vietnam.

	Bui Xuong Trach	Van Quan	Hung Yen	Tan Linh	Thai Ha	Vong Thi
Bui Xuong Trach	430	99%	100%	99%	99%	100%
Van Quan	426	430	99%	98%	99%	99%
Hung Yen	430	426	430	99%	99%	100%
Tan Linh	430	424	430	430	99%	99%
Thai Ha	430	430	430	426	430	99%
Vong Thi	430	426	430	430	430	430

Table 2. Summary of the alignment of the 430-bp DNA sequences (Accession No KF703756 - KF703761) of the 16S rRNA gene from Vietnamese *Coptotermes* samples and the corresponding sequences in GenBank BXT: Bui Xuong Trach, TH: Thai Ha, VT: Vong Thi, VQ: Van Quan, TL: Tan Linh, VL: Van Lam.

Species or strains	Accession No (reference)	Location	Percentage of homology with sequences from					
			BXT	VL	TL	TH	VQ	VT
<i>C. formosanus</i>	AB626145 (Tokuda <i>et al.</i> , 2012)	Okinawa, Okinawa Island Japan	93	93	93	94	93	93
<i>C. formosanus</i>	AB626147 (Tokuda <i>et al.</i> , 2012)	Okinawa, Iriomote Island, Japan	93	93	93	94	93	93
<i>C. formosanus</i>	AY558911.1 (Scheffrahn <i>et al.</i> , 2004)	Guangzhou, China	94	94	93	94	94	94
<i>C. formosanus</i> Cf1	DQ007344.1	Norcross, Georgia, USA	94	94	93	94	94	94
<i>C. formosanus</i>	GU075666	China	93	93	93	93	93	93
<i>C. formosanus</i>	U17778 (Kambhampati., 1995)	USA	93	93	93	93	93	93
<i>C. gestroi</i>	AY558905 (Scheffrahn <i>et al.</i> , 2004)	Antigua and Barbuda	99	99	98	99	98	99
<i>C. gestroi</i>	AY558906 (Scheffrahn <i>et al.</i> , 2004)	Grand Turk, Turks and Caicos Islands	98	98	98	98	98	98
<i>C. gestroi</i> CgS3	DQ004478.1	Sime Avenue, Singapore	99	99	99	99	99	99
<i>C. gestroi</i> CgA2	DQ004487.1	Hamilton QLD, Australia	98	98	97	98	97	98
<i>C. gestroi</i> CgS6	DQ915942 (Jenkins <i>et al.</i> , 2007)	Kim Keat Rd, Singapore	99	99	99	99	99	99
<i>C. gestroi</i> CgS7	EF092285 (Jenkins <i>et al.</i> , 2007)	Singapore	99	99	99	99	99	99
<i>C. gestroi</i> CgF2	EF156760.1 (Jenkins <i>et al.</i> , 2007)	Key West, Florida, USA	98	98	98	98	98	98
<i>C. kalshoveni</i>	AY683211.1	Malaysia	95	95	94	95	94	95
<i>C. lacteus</i>	AY558912.1 (Scheffrahn <i>et al.</i> , 2004)	Beerburrum, Australia	93	93	92	93	93	93
<i>C. vastator</i>	AY558898.1 (Scheffrahn <i>et al.</i> , 2004)	Oahu, HI, USA	93	93	92	93	93	92

The nucleotide alignment of six sequences derived from the amplified 430-bp region by GeneDoc indicated no genetic variation between the three *Coptotermes* isolated from Vong Thi, Hung Yen, Bui Xuong Trach. However, we found 0.45-0.9% variation in two *Coptotermes* termites from Van Quan and Thai Ha and 1.5% variation in *Coptotermes* from Tan Linh when compared to other samples (table 1). The analysis of the nucleotide sequences of these fragments by BlastN revealed that a very high homology (97-99%) was found between all six nucleotide sequences and the corresponding sequences of *C. gestroi*, while only 93-94% of 430 nucleotides from every sequence were identical to the corresponding sequences of *C. formosanus*. The six sequences also showed 92-93% homology with the corresponding sequences of *Coptotermes lacteus* (Froggatt) or *Coptotermes vastator* Light (table 2).

For analyzing the amplified 151-bp regions of the 16S rRNA genes from termites collected in Tan Linh and Van Quan, we used internal 126-bp sequences (primer

regions were eliminated). The 126-bp sequences from *Coptotermes* isolates from Van Quan, Thai Ha were 99-100% homologous to the corresponding regions of *C. gestroi* [GenBank Accession EU805727 (Li *et al.*, 2009), FJ376672, HQ231234 (Ghesini *et al.*, 2011a), JX128688, JX128689, KC887123], 95-96% homologous to the corresponding regions of *C. formosanus* [GenBank Accession No AB626145 (Tokuda *et al.*, 2012), EU805741, EU805742 (Li *et al.*, 2009), JN615270 (Djernæs *et al.*, 2012), KC887106], 97-98% identity to the corresponding regions of *Coptotermes heimi* (Wasmann) (GenBank Accession No AY558908, GQ422885) (Salunke *et al.*, 2010; Scheffrahn *et al.*, 2004) (figure 3). *C. heimi* used to proposed to the synonym of *C. gestroi* (Li *et al.*, 2013; Yeap *et al.*, 2010), but we found 2-3% genetic variation occurred in 126 bps regions of the investigated *C. gestroi* and *C. heimi* from GenBank Accession No AY558908, GQ422885. There was only one nucleotide variation in the 126-bp regions between *Coptotermes* isolates from Van Quan

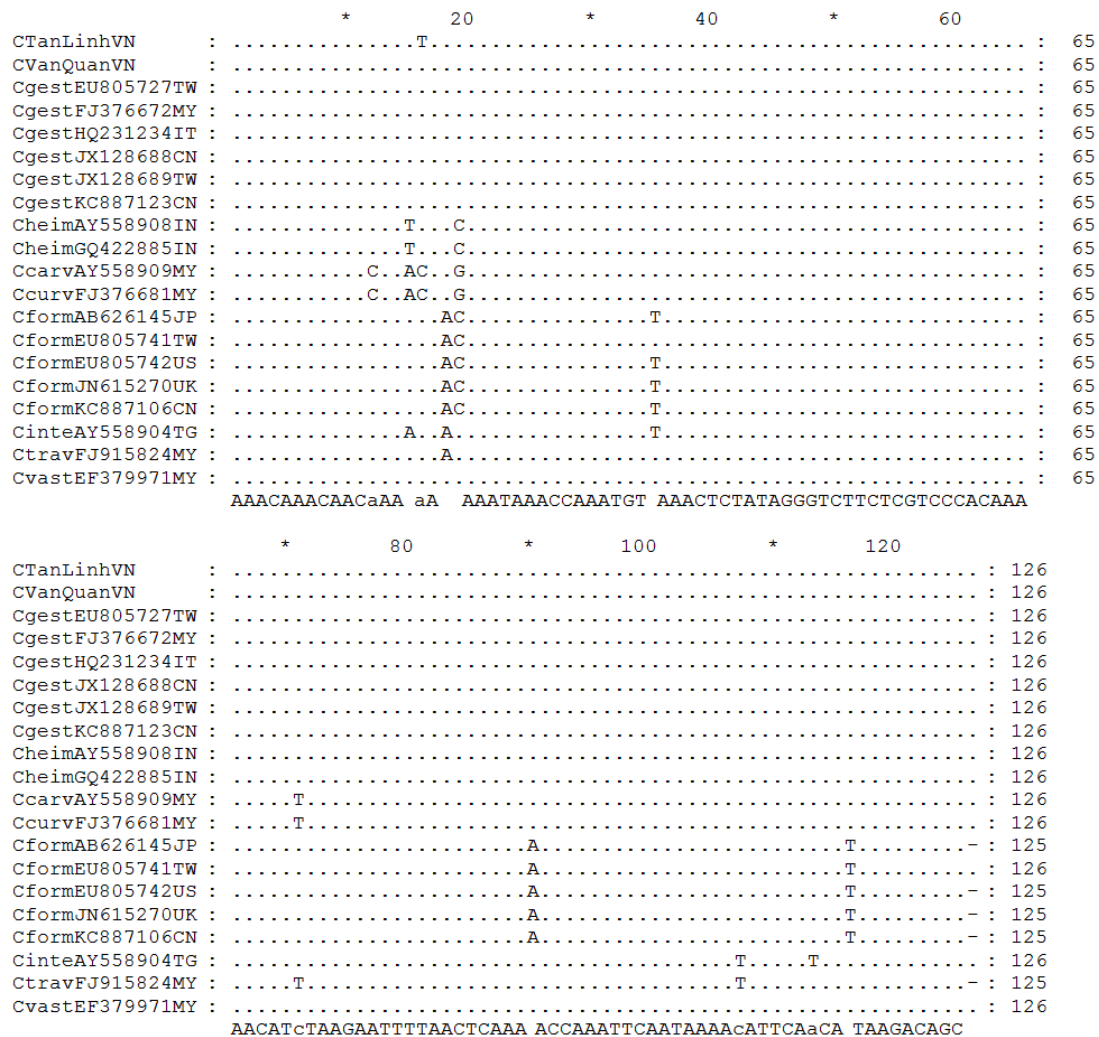


Figure 3. Alignment of 126-bp sequences of 16S rRNA from *Coptotermes* collected in Tan Linh, Van Quan and the corresponding sequences in GenBank. Sequence alignments were generated by GeneDoc. Dot areas denote sequence homologies. In ID names: Cgest, Cheim, Ccarv, Ccurv, Cform, Cinte, Ctrav, Cvast are abbreviation for *C. gestroi*, *C. heimi*, *Coptotermes carvinatus* Scheffrahn *et al.*, *C. curvignathus*, *C. formosanus*, *Coptotermes intermedius* Silvestri, *C. travians*, and *C. vastator* followed by GenBank Accession No; the two characters at the ends are international standard of countries abbreviation.

Primer FST-F	TAAACAAACAAACAACAAACAAAC	
KC887111 273	ACAAACAACAAACAACAAACAAGTAAATAAACCAAATGTCAAACCTCTATAGGGTCT	214
JX12869 236	ACCAAAAATAAACAAACAACAAACAAGTAAATAAACCAAATGTCAAACCTCTATAGGGTCT	177
KC887125 273	ACCAAAAATAAACAAACAACAAACAAGTAAATAAACCAAATGTCAAACCTCTATAGGGTCT	214
GU075665 293	ACCAAAAATAAACAAACAACAAACAAGTAAATAAACCAAATGTCAAACCTCTATAGGGTCT	234
AY558906 137	ACCAAAAATAAACAAACAACAAACAAGTAAATAAACCAAATGTCAAACCTCTATAGGGTCT	196
	** * * * *	
KC887111 213	TCTCGTCCCACAAAAACATCTAAGAATTTTAACTCAAAGACCAAATTC AATAAAACATTC	154
JX12869 176	TCTCGTCCCACAAAAACATCTAAGAATTTTAACTCAAAGACCAAATTC AATAAAACATTC	117
KC887125 213	TCTCGTCCCACAAAAACATCTAAGAATTTTAACTCAAAGACCAAATTC AATAAAACATTC	154
GU075665 233	TCTCTTCCCACAAAAACATCTAAGAATTTTAACTCAAAGACCAAATTC AATAAAACATTC	174
AY558906 197	TCTCGTCCCACAAAAACATCTAAGAATTTTAACTCGAAGACCAAATTC AATAAAACATTC	256
	**** * * * * *	
Primer FST-R	TTGTGCCTCGTCAAGCCAT	
GU075665 173	AACACTAAGACAGCCCGTGCCTCGTCAAGCCAT	141
JX12869 116	AACACTAAGACAGCCCGTGCCTCGTCAAGCCAT	84
KC887125 153	AACACTAAGACAGCCCGTGCCTCGTCAAGCCAT	121
KC887111 154	AACACTAAGACAGCCCGTGCCTCGTCAAGCCAT	121
AY558906 257	AACACTAAGACAGCCCGTGCCTCGTCAAGCCAT	289

Figure 4. Occurrence of mismatches between *C. formosanus* specific primers FST-F, FST-R and the 151-bp regions of 16S rRNA genes from *C. gestroi*. The shaded characters indicate the nucleotide variation

and Tan Linh (figure 3). In agreement with previous studies, *C. formosanus* was not reported as an invasive termite species in Vietnam period 1969-2013 (Evans *et al.*, 2013) but *C. gestroi* has been known to be distributed in Ha Noi (Trinh *et al.*, 2010).

To explain the reason why the 151-bp region of *C. gestroi* was amplified by the specific primers for *C. formosanus*, we raise a hypothesis that there are too few mismatches between the primers and the target sequence of *C. gestroi*. We performed the PCR experiments under the condition optimized by Szalanski *et al.* (2004); however, the forwards primer (FST-F) containing 24 nucleotides, of which 19 nucleotides, especially seven nucleotides at 3' terminal were conserved with the corresponding sequences of *C. gestroi* (GenBank Accession No KC887111, JX12869, KC887125, GU075665, AY558906, figure 4), and the reverse primer (FST-R) consisting of 19 nucleotides, of which 17 nucleotide matched very well with the corresponding sequences in *C. gestroi* except two nucleotides at the end of 3' terminal (figure 4). Mismatching between primers and template are known to affect on the stability of primer-template duplex and the efficiency of polymerase to extend primers (Kwok *et al.*, 1990). It is not easy to design a specific primer pair to distinguish very close relative species, because specific regions are often too small. Mismatches occurring in 5' terminal usually does not have a strong impact on the PCR efficiency (Kwok *et al.*, 1990; Stadhouders *et al.*, 2010). On the other hand, mismatches located in the 3' end region (defined as the last 5 nucleotides of the 3' end region) of primer has been shown to affect priming efficiency significantly. However, depending on the type of mismatches, PCR products can be obtained, although the amplified amount could be reduced to 1/10 to 1/100 fold (Kwok *et al.*, 1990). A:G, G:A, and C:C mismatches cause 100 times reduction in PCR product yield, but mismatches of T with either G, C, or T have a minimal effect on PCR product yield (Kwok *et al.*, 1990). Stadhouders *et al.* (2010) also observed the same. Thus, despite two mismatches of T:C between reverse primer FST-R and the template, we still obtained PCR products from template DNA of *C. gestroi* collected from Van Quan and Tan Linh. We did not amplify the 151-bp region from the template DNAs of other four samples collected from Thai Ha, Vong Thi, Bui Xuong, and Van Lam (figure 2). The entire nucleotide sequences of the 151-bp region of these samples are unknown; however, they might be identical to those of samples obtained from Van Quan and Tan Linh. The mismatches may reduce the PCR efficiency, and perhaps the amplification products could be obtained by chance at the condition we employed in this study. Therefore, for the identification of closely related *Coptotermes* species by 16S rRNA gene, determination of nucleotide sequences is essential.

holders *et al.* (2010) also observed the same. Thus, despite two mismatches of T:C between reverse primer FST-R and the template, we still obtained PCR products from template DNA of *C. gestroi* collected from Van Quan and Tan Linh. We did not amplify the 151-bp region from the template DNAs of other four samples collected from Thai Ha, Vong Thi, Bui Xuong, and Van Lam (figure 2). The entire nucleotide sequences of the 151-bp region of these samples are unknown; however, they might be identical to those of samples obtained from Van Quan and Tan Linh. The mismatches may reduce the PCR efficiency, and perhaps the amplification products could be obtained by chance at the condition we employed in this study. Therefore, for the identification of closely related *Coptotermes* species by 16S rRNA gene, determination of nucleotide sequences is essential.

Conclusions

The *Coptotermes* from six places in Vietnam were identified to be *C. gestroi* based on the sequences of 430-bp and 151-bp regions of 16S rRNA. The observation of both PCR products of the 430-bp and 151-bp regions on agarose gel did not indicate the DNA template originated from *C. formosanus*.

Acknowledgements

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corrigenda

Identification of Vietnamese *Coptotermes* pest species based on the sequencing of two regions of 16S rRNA gene

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The name of one author was incorrect.

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