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Molecular Diagnosis of the Biological Control Agent *Nesidiocoris tenuis* (Tobacco Plant Bug) and Its Allied Species (Insecta: Hemiptera) Using COI Barcoding

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DNA barcoding is a useful molecular method for identification of certain animal groups. It uses partial DNA sequences of mitochondrial genes such as the cytochrome c oxidase subunit I (COI) gene. In this study, effectiveness of the COI barcoding as an identification tool for *Nesidiocoris tenuis*, which is used as a biological control agent, and for its allied species, was evaluated. All the species used in this study had a distinct COI barcode sequence, and the Neighbor–Joining (NJ) tree based on COI sequences almost matched the morphological classification for most genera investigated in this study, except for the genus *Cyrtopeltis*. The average interspecific genetic distance between *N. tenuis* and its allied species was 111 times higher than the average intraspecific genetic distance. The tree showed shallow intraspecific divergences and deep interspecific divergences. Therefore, our results suggested that COI barcode for *N. tenuis* and its allied species can be used as an effective identification tool by entomologists, quarantine experts and other related researchers, and can provide directions to taxonomists for further taxonomic studies.

Key words: Barcoding, Biological control agent, COI, Diagnosis, Miridae, *Nesidiocoris tenuis*,

INTRODUCTION

DNA barcoding is a useful molecular method for identification of certain animal groups using partial DNA sequences of mitochondrial genes such as the cytochrome c oxidase subunit I (COI) gene (Hebert *et al.*, 2004a; Hebert *et al.*, 2004b; Ward *et al.*, 2005; Jung *et al.*, 2011). This method can be applied to eggs, nymph, body fragments, and morphologically cryptic groups (Hebert *et al.*, 2003; Jung *et al.*, 2011; Park *et al.*, 2011). In previous studies, there have also been reports of successful COI barcoding in heteropteran species (Jung *et al.*, 2011; Part *et al.*, 2011; Raupach *et al.*, 2014).

Many species of the tribe Dicyphini (Hemiptera: Cimicomorpha: Miridae: Bryocorinae) can feed on other insects as well as their host plants for survival (Gemeno *et al.*, 2007; Namyatova *et al.*, 2015). Amongst them, some species, such as *Nesidiocoris tenuis* (Reuter, 1895), also called tobacco plant bug, are well known biological control agent in agro–ecosystems in many countries. They have predatory preference feeding habits, and they can control major insect pests such as whiteflies, aphids, thrips and moths in greenhouses (Wheeler, 2000a; Wheeler, 2001; Lins Jr *et al.*, 2014). On the other hand, these bugs also have a wide range of host plants, including certain greenhouse crops (Sanchez *et al.*, 2008). Therefore, they are classified as insect pests, as they can

damage crops by directly feeding on host plants such as tomato and pepper (Schuh and Slater, 1995; Wheeler, 2000b; Yasunaga, 2000; Wheeler, 2001; Arnó *et al.*, 2009). Despite their high economic importance, their morphological identification is very difficult (Raupach *et al.*, 2014). The aim of this study was to evaluate the effectiveness of COI barcoding as identification tool for discrimination between *N. tenuis*, which is used as a biological control agent, and its allied species, and to construct a COI barcode data of heteropterans found in a paprika farm for use in molecular diagnosis.

MATERIALS AND METHODS

Sampling focused on *N. tenuis* in Korea and Japan. In particular, Korean *N. tenuis* was separately collected from populations of natural field and paprika farms to evaluate the genetic divergences between them. Information of individuals used in this study is shown in Table 1. Most samples used in this study were directly placed in vials containing 99.9% ethanol after capturing, to preserve the DNA. All the species were identified based on morphological characters, including the parameres and genitalia. Taxonomic references for identification are as follows: *N. tenuis*: Hernandez and Henry (2010); *Cyrtopeltis miyamotoi*: Yasunaga (2000); *Cyrtopeltis rufobrunnea*: Lee and Kerzhner (1995); *Adelphocoris suturalis*: Yasunaga (1990); *Deraeocoris ulmi*: Josifov (1983); *Orius laevigatus*: Jung *et al.*, (2011); *Nabis stenoserus*: Kerzhner (1981); *Nezara antennata*: Freeman (1940).

Genomic DNA was extracted from tissues or whole body using the QIAamp DNA Mini Kit in accordance with the protocol of the manufacturer (Qiagen, Germany), after identification based on genital morphology.

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Polymerase chain reactions were performed using the Solg 2X Taq PCR Pre-mix (SolGent, Korea) with the primer pair LCO1490 and HCO2198 (Folmer *et al.*,

1994). The thermal cycling program consisted of an initial step of 95°C/2 min followed by 35 cycles of 95°C/20 s, 50°C/40 s and 72°C/1 min, and then followed by a final

Table 1. Information of individuals used in this study. Population types are coded as follows: B: biological control agent; I: introduced population from natural field; N: natural species.

Taxa	Location	Collecting site	Population type	Collectingdate	Host plant / Habitat type
Miridae					
<i>Nesidiocoris tenuis</i>	Korea	Daejeon	N	11.viii.2014	–
	Korea	Daejeon	N	22.viii.2014	<i>Humulus japonicus</i>
	Korea	Daejeon	N	12.ix.2014	Forbs
	Korea	Daejeon	N	5.x.2014	<i>Lycopersicon esculentum</i>
	Korea	Daejeon	N	5.x.2014	<i>Lycopersicon esculentum</i>
	Korea	Daejeon	N	5.x.2014	<i>Lycopersicon esculentum</i>
	Korea	Nonsan	B	21.iii.2014	<i>Nicotiana tabacum</i>
	Korea	Hwasun	B	16.vi.2014	<i>Capsicum annuum</i> ^a
	Korea	Hwasun	N	16.vi.2014	Forbs
	Korea	Jeju	B	17.vi.2014	<i>Capsicum annuum</i> ^a
	Korea	Jeju	B	17.vi.2014	<i>Capsicum annuum</i> ^a
	Korea ^b	–	–	–	–
	Japan	Tokushima	N	29.vii.2014	<i>Sesamum indicum</i>
	Japan	Tokushima	N	29.vii.2014	<i>Sesamum indicum</i>
	Japan	Tokushima	N	29.vii.2014	<i>Sesamum indicum</i>
	Japan	Tokushima	N	29.vii.2014	<i>Sesamum indicum</i>
	Japan	Tokushima	N	29.vii.2014	<i>Sesamum indicum</i>
	Japan ^b	–	–	–	–
	Spain ^b	–	–	–	–
	Korea	Guemsan	N	5.vi.2014	<i>Rosa multiflora</i>
	Korea	Jeju	N	19.vi.2014	<i>Rosa multiflora</i>
<i>Cyrtopeltis rufobrunnea</i>	Korea	Hwacheon	N	17.vii.2015	<i>Rubus phoenicolasius</i>
<i>Macrolophus melanotoma</i>	Greece ^b	–	–	–	–
<i>Macrolophus pygmaeus</i>	Germany ^b	–	–	–	–
<i>Adelphocoris suturalis</i>	Korea	Daejeon	N	11.viii.2014	Forbs
<i>Deraeocoris ulmi</i>	Korea	Daejeon	N	07.x.2014	<i>Zelkova serrata</i>
Anthocoridae					
<i>Orius laevigatus</i>	Korea	Hwasun	B	16.vi.2014	<i>Capsicum annuum</i> ^a
		Jeju	B	17.vi.2014	<i>Capsicum annuum</i> ^a
Nabidae					
<i>Nabis stenoferus</i>	Korea	Daejeon	N	15.ix.2014	Forbs
Pentatomidae					
<i>Nezara antennata</i>	Korea	Daejeon	N	27.ix.2014	<i>Capsicum annuum</i> ^a
	Korea	Jeju	I	17.vi.2014	<i>Capsicum annuum</i> ^a

^a Full scientific name: *Capsicum annuum* var. *angulosum*

^b Sequences from NCBI. Korea: GU194801; Japan: AB587603; Spain: HQ291844; Germany: KM022848; Greece: HQ707832.

extension step of 72°C/5 min. The product yield was monitored by electrophoresis with 1.4% agarose gel. The amplified products were purified using a MG™ PCR SV purification kit (MGmed, Inc.), and were sequenced using an ABI PRISM 3730xl analyzer (96 capillary type) (Macrogen, Korea). All the sequences obtained were aligned with certain sequences from National Center for Biotechnology Information (NCBI) (Table 1) using Megalign (DNA-star) and MEGA version 5.2 (Tamura *et al.*, 2011), and were found to have no INDELs. Sequence divergences were calculated using the Kimura-2-parameter model (K2P) (Kimura, 1980), and the trees

were generated using the neighbor-joining method (NJ) (Saitou and Nei, 1987).

RESULTS

All the species used in this study had a distinct COI barcode sequence (480 bp). Intraspecific sequences from individuals of 4 species were identical or very similar. The average interspecific genetic distance between *N. tenuis* and its allied species (22.3%) was 111 times higher than the average intraspecific genetic distance (0.2%). The tree constructed based on the NJ method showed

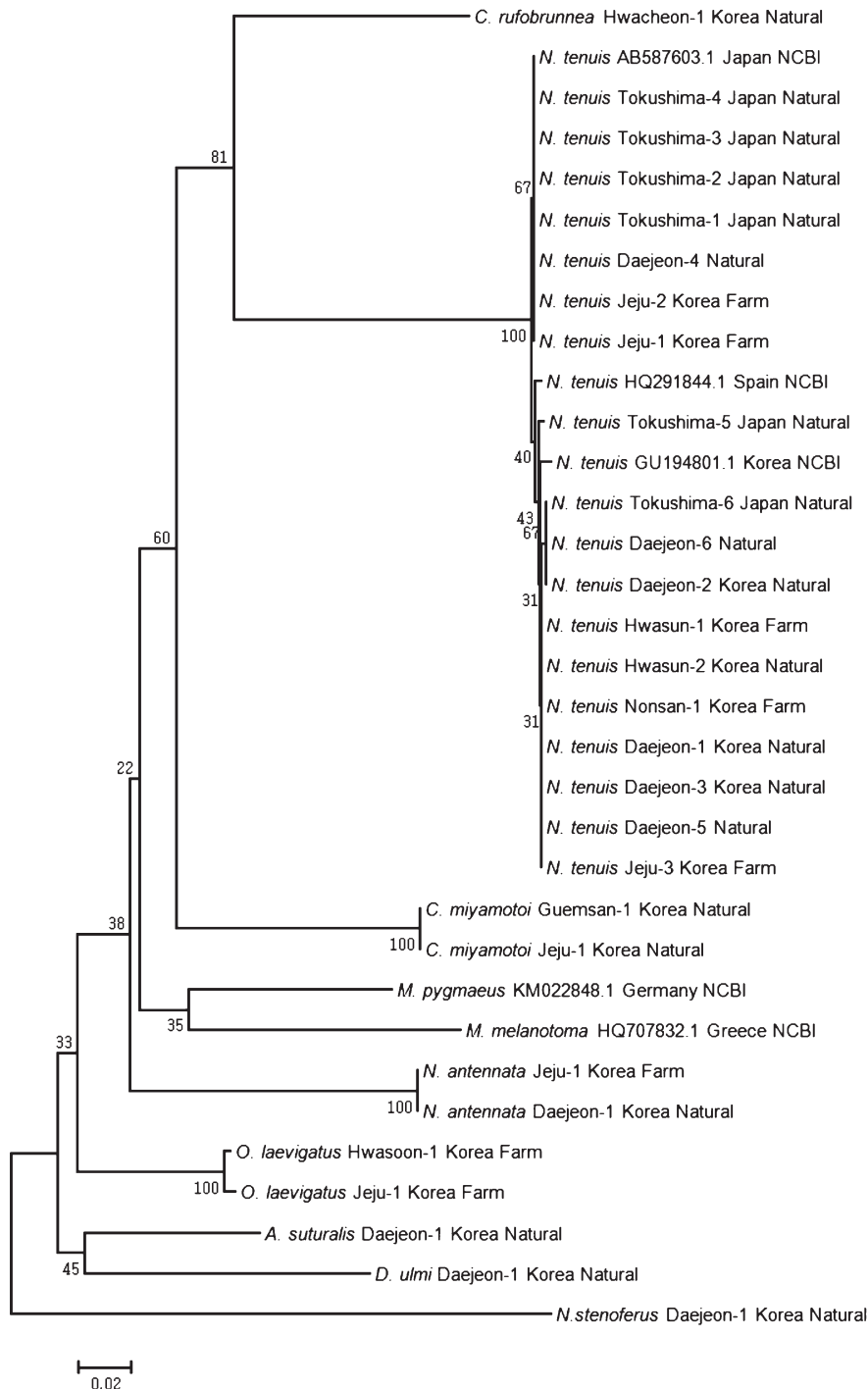


Fig. 1. Neighbor-Joining tree based on 33 COI sequences of ten species in this study.

shallow intraspecific divergences and deep interspecific divergences.

In case of *N. tenuis*, all the individuals used in this study were clustered together regardless of the populations (Fig. 1). The average distance of *N. tenuis* (0.3%) was lower than the average distance of *Orius laevigatus* (0.6%).

The NJ tree based on COI barcode sequences was very similar to the morphological classification for most genera analyzed in this study (Fig. 1). As an exception, the two species of the genus *Cyrtopeltis* in this study were separated into two clades based on the NJ tree, indicating that *C. rufobrunnea* was clustered with *N. tenuis* in the same clade. The maximum K2P distance was 20.8%, and the average K2P distance was 20.4% between *C. rufobrunnea* and *N. tenuis*.

DISCUSSIONS

To investigate the effectiveness of a COI barcode of *N. tenuis* as a molecular diagnosis tool, the levels of intraspecific variation of *N. tenuis* and interspecific distances between *N. tenuis* and its allied species were evaluated. The level of interspecific distance of all the species used in this study was found to exceed the level of intraspecific distance, and each genus consisted of distinct clades, except for the two species in the genus *Cyrtopeltis*. This result suggested that *C. rufobrunnea* was more closely related to *N. tenuis* than related to *C. miyamotoi*. To confirm their taxonomic positions, further studies would be required of additional samples of these species for morphological and molecular analysis. Furthermore, all the individuals of *N. tenuis* from different populations of natural fields and paprika farm, and Korea and Japan were clustered together (Fig. 1). This might be a result of the shorter length of the COI barcode (480 bp) used in this study, as compared to the previous barcode studies (Jung *et al.*, 2011; Park *et al.*, 2011).

To confirm the effectiveness of the COI barcodes, the minimum interspecific distance of congeners was compared with the maximum intraspecific distance. The average maximum intraspecific distance of *N. tenuis* and *O. laevigatus* was 0.8%, whereas the average minimum interspecific distance of congeners was 18.5%. Some previous studies on barcode for diverse animal groups have reported a minimum interspecific distance of >2% for sister species (Klicka and Zink, 1997; Johns and Avise, 1998; Hebert *et al.*, 2004a; Jung *et al.*, 2011). Therefore, *N. tenuis* and its allied species can be easily distinguished using the COI barcode.

Some Dicyphini species have been used as the major biological control agents in agriculture in several countries, and they play an important role in ecosystem as predator or pests because they are zoophytophagous (Schuh and Slater, 1995; Wheeler, 2001). Among them, *N. tenuis* is one of the most popular species in many countries as a biological control agent (Wheeler, 2000; Bueno and van Lenteren, 2012). Nevertheless, this tiny bug is often difficult to identify without the help of tax-

onomist. Furthermore, this species usually leads to problems for the development and maintenance of an effective quarantine system, when agricultural products and nurseries are imported and exported because of the difficulty of identification. Therefore, our results suggested that the COI barcode for *N. tenuis* and its allied species can be used as an identification tool by entomologists, quarantine experts and other related researchers, and can provide directions to taxonomist for further taxonomic studies of these species.

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