

# A multiplex polymerase chain reaction based method for rapid identification of two species of the genus *Scolytus* Geoffroy (Col: Curculionidae: Scolytinae) in Iran

S. Amini, R. Hosseini

Department of Plant Protection, University of Guilan, Rasht, Iran

## Abstract

Molecular identification is going to be more widespread in taxonomic studies of insects when traditional tools are problematic and time consuming. Identification of bark beetles, as one of the most important pests of forests, based on morphological characteristics is difficult because of their small size and morphological similarities. In the current study, species-specific primers were designed to identify two most abundant and morphologically similar bark beetle species *Scolytus ensifer* Eichhoff 1881 and *S. ecksteini* Butovitsch 1929, both found on *Ulmus minor* Miller in north of Iran. These species-specific primers successfully produced a fragment size with 318 bp and 465 bp of mitochondrial cytochrome oxidase I (COI) gene in *S. ensifer* and *S. ecksteini* respectively. The results revealed that the multiplex polymerase chain reaction using the species-specific primers could amplify a unique band to distinguish these two species so confirmed this method as a convenient and quick tool to identify those two bark beetle species.

Correspondence: Sudabe Amini, Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran.  
Tel: +98.9151602647 - Fax: +98.563242202.  
E-mail: sudabe.amini@ut.ac.ir.

Key words: Bark beetle; cytochrome oxidase I; molecular identification; multiplex polymerase chain reaction.

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## Introduction

Bark beetles of the subfamily Scolytinae (Coleoptera: Curculionidae) contain nearly 6000 described species worldwide by small size less than <1 mm to 1 cm in length (Jordal, 2007). The majority of Scolytids are decomposer of plant tissues but some are aggressive pests of forests by significant economic and ecological damage in this ecosystem (Furniss & Carolin 1977; Kuschel, 1995). The pest species of this group include those feed on phloem (true bark beetles), bore into the xylem and cultivate a fungal garden (Ambrosia beetles). Some species in the genus *Scolytus* Geoffroy, 1762 can transfer pathogenic ophiostomatoid fungi, which cause lethal Dutch elm disease (DED) in *Ulmus* (elm) trees (Gibbs, 1978). At least eight *Scolytus* species are known as vectors of DED including *Scolytus ensifer* Eichhoff, 1881 and *S. pygmaeus* F. 1787 (Gibbs, 1978; Faccoli *et al.*, 1998).

Identification of bark beetles is problematic since there are paucity in morphological characteristics. Meanwhile, identification of scolytine immature stages would be more challenging if a control strategy needed to be implemented for the pest species, such as vectors of Dutch elm disease (Santini and Faccoli, 2015). *S. ensifer* and *S. ecksteini* Butovitsch, 1929 are two abundant bark beetles species in Guilan province (north of Iran) that utilize *Ulmus* species (Ulmaceae) (Amini *et al.*, 2012). Traditional identification of *S. ensifer* and *S. ecksteini* based on morphological characteristics is difficult due to wide inter- and intra-specific variability of their general size, shape, and color of adults. For most *Scolytus* species, diagnostic characteristics are the presence and shape of spines on the abdominal sternites. In both species, location of spines on basal margin of the second abdominal sternite is identical (Pfeffer, 1994). However, shape of the spine is slightly different and taxonomic training is necessary for its correct identification (Amini & Hosseini, 2012).

During the last decade, molecular techniques have been used for identification of insects that lack diagnostic morphological characteristics (Caterino *et al.*, 2000) but a few molecular methods have been developed for identification of Bark beetles. Mitochondrial cytochrome oxidase I (COI) sequences has provided tools for identification of scolytines (Stauffer *et al.*, 1997; Kelley *et al.* 1999; Cognato & Sperling, 2000; Cognato & Sun, 2007). Johnson *et al.* (2008) used random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) for identification of the two non-native bark beetles. Jordal and Kambestad (2013) used DNA Barcoding for identification of bark and ambrosia beetles. Among molecular diagnostic techniques, the multiplex PCR method uses multiple primers in a single reaction for amplification of species-specific PCR products. This method amplifies specific sequences of genome by PCR in a single reaction simultaneously to identify any life stage of numbers of species (Bej *et al.*, 1991; Henson & French, 1993). This method has been proven as a reliable

identification method for economically important pests or predators (Szalanski *et al.*, 2003; Hosseini *et al.*, 2007; Szalanski & McKern, 2007; Hosseini & Hajizadeh, 2011). So, objectives of the current study were to design species-specific primers and to develop a multiplex PCR method for rapid and accurate identification of the two common *Scolytus* species present on *Ulmus minor*.

## Materials and methods

### Bark beetle sampling

Bark beetle specimens were collected on the trunks and twigs of *U. minor* in three different parts of Guilan province during May - September 2011-2012. In total 160 samples were collected on *Ulmus minor*. Specimens were placed in 1.5 mL micro tubes, transferred to the laboratory and immediately stored at  $-20^{\circ}\text{C}$ . All specimens were identified under a stereomicroscope (Olympus<sup>TM</sup>, model SZX-12) by using taxonomic keys (Pfeffer, 1994) and sent to Sarah Smith and Anthony Cognato (United States of America, Michigan State University) to check the identifications based on morphological characters. All voucher specimens were deposited in Natural History Museum, University of Guilan (Rasht, Iran).

### DNA extraction

DNA was extracted from whole body of each identified species, separately. Each species individually was homogenized in 1.5 mL tubes using a sterile plastic pestle in 50  $\mu\text{L}$  of phosphate buffer saline pH 7.4 Samples were incubated at  $56^{\circ}\text{C}$  for 4-5 h followed by adding 500  $\mu\text{L}$  of Chelex 5% (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and placed in water bath at  $94^{\circ}\text{C}$  for 15 min. After centrifugation at 13,000 g for 5 min, DNA was transferred to a new tube and stored at  $-20^{\circ}\text{C}$  (Hosseini, 2010).

### Polymerase chain reaction and sequencing

Three universal primers including C1-J-1718 (5'-GGAGGATTG-GAAATTGATTAGTTCC-3') as forward primer, C1-N-2191 (5'- CCCG-GTAAATTAATAATAACTTC -3') (Simon *et al.*, 1994) and C1-J-2411 (5'-GCTAATCATCTAAAACTTTAATCCWGTWG-3') as reverse primers (Normark *et al.*, 1999) were used to amplify a part of CO1 in each species. PCR cocktails contained a total volume of 25  $\mu\text{L}$  including 15.05  $\mu\text{L}$  ddH<sub>2</sub>O, 2.5  $\mu\text{L}$  reaction buffer, 0.25  $\mu\text{L}$  dNTPs (15 mM, Fisher Scientific Inc., Hampton, New Hampshire, USA), 1  $\mu\text{L}$  MgCl<sub>2</sub> (50 mM Cinnagen Co., Tehran, Iran), 1  $\mu\text{L}$  of each primer (forward and reverse), 10  $\mu\text{M}$  (Bioneer Co., Daejeon, South Korea), 0.2  $\mu\text{L}$  of Taq DNA polymerase (5U/  $\mu\text{L}$ , Cinnagen Co.) and 4  $\mu\text{L}$  of DNA template (50-70 ng/  $\mu\text{L}$ ). The master mix was placed in a 0.2 mL PCR tube and amplified in a thermocycler (Mastercycler gradient, Eppendorf, Germany) with the relevant temperature profile shown in Table 1. The amplified fragments were visualized by 1.4% agarose gel in TAE or TBE buffer

stained in SYBER Safe DNA Gel Stain% (Bio-Rad Laboratories, Inc.). PCR products were sequenced using the Sanger sequencing method by ABI3730XL sequence analyzer (Applied Biosystems, Foster City, CA, USA) from Bioneer Co.

### Sequence alignment and primer design

CO1 fragments were sequenced from three individuals per species in forward direction. Sequencing results were reviewed by the Finch Tv Soft-pedia software (<http://www.geospiza.com/ftvdlinfo.html>) and edited manually for each species individually. The *basic local alignment search tool* (BLAST) was used to compare similarity of our nucleotide sequences with other sequences present in GenBank database, (<http://www.ncbi.nlm.nih.gov/blast>). Edited sequences were deposited in GenBank database with specific accession numbers (Tables 2 and 3). Sequences were aligned using MEGA ver. 5.0 software (Tamura *et al.*, 2011) to reveal their nucleotide variation. Forward primers were designed for *S. ensifer* and *S. ecksteini* according to their sequence differences by using universal reverse primer (C1-J-2411). Guidelines proposed by Innis and Gelfand (1990) and Saiki (1990) were followed to design efficient and specific primers. The primer-primer interactions were analyzed using Integrated DNA technologies (IDT 2010; PrimerQuest: <http://eu.idtdna.com/Scitools/Applications/Primer-quest/Default.aspx>) software and those were synthesized by Bioneer Co. A gradient PCR program was performed using a gradient thermocycler as 35 cycles at  $94^{\circ}\text{C}$  for 1 min,  $50^{\circ}\text{C}$  as the lower temperature and  $60^{\circ}\text{C}$  as the higher temperature for 45 s, and  $72^{\circ}\text{C}$  for 1 min 30 s to optimize annealing temperature of primer pairs. A first cycle of denaturation was  $94^{\circ}\text{C}$  for 2 min and a final extension was performed at  $72^{\circ}\text{C}$  for 5 min. Specificity of designed primers were evaluated using both singleplex and multiplex PCR compared with other bark beetle species including; *S. pygmaeus* (F.), *S. rugulosus* (Müller), *Hypothenemus eruditus* Westwood, and *Taphrorychus lenkoranus* Reitter found on other hosts in Guilan province (Amini *et al.*, 2012).

### Multiplex polymerase chain reaction

A multiplex PCR was conducted to amplify specific fragments from each bark beetle species. PCR cocktails contained a total volume of 25  $\mu\text{L}$  including 4  $\mu\text{L}$  of DNA (20-50 ng/ $\mu\text{L}$ ), 0.25  $\mu\text{L}$  of dNTP's (15 mM), 1  $\mu\text{L}$  of MgCl<sub>2</sub> (50 mM), 4  $\mu\text{M}$  of equal molar of each specific forward primers with one reverse universal primer (C1-J-2411) and 0.2  $\mu\text{L}$  of Taq DNA polymerase (5 U/ $\mu\text{L}$ ). Cycling condition of PCR has been shown in Table 1. PCR products were mixed with loading buffer and run on 1.4% agarose gel prior to be stained by SYBER Safe DNA Gel Stain solution (Cinnagen Co.). A 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) was used for determining of fragment size.

### Application of multiplex polymerase chain reaction

To test efficiency of multiplex PCR for identification of the collected specimens, 70 samples of unknown adults and larvae (58 and 12

**Table 1. Temperature profile for amplification of cytochrome oxidase one fragments and multiplex polymerase chain reaction.**

C1-J-1718/ C1-J-2411		C1-J-1718/ C1-N-2191		Multiplex PCR	
1	$94^{\circ}\text{C}$ for 2 min	1	$94^{\circ}\text{C}$ for 2 min	1	$94^{\circ}\text{C}$ for 2 min
2	$94^{\circ}\text{C}$ for 1 min	2	$94^{\circ}\text{C}$ for 1 min	2	$94^{\circ}\text{C}$ for 1 min
3	$52^{\circ}\text{C}$ for 45 s	3	$50^{\circ}\text{C}$ for 1 min	3	$53^{\circ}\text{C}$ for 1 min
4	$72^{\circ}\text{C}$ for 1 min	4	$72^{\circ}\text{C}$ for 1 min	4	$72^{\circ}\text{C}$ for 1 min 30 s
5	Go to 2 for 34 cycle	5	Go to 2 for 34 cycle	5	Go to 2 for 35 cycle
6	$72^{\circ}\text{C}$ for 5 min	6	$72^{\circ}\text{C}$ for 5 min	6	$72^{\circ}\text{C}$ for 5 min

PCR, polymerase chain reaction.

respectively) were collected on *U. minor* from different locations of Guilan province and identified morphologically by expert entomologist (Sarah M. Smith Ph.D. in Michigan State University). Four adults and two larvae were selected for the multiplex PCR test. All specimens were individually placed in 1.5 mL tubes and kept at  $-20^{\circ}\text{C}$  for subsequent molecular assay. DNA extractions were performed using the Chelex 5% (Bio-Rad Laboratories, Inc.) method described earlier.

## Results

In this study, adults of three *Scolytus* species including *S. ecksteini*, *S. ensifer*, and *S. pygmaeus* were identified morphologically and a part of their CO1 was successfully amplified. The universal primers, C1-J-1718/ C1-J-2411, amplified fragments of approximately 650 bp of CO1 gene in *S. ecksteini*, *S. pygmaeus* and *S. rugulosus*, but amplification was not successful for *S. ensifer*. Therefore primers C1-J-1718/ C1-N-2191 were used to amplify a shorter fragment in *S. ensifer*. These universal primers amplified first 465 bp of CO1 upstream region in *S. ensifer* but those amplified nearly 650 bp barcode region in other

*Scolytus* species (Hebert *et al.*, 2003). Amplified fragments varied in size because of using different primers. Size of fragments was 610, 650, 661 and 465 bp in *Scolytus rugulosus*, *S. pygmaeus*, *S. ecksteini* and *S. ensifer*, respectively.

BLAST searches on Genbank show high similarity to others *Scolytus* species. Obtained sequences were submitted to Genbank and were provide accession number (Table 2).

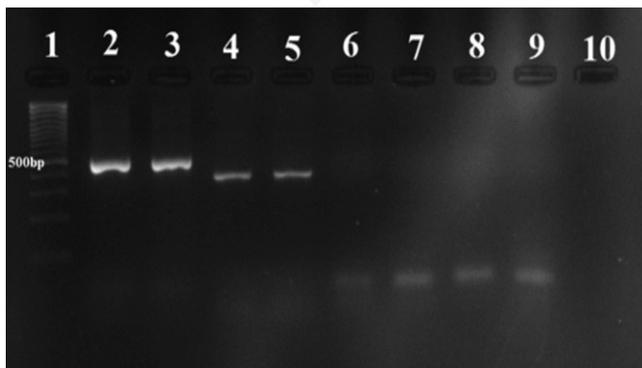
Three equal molar primers including two specific forward primers and one universal reverse primer (C1-N-2191) were combined in a multiplex PCR assay to achieve a quick and robust molecular technique for identification two species of bark beetles on *Ulmus* trees in Guilan province. The optimal annealing temperature for multiplex PCR was determined to be  $53^{\circ}\text{C}$ . Results indicated that the specific used primers developed were successfully amplified the expected specific fragment for *S. ensifer* and *S. ecksteini*. The size of PCR products for *S. ensifer* and *S. ecksteini* were 318 bp and 465 bp respectively. Specificity tests for primer pairs showed amplification of fragments for target species although no amplification was observed in *S. pygmaeus* and *S. rugulosus* as non-target species (Figure 1). Our results also verified the application of multiplex PCR for successful identification of field-collected specimens (Figure 2).

**Table 2. Species, primers and GenBank accession numbers for mitochondrial cytochrome oxidase one sequences.**

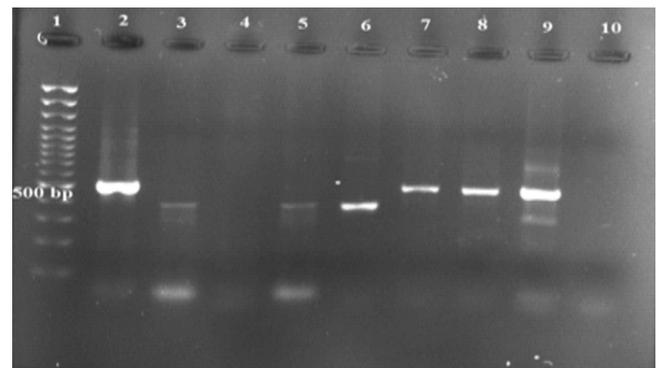
Species	Primers	Accession numbers
<i>Scolytus ensifer</i>	(1718/2191)	JX913804
<i>Scolytus ecksteini</i>	(1718/2411)	JX416909, JX416907, JX416902
<i>Scolytus pygmaeus</i>	(1718/2411)	JX089346, JX089347, JX089348
<i>Scolytus rugulosus</i>	(1718/2411)	JX089345, JX089343, JX089342
<i>Scolytus pygmaeus</i>	(1718/2411)	JX089346, JX089347, JX089348

**Table 3. Species-specific forward primers for cytochrome oxidase one, length of primer and fragment size.**

Species	Primer name	Primer sequence (5' to 3')	Primer length (bp)	Fragment size (bp)
<i>Scolytus ecksteini</i>	EC-S19	GATTCCTCTATTTTGGTGCTA	22	465
<i>Scolytus ensifer</i>	EN-S20	TTTACTCTCGTTGCCCGTAC	20	318



**Figure 1. Multiplex polymerase chain reaction by combination of three equimolar primers for CO1. 1: 100 bp DNA ladder; 2 and 3: *Scolytus ecksteini*; 4-5: *S. ensifer*; 6: *S. pygmaeus*; 7: *S. rugulosus*; 8: *Hypothenemus eruditus*; 9: *Taphrorychus lenkoranus*; 10: negative control.**



**Figure 2. Multiplex polymerase chain reaction test for identification of field collected specimens. 1: 100 bp DNA ladder; 2 and 3: positive control (*Scolytus ensifer* and *S. ecksteini*); 4: unknown species larvae; 5-6: *S. ensifer* adults; 7-8: *S. ecksteini* adults; 9: *S. ecksteini* larvae; 10: negative control.**

## Discussion and conclusions

Accurate identification of pests is fundamental in control strategy of them. In this study in the first step part of mitochondrial genome sequenced successfully and then designed primers and test primers to amplify specific fragments. The major problem in identification of bark beetles species is similarity of morphological characters such as similarity in shape of spine that is exactly on the same sternites of abdomen in both species (Amini *et al.*, 2012) that makes identification difficult, time consuming, and morphologic method needs good quality specimens and expertise in specialized taxonomy (Leclercq & Lecomte, 1978). Above all *Scolytus* species are vectors of fungi *Ophostoma ulmi* that lead to Dutch elm disease and implementation of a specific control strategy for species would be a crucial challenge if identification should be made in their immature stages. According to recent studies PCR-based methods have shown to be a powerful tool in the identification of quarantined pests that have a similar morphology (Hosseini *et al.*, 2007), but the molecular technology using mtDNA is easier to perform and saves time. This method can also identify damaged specimens with lack of morphological characters (Judith & Nicola, 2008).

As Bark beetles life cycle is under the bark of tree, in most of time they are not accessible in numerous and just their galleries or their vouchers are represented. So multiplex-PCR method could be an efficient identification tool when a specimen is one of only a handful of species (Garipey *et al.*, 2008). Results of this study indicated that different *Scolytus* species could utilize and simultaneously live on the same branches of elm trees. Those are very similar so their discrimination might be difficult mainly in immature stages. A rapid identification method develop for diagnosis of two important similar species of bark beetles among the bark beetles that attack elm trees.

Our experiments confirmed efficiency of multiplex PCR method for identification of field collected *Scolytus* in both larval and adult stages in same time that might be compared with findings of Hosseini *et al.* (2007) and Hosseini and Hajizadeh (2011). Hosseini and Hajizadeh (2011) designed species-specific primer and identified three mealybug species belong to different geographic location in a single reaction by multiplex PCR.

In this study CO1 in mtDNA region is selected for design of primers, because studies have shown that this region is useful for identification of Coleoptera species (Paul *et al.*, 2009; Dirk *et al.*, 2007; Fang, 2009) as result showed high potential of this region of genome in discrimination of *Scolytus* species.

Johnson *et al.* (2008) developed a RAPD-PCR method to identify collected *Scolytus* species as the two non-native bark beetles. Although, the authors insist on using selected oligonucleotide primers in the RAPD-PCR analysis but other studies showed RAPD-PCR technique as a notoriously laboratory-dependent that could not be reproducible in other conditions (Johnson *et al.*, 2008). Our study, the specific primers were successfully designed according differentiates in sequences and developed from Barcoding region of *S. ensifer* and *S. ecksteini* species (CO1, 5' upstream region) and distinguished species from the other scolytid species accurate, in one reaction simultaneously.

Chen *et al.* (2013) designed species-specific primers according to CO1 region of an invasive species *Dendroctonus valens* Leconte by using Nested PCR method. The authors had chosen the specific region of genome by Nested PCR method to design the high sensitive and specific primers for rapid and accurate distinguish beetles from the others that are abundant in China ports. Chen *et al.* (2013) result is compared with our essay, but because in *S. ensifer* species PCR was not successful and DNA could not amplified by universal primers, this led to choose a shorter region of CO1 genome to amplify. Combination of the two specific primers along with a reverse primer could successfully generated two identical different fragments, which is a major necessity in designing

multiplex PCR primers. The species-specific associated primers could be successfully used in multiplex PCR to amplify products with two different sizes, which allows identification of the two bark beetle species in a single reaction. It is possible to identify a large number of *Scolytus* species even in immature developmental stages, which might be a rapid and relatively low cost. This method proved to be an applicable technique for forestry studies, which is rapid and accurate particularly when the issue involves quarantine pests. Multiplex PCR has ability to detect detecting pathogens in insect vectors (Roy *et al.*, 2005; Ravikumar *et al.*, 2011) like ambrosia beetles and it would be applicable in future study to detect fungi transported by ambrosia beetle by this innovative methods.

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