Comparison of DNA Sequence Encoding Hydroxynitrile Lyase from the Invasive Millipede, *Chamberlinius hualienensis,* collected at Kagoshima, Shizuoka, and Hachijojima, Tokyo

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Abstract

The invasive millipede, *Chamberlinius hualienensis,* has been expanding its habitat from Okinawa to Kagoshima, Shizuoka, and Hachijojima, Tokyo, within a short period of time. It is thought that the animals in the three places had come from the same original population. The gene for hydroxynitrile lyase from *Chamberlinius hualienesis* (*ChuaHNL*) consists of 5 exons: 16-123, 186-316, 370-440, 499-594, and 653-798. The ChuaHNL cDNA sequence of the three samples was identical to each other. These results suggest that the wide distribution of *C. hualienensis* in southern Japan is likely caused by soil contamination and/or millipedes clinging to wooden boxes or timber as a result of transportation by human activities.

Key words

Chamberlinius hualienensis, hydroxynitrile lyase, genomic sequence, cDNA

1. Introduction

Non-indigenous species are unintendedly imported as results of international trade and human mobility and recognized as a major threat to biodiversity in environments. In the United States, these invasive arthropods such as bark beetles (Coleoptera: Curculionidae), the gypsy moth *Lymantria dispar,* the potato psyllid *Bactericera cockerelli,* are responsible for severe damages in agricultures and forest industries (Hanner et al, 2009; Liu & Trumble, 2007).

The invasive millipede, *Chamberlinius hualienensis,* originally from Hualien, Taiwan (Chen et al, 2011), invaded Okinawa Island, Japan in 1983 (Higa & Kishimoto, 1987). Large swarms of the millipedes enter houses and sometimes cause train delays (Niijima & Arimura, 2002). Swarming of these millipedes has been observed in Kagoshima in 1999, Shizuoka in 2003, and Hachijojima, Tokyo, Japan in 2002 (Fujiyama, 2009; Ishida et al, 2016; Meyer-Rochow, 2015) (https://www.nies.go.jp/biodiversity/invasive/DB/detail/70490.html). Since the millipede species has been expanding its habitat over a short period of time, it is thought that the animals in the three places had come from the same original population. However, its genetic diversity had remained to be elucidated.

Hydroxynitrile lyase from *C. hualienensis* (ChuaHNL) is identified as an enzyme, which plays a role in decomposing (*R*)-mandelonitrile in the storage chamber into benzaldehyde and hydrogen cyanide as defensive chemicals. This enzyme also catalyzes stereoselective cyanohydrin synthesis from aldehydes or ketones and potassium cyanide with the highest specific activity among known HNLs, and is considered as a biocatalyst for the synthesis

of intermediates in the fine chemical and pharmaceutical industries. In addition, no homologous protein is identified by Blast search analysis (Dadashipour et al, 2015). Thus, the gene and cDNA are appropriate DNA sequences for identification of its species and evaluation of its genetic diversity.

In this short report, we sought to determine ChuaHNL genomic and cDNA sequences and compare the cDNA sequence among millipedes of *C. hualienensis,* collected in Kagoshima, Shizuoka, and Hachijojima, Tokyo, and analyze their genetic diversity.

2. Materials and Methods

2.1 Animal collection

Invasive millipedes of *C. hualienensis* [Polydesmida: Paradoxomatidae] were collected in Kagoshima, Shizuoka, and Hachijo, Tokyo, Japan and brought to our laboratory. The colonies were maintained by supplying them with sliced sweet potatoes and spraying water *ad libitum* at 18℃ and 70-90% relative humidity.

Metridia pacifica and the sea firefly, *Vargula hilgendorfii,* were collected at Himi and Hotaruika museum at Namerikawa, Toyama, Japan, respectively.

2.2 Genomic DNA and RNA extraction

Each millipede was anesthetized on ice. Legs and antennae were removed using clean fine forceps under a microscope. The body was immobilized with insect pins and soaked in phosphate buffered saline (Nacalai Tesque, Kyoto, Japan). Paratarga containing a storage chamber and a reaction chamber were collected and immediately homogenized in a TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) using a disposable pellet pestle (Nippi, Tokyo, Japan). DNA and RNA were isolated according to the manufacturer's instruction. *M. pacifica* was directly homogenized in a TRIzol Reagent and *V. hilgendorfii* after removing the shells and the gut was homogenited in the same reagent. Other procedures were carried out as described above.

2.3 cDNA synthesis

cDNA for 3'-rapid amplification of cDNA ends (RACE) was synthesized using a SMART RACE cDNA Amplification Kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's instruction.

2.4 PCR

The following PCR program was carried out using KOD plus neo or KOD FX neo (Toyobo, Osaka, Japan) as *Taq* DNA polymerases and gene-specific primers, ChuaHNL-4 gene-specific primer, 5'-ATAATCCCTTTAAAGTTCAGGTGCAATTAG-3' and ChuaHNL-5 gene specific primer, 5'-ATACCAACACATCAAACTTACCAAGCTTAG-3' (These primers are able to anneal 5'- and 3'-untranslated regions of ChuaHNL cDNA and to PCR-amplify the open reading frame.) (Dadashipour et al, 2015); 94℃ for 2 min; 40 cycles of 98℃ for 10 s, 64℃ for 30 s, and 68℃ for 30 s.

The following gene-specific primers were used for PCR amplification of cDNAs encoding luciferases from *M. pacifica* and *V. hilgendorfii:* MpacLUC1-1, 5'-GGATCCAAAAGGAAAGGAGCTAAATCTACAG-3'; MpacLUC1-2, 5'-AATTTCATTCTGTTTGACAAAAAAATGGCCAC-3'; VhilLUC-1, 5'-CTATCAGATGGACTGTGTGAAAATAAACCAGG-3'; VhilLUC-2, 5'-GACAAAGCAAGATATCAATTCCAGGG-3'; VhilLUC-3, 5'-CCCTGGAATTGATATCTTGCTTTGTC-3'; VhilLUC-4, 5'-CCTTGTTGTCCCCTCAGGCAATACTCGTAC-3'; VhilLUC-5, 5'-GAGTGGATGTATCTATCCCGTACAGCTCTG-3'; VhilLUC-6, 5'-GGGGGTCAGAGCGCATGCTCCTTCTGCGTC-3'; VhilLUC-7, 5'-GGTAAATATGTGCTGGCTCGAGGAACCAAG-3'; VhilLUC-8, 5'-

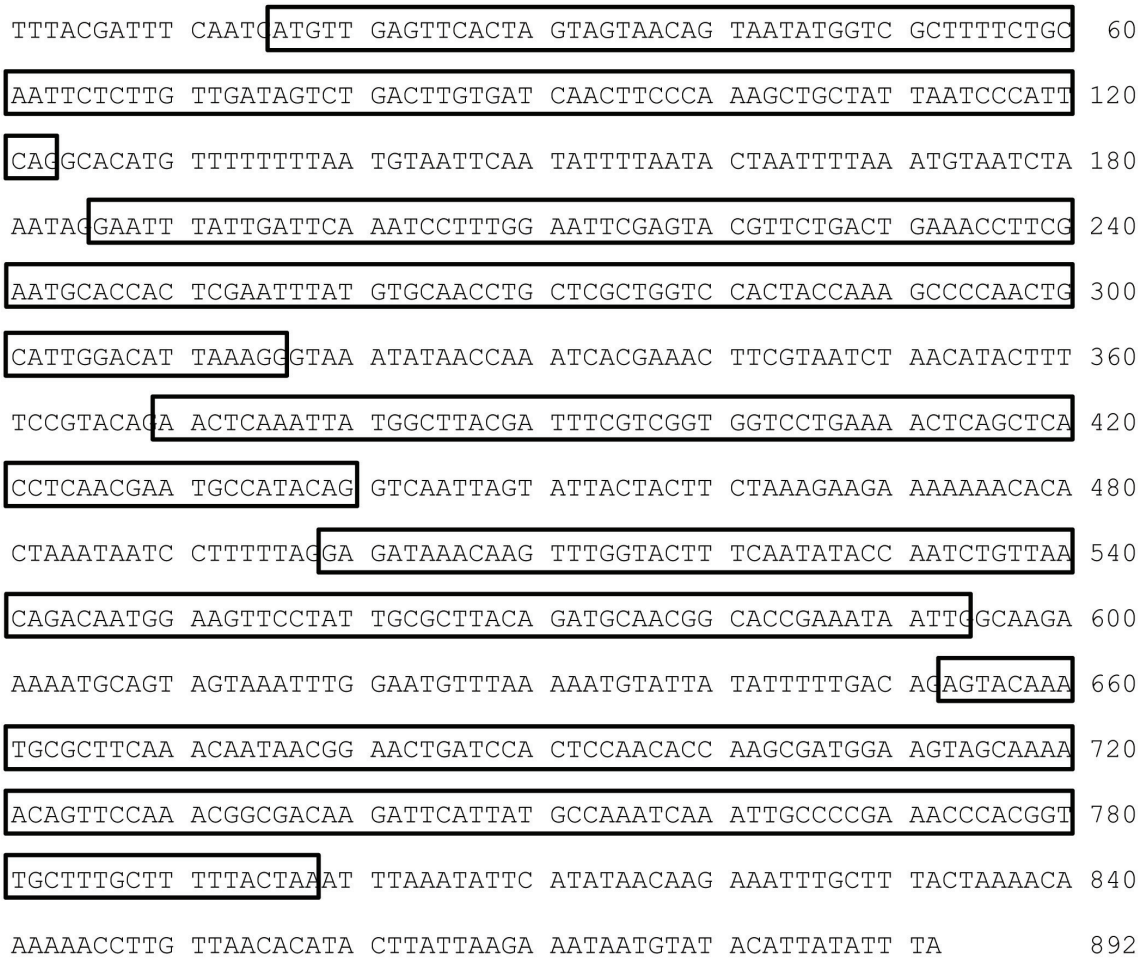

Fig. 1 DNA sequence of *ChuaHNL*. Exons are boxed. The DNA sequence was determined from 4 independent clones.

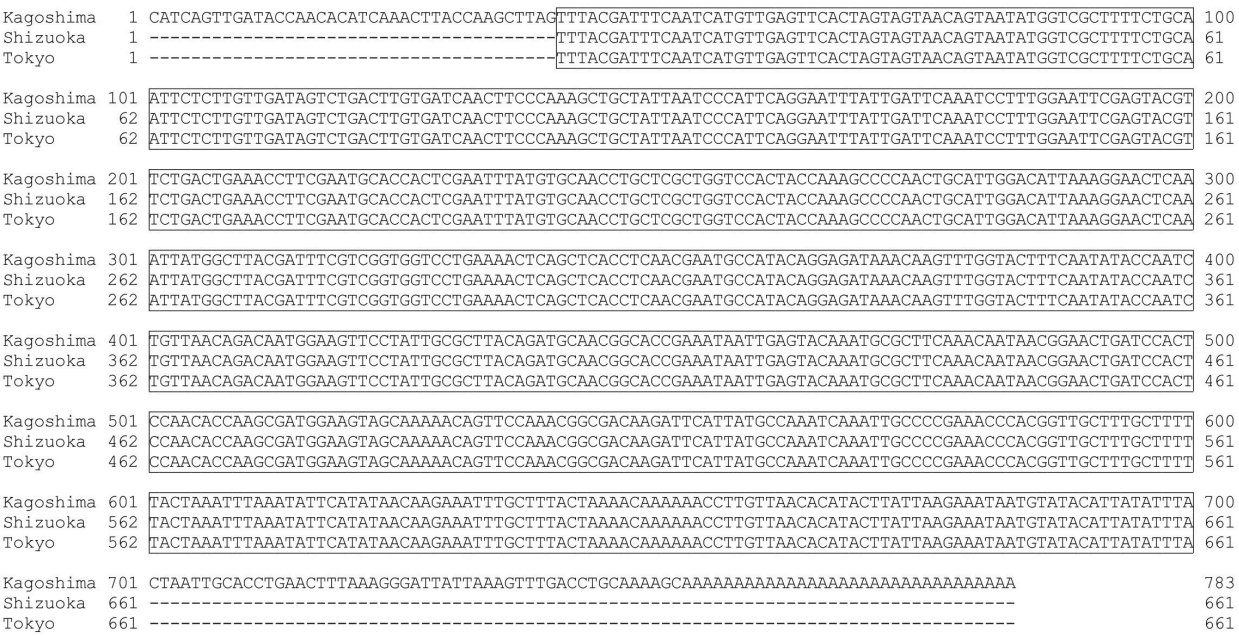

Fig. 2 ClustalW alignment of ChuaHNL cDNA sequence among samples collected at Kagoshima, Shizuoka, and Hachijo, Tokyo. Each cDNA fragment was PCR-amplified using ChuaHNL-4 gene-specific primer and ChuaHNL-5 gene-specific primer. The DNA sequence was identical among three samples. Collection sites were presented in the left column.

GTCTGGAGCAATTCTCACAGATCTTCCTCC-3'; VhilLUC-9, 5'-GTTTATTGGCATTCAGGTGGTACTTCTAGAG-3'; VhilLUC-10, 5'-GTTTATTTTCACACAGTCCATCTGATAG-3'; VhilLUC-11, 5'-GAAGACATGCTGTAGAATGTGCCAGTATG-3'; P-VhilLUC-12, P-5'-CTGCTTCTACTCTGC-3' (P indicates 5'-phosphorylation.); VhilLUC-13, 5'-CCCGGTGATCCGTACAAACCGACATCACC-3'; VhilLUC-14, 5'-GTAGTAGTCTTAGCTTCTGTATCTTTGTTCG-3'.

2.5 Determination of DNA sequence

PCR amplicon was separated using agarose gel electrophoresis, purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and ligated into EcoRV recognition site of pBluescript II SK (+) (Agilent Technologies, Santa Clara, CA, USA) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The ligated DNA was transformed into Competent high DH5α (Toyobo, Osaka, Japan). Plasmid DNA was purified using a GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). DNA sequence was determined using a 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). DNA sequence was analyzed using ATGC and GENETYX Ver. 12.0.5 (Genetyx, Tokyo, Japan). Genomic DNA and cDNA sequences were determined using 4 independent clones to avoid PCR-derived sequence errors, respectively. Homology search and ClustalW alignment were performed using GENETYX Ver. 12.0.5.

3. Results and Discussion

Blast search shows that there is no protein homologous to ChuaHNL in 2016, indicating that gene-specific primers for PCR-based cloning of ChuaHNL cDNA (Dadashipour et al, 2015) presumably amplify its related DNA fragments. A combination of ChuaHNL-4 gene-specific primer and ChuaHNL-5 gene-specific primer, PCR-amplified a 952 nt-long DNA fragment (accession no. LC203137) from genomic DNA prepared from the millipede. By comparing the DNA sequence between the amplified genomic DNA and ChuaHNL cDNA (accession no. LC004755) (Dadashipour et al, 2015), we identified 5 exons in the gene sequence: 16-123, 186-316, 370-440, 499-594, and 653-798 (Fig. 1).

On the basis of results of the RACE, DNA sequences of ChuaHNL cDNA collected at Kagoshima, Shizuoka, and Hachijojima, Tokyo were 100% identical to each other (Fig. 2). These results of the genetic analysis suggest that the millipedes in Shizuoka and Hachijojima, Tokyo are identified as *C. hualienensis* at the nucleotide level.

It is well known that there can be polymorphism at the nucleotide level in various arthropods collected in the field. For example, the wild silkmoth, *Antheraea polyphemus,* has two types of cDNA sequences encoding a pheromone-degrading enzyme (accession nos. AY866480 and AY866481) (Ishida & Leal, 2005). *M. pacifica* and *V. hilgendorfii,* have 6 and 12 cDNA sequences each encoding the respective luciferase (accession nos. LC203027-LC203033 and LC164230-LC164243; Ishida and Asano, unpublished data). In our study, we were not able to observe any variances of the three ChuaHNL cDNA sequences, suggesting that *C. hualienensis'* wide expansion in southern Japan over a short period of time was likely caused by soil contamination as a result of transportation by human activities during 1999-2003.

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鹿児島、静岡、八丈島（東京）で採集されたヤンバルトサカヤスデ *Chamberlinius hualienensis*由来ヒドロキシニトリルリアーゼのDNA塩基配列の比較

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要旨

ヤンバルトサカヤスデ*Chamberlinius hualienensis*は、短期間に沖縄から鹿児島、静岡、八丈島（東京）とその生息域を拡大してきた。そのため、３地域に生息するこのヤスデは同一起源の個体群由来と考えられている。ヤンバルトサカヤスデ由来ヒドロキシニトリルリアーゼ遺伝子（*ChuaHNL*）は、５つのエクソン（16-123、186-316、370-440、499-594、653-798）で構成されていた。それぞれの地域に生息するヤンバルトサカヤスデから調製したChuaHNL cDNAの塩基配列は同一であった。以上から、ヤンバルトサカヤスデの南日本での広い分布は、土壌運送に起因する卵の混入が原因と推察される。

Key Words

Chamberlinius hualienensis、ヒドロキシニトリルリアーゼ、ゲノム配列、cDNA