Journal of Asia-Pacific Entomology 17 (2014) 617–627

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Developing a new molecular marker for aphid species identification: Evaluation of eleven candidate genes with species-level sampling

Wonhoon Lee [a,1], Yerim Lee [b,1], Hyojoong Kim [c], Shin-Ichi Akimoto [a,*], Seunghwan Lee [b,*]

[a] Laboratory of Systematic Entomology, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan
[b] Insect Biosystematics Laboratory, Research Institute for Agricultural and Life Sciences, Seoul National University, Seoul 151-921, Korea
[c] Animal Phylogeny and Systematics Lab., Department of Biology, Kunsan National University, Gunsan 573-701, Korea

ARTICLE INFO

Article history:
Received 28 February 2014
Revised 12 June 2014
Accepted 14 June 2014
Available online 21 June 2014

Keywords:
Aphididae
Species identification
ATP6
COI

ABSTRACT

The mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene has been utilized as a molecular marker for aphid species identification. However, this gene has sometimes resulted in misidentification because of low interspecific genetic divergences between some species pairs. In this study, to propose new molecular markers for the family Aphididae, we first screened 2289 sequences of 11 genes (*COI*, *COII*, *CytB*, *ATP6*, *lrRNA*, *srRNA*, *ITS1*, *ITS2*, *EF1a*, *18S*, and *28S*) collected from the GenBank. Among the 11 genes, *ATP6* gene revealed the largest genetic divergence among congeneric species with the smallest divergence among conspecific individuals; in contrast, species pairs with low genetic divergences (<1%) were not observed. Secondly, for statistically testing the usefulness of *ATP6* gene in species identification, we analyzed genetic distances between all of the combinations of 32 individuals of 20 species for both *COI* and *ATP6* genes. The *ATP6* gene showed lower intraspecific (on average 0.08%) and higher interspecific (on average 8.28%) genetic distances than the *COI* gene (on average 0.19% and 6.24%, respectively) for the same pairs of individuals. This study corroborates the usefulness of the *ATP6* gene as a new molecular marker that could improve the misidentification problems that are inherent with the *COI* gene.

Introduction

Since 2003 (Hebert et al., 2003), DNA barcode has been reported as a useful method for identifying species in various hexapod orders (Ball et al., 2005; Löbl and Leschen, 2005; Hajibabaei et al., 2006; Scheffer et al., 2006; Smith et al., 2008; Lee et al., 2011). Especially, DNA barcode has been reported to show high efficiency in quarantine and pest monitoring for detecting invasive species (Scheffer et al., 2006). However, as the coverage of barcode studies expands to the levels of genus, family, or suborder, misidentification problems have also been reported repeatedly (Meyer and Paulay, 2005; Cognato, 2006; Whitworth et al., 2007; Song et al., 2008). For example, in the order Orthoptera, nuclear mitochondrial pseudogenes were coamplified by the universal primers, leading researchers to wrong results (Song et al., 2008). In the order Hemiptera, species groups undistinguished by *cytochrome c oxidase subunit I* (*COI*) sequences were detected (Lee et al., 2011). These suggest that the current protocol of DNA barcode studies should be improved.

Several DNA barcode studies have been performed within the family Aphididae. Foottite et al. (2008) analyzed 690 *COI* sequences from 690 samples (335 species, 134 genera and 16 subfamilies), and Lee et al. (2011) analyzed 249 *COI* sequences from 249 samples (154 species, 72 genera and 11 subfamilies) to evaluate the utilities of DNA barcode in aphid species identification. They proved the usefulness of DNA barcode by which 93% of the examined aphid species were successfully identified. However, some species were hardly identified due to no or very low genetic divergences based on the *COI* sequences in them. Especially, Lee et al. (2011) indicated that 24 species pairs from 33 species showed low genetic divergences (<1%) in *COI* sequences which sometimes could lead to species identification problems in Aphididae. Therefore, the improvements in the barcode method are required for more precise identification.

One way to overcome this problem is to develop a new molecular marker that can provide enough sequence diversity between different species. In this study, to isolate new molecular markers in Aphididae, 1270 sequences of ten genes were screened together with 1019 sequences of the *COI* gene. From comparing genetic divergences among the 11 genes and detecting species pairs with low interspecific divergences (<1%), one mitochondrial gene, *subunit 6 of mitochondrial F-ATPase* (*ATP6*), was selected as a candidate for a new molecular marker. The usefulness of the *ATP6* gene was statistically tested in comparison to the *COI* gene by calculating divergences between the sequences of both genes from 32 individuals of 20 aphid species for both genes.

* Corresponding authors.
E-mail addresses: akimoto@res.agr.hokudai.ac.jp (S.-I. Akimoto), seung@snu.ac.kr (S. Lee).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.aspen.2014.06.008
1226-8615/

Materials and methods

Comparison of genetic divergences among 11 genes

Using three keywords, "Aphididae", "mitochondrial", and "nuclear", we downloaded a total of 2289 sequences of six mitochondrial genes (*ATP6*, *COI*, *cytochrome c oxidase subunit II* (*COII*), *cytochrome b* (*CytB*), *large subunit ribosomal RNA* (*lrRNA*), and *small subunit ribosomal RNA* (*srRNA*)) and five nuclear genes (*elongation factor 1 alpha* (*EF1a*), *internal transcribed spacer 1* (*ITS1*), *internal transcribed spacer 2* (*ITS2*), *18S ribosomal RNA* (*18S*), and *28S ribosomal RNA* (*28S*)) from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The numbers of species, genera, and subfamilies included in the 2289 sequences were summed up for each of the 11 genes, using the Insect Mitochondrial Genome Database (IMGD; http://www.imgd.org/) (Lee et al., 2009).

The alignments of the 2289 sequences were conducted for the 11 genes by using the default parameters of CLUSTALW 1.83 (Thompson et al., 1997) in the IMGD. Genetic divergences for the 11 genes were calculated using the "distance matrix analysis function" implemented in the IMGD at two taxonomic levels, among conspecific individuals and among congeneric species. Because the number of sequences and species included in each gene varied, all the pairwise distances were compared among the 11 genes using the Kruskal–Wallis test ($P < 0.05$) and where significant differences were observed, Mann–Whitney U-tests were used to identify significantly different pairs ($P < 0.05$).

Testing the usefulness of ATP6 gene in species identification

Of the 11 genes, the superiority of *ATP6* as a new molecular marker was suggested based on the sequences collected from the databases (see Results). To test this hypothesis using pairwise statistical test, we compared the extent of the genetic divergences of *ATP6* and *COI* in the same pairs of species. A total of 32 *COI* sequences and 32 *ATP6* sequences were generated from 32 individuals of 20 species belonging to the family Aphididae. All the samples were collected in two countries, Korea and Japan, from 2003 to 2012 and stored in 80% ethanol (Table 1).

The genomic DNA extraction was performed using the DNeasy® Blood and Tissue kit (Qiagen, Inc., Dusseldorf, Germany), according to the manufacturer's protocol. Samples for extraction consisted of single or several individuals from the same colony. We prepared 32 *COI* and 32 *ATP6* sequences from the same samples. The target *COI* fragment of 658 bp and *ATP6* fragment of 655 bp were amplified by performing polymerase chain reaction (PCR), using two primer datasets, LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′, PCR/seq) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′, PCR/seq) (Folmer et al., 1994), and CO2Af (5′-AATCAYAGWTTTATRCCWATTCA-3′, PCR/seq) tRNALysAf2 (5′-GACTGAAAAGCAAAGTAATGATCTCT-3′, seq), and CO3WWRD (5′-TCWCGAATWACATCWCGTCATCA-3′, PCR/seq) (Martinez-Torres et al., 2001). PCRs were performed in 5 μl reaction volumes using TaKaRa *Ex Taq* (TaKaRa Bio). PCR amplification with 35 cycles each consisting of 30 s at 94 °C, 30 s at 42 — 46 °C and 1 min at 65 °C was performed after an initial denaturing step of 3 min at 94 °C. The PCR products were tested by electrophoresis on an agar gel and, if a single band was observed, were purified using the QIAquick PCR purification kit (QIAGEN Inc). The PCR products were sequenced in both directions by using an ABI 3730xl sequencer (Applied Biosystems). Resulting chromatograms were evaluated for miscalls and ambiguities and were assembled into contigs in SeqManTMPro (version 7.1.0, 2006; DNAstarInc., Madison, WI, USA). The sequences were individually checked by eye and verified for protein coding frame-shifts to avoid pseudogenes (Zhang and Hewitt, 1996). Consensus files were aligned using Clustal X

Table 1
The list of Aphididae specimens analyzed in this study.

Species	Collection date	Collection region	Host plant	Specimen voucher number	Accession number	
					ATP6	*COI*
Aphis fukii	27.v.2004	Jeju, Korea	*Petasites japonicus*	040527HJ10-1	KJ502208	KJ502179
Aphis fukii	31.vii.2011	Jozanke, Hokkaido, Japan	*Petasites japonicus*	110731WH09	KJ502209	KJ502180
Aphis glycines	28.iv.2011	Osan, Gyeonggido, Korea	*Rhamnus davurica*	110428YR3-1	KJ502210	KJ502181
Aphis gossypii	22.iv.2011	Wando, Jeollanamdo, Korea	*Hibiscus syriacus*	110422HJ1-1	KJ502211	KJ502182
Aphis gossypii	19.v.2011	Seodaemun, Seoul, Korea	*Celastrus orbiculatus*	110519HJ5-1	KJ502212	KJ502183
Aphis gossypii	5.vi.2011	Gimje, Jeollanamdo, Korea	*Cucumis sativus*	110605YR23-1	KJ502213	KJ502184
Aphis gossypii	29.vi.2011	Jumunjin, Gangwondo, Korea	*Capsicum annum*	110629YR53-1	KJ502214	KJ502185
Aphis gosypii	21.ix.2011	Utoro, Hokkaido, Japan	*Capsicum annum*	110921WH17	KJ502215	KJ502186
Aphis hederae	7.iv.2006	Namhae, Gyeongsangnamdo, Korea	*Hedera japonica*	060407SH24	KJ502216	KJ502187
Aphis horii	8.vi.2004	Urleung, Gyeongsangnamdo, Korea	*Sambucus williamsii*	040608HJ24	KJ502217	KJ502188
Aphis newtoni	3.vi.2005	Anyang, Gyeonggido, Korea	*Iris louisiana*	050603SH10	KJ502218	KJ502189
Aphis oenotherae	25.vi.2003	Hongcheon, Gangwondo, Korea	*Oenothera biennis*	030625SH67	KJ502219	KJ502190
Aphis oenotherae	21.ix.2011	Utoro, Hokkaido, Japan	*Oenothera biennis*	110921WH20	KJ502220	KJ502191
Aphis oenotherae	18.vii.2011	Daisetsu, Hokkaido, Japan	*Oenothera biennis*	110718WH04	KJ502221	KJ502192
Aphis sanguisorbicola	17.ix.2004	Pyeongchang, Gangwondo, Korea	*Sanguisorbs officinalis*	040917HJ06	KJ502222	KJ502193
Aphis spiraecola	28.vii.2011	Sapporo, Hokkaido, Japan	*Spirae prunifolia*	110728WH04	KJ502223	KJ502194
Aphis spiraecola	18.vii.2011	Daisetsu, Hokkaido, Japan	*Spirae prunifolia*	110718WH39	KJ502224	KJ502195
Aphis sp.1	3.v.2012	Gongju, Chungcheongnamdo, Korea	*Rhamnus davurica*	120503YR1-1	KJ502225	KJ502196
Aphis sp.1	3.v.2012	Gongju, Chungcheongnamdo, Korea	*Rhamnus davurica*	120503YR1-2	KJ502226	KJ502197
Acyrthosiphon pisum	18.vii.2011	Daisetsu, Hokkaido, Japan	*Lathyrus japonicus*	110718WH72	KJ502227	KJ502198
Aulacorthum solani	9.x.2003	Boryeong, Chungcheongnamdo, Korea	*Ranunculus* sp.	031009SH15	KJ502228	KJ502199
Aulacorthum solani	17.viii.2011	Jozanke, Hokkaido, Japan	*Prunus* sp.	110817WH14	KJ502229	KJ502200
Aulacorthum vandenboschi	31.vii.2011	Jozanke, Hokkaido, Japan	*Cirsum japonicusa*	110731WH05	KJ502230	KJ502201
Aulacorthum vandenboschi	31.vii.2011	Jozanke, Hokkaido, Japan	*Cirsum japonicusa*	110731WH12	KJ502231	KJ502202
Macrosiphoniella yomogicola	28.vii.2011	Sapporo, Hokkaido, Japan	*Artemisia montana*	110728WH26	KJ502232	KJ502203
Macrosiphoniella yomogicola	21.ix.2011	Utoro, Hokkaido, Japan	*Artemisia montana*	110921WH32	KJ502233	KJ502204
Macrosiphum rosae	21.ix.2011	Utoro, Hokkaido, Japan	*Rosa rugosa*	110921WH12	KJ502234	KJ502205
Myzus sp.1	21.ix.2011	Utoro, Hokkaido, Japan	*Prllnlls mllme*	110921WH22-1	KJ502235	KJ502206
Myzus sp.1	21.ix.2011	Utoro, Hokkaido, Japan	*Prllnlls mllme*	110921WH13	KJ502236	KJ502207
Tetraneura radicicola	19.vi.2012	Iwamizawa, Hokkaido, Japan	*Ulmus davidiana*	AK12150	KC197285	KC197252
Tetraneura nigriabdominalis	15.vi.2012	Sapporo, Hokkaido, Japan	*Ulmus davidiana*	LW12001	KC197275	KC197242
Tetraneura nigriabdominalis	26.v.2012	Yeongwol, Gangwondo, Korea	*Ulmus davidiana*	AK12157	KC197280	KC197247