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Molecular identification of AFLP fragments associated with elytra color variation of the Asian ladybird beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae)

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ABSTRACT

The Asian ladybird beetle, *Harmonia axyridis* shows polymorphism in elytra color patterns. However, it is uncertain whether these color patterns are regulated by genetic factors. This investigation used amplified fragment length polymorphism (AFLP) analysis to determine any genetic causes of the variability of color patterns. Using four individuals of each group, AFLP analysis produced 37 polymorphic bands. Among several polymorphic bands, six AFLP markers were associated with elytra color patterns after further analysis using six additional individuals of each group. These polymorphic sites were sequenced but did not match DNA sequence data deposited in GenBank. Based on the color-associated AFLP markers, SCAR primers were designed for PCR amplification of genomic DNA. These primers (SCAR 12 and SCAR 44) were used to analyze color-associated loci and/or alleles of *H. axyridis* DNA. SCAR 12 primers designed from a Spectabilis type-specific fragment (AFLP 12) amplified a specific band of 530 bp in four Spectabilis individuals, but not in the insects with other color patterns.

Introduction

The multicolored Asian ladybird beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae), is a generalist predator of aphids and other soft-bodied insects. It is native to temperate and subtropical parts of Asia. Since the beginning of the 20th century, this species has been introduced several times into different parts of the world as a biological control agent. International releases for biological control purposes (Gordon, 1985) coupled with potential unintentional introductions via international trade (Day et al., 1994; Roy et al., 2005) have contributed to the expansion of its range to include North and South America, Europe, and Africa. Based on the CLIMEX prediction (Poutsma et al., 2008), a large part of Mediterranean Europe, South America, Africa, Australia, and New Zealand seem highly suitable for the long-term survival of *H. axyridis*. There are at least three adverse impacts if they become established: impacts on nontarget arthropods, impacts on fruit production, and impacts as household invaders (Koch and Galvan, 2008). However, *H. axyridis* is still the most popular biological pest control agent used worldwide.

Interestingly, *H. axyridis* shows a high level of phenotypic polymorphism in elytra color pattern. The phenotypes are classified into two groups: the Succinea type, which is characterized by a light reddish or brownish ground color, and the melanic type, which is characterized by melanic elytral patterns (Komai, 1956). Komai (1956) also reported that *H. axyridis* had four color patterns, Succinea, Conspicua, Spectabilis and Axyridis. Seo et al. (2007, 2008) indicated that the Succinea group comprised two subgroups according to the number of spots on the elytra. Many factors regarding these color polymorphisms have been investigated. Some researchers found that the frequency of each color-morph varies according to the geographical location of the populations. Macro-geographical variations in Asian populations of *H. axyridis* are related mainly to climatic factors because light and melanic phenotypes have different physiological responses (Komai, 1956). Also, one factor that seems of major importance for the selection of different color patterns is the efficient absorption of solar energy by the melanic type. Stewart and Dixon (1998) reported that temperature excess led to black elytra surfaces. Thus, difference in solar energy absorption between melanic and nonmelanic ladybirds results in different body sizes in males (Stewart and Dixon, 1998), but not in females (Seo et al., 2008).

Other researchers found that color-morph variation results from genetic polymorphisms. Tan and Li (1934) showed that color variations are controlled by alleles on a single locus and that the nonmelanic Succinea type of elytron is due to a simple genetic recessive to any form with melanic patterns (Tan and Li, 1934). In addition, in crossbreeding experiments with both nonmelanic forms, all progeny were nonmelanic. In crossbreeding experiments with both melanic forms, Conspicua males and Spectabilis females, none of the progeny showed the parent's color patterns (Seo et al., 2007). This might support the idea of Tan and Li (1934) that the nonmelanic pattern is genetically recessive to any melanic pattern. Recently, a

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study to elucidate the developmental mechanisms of color pattern formation was performed using germline transformation and RNA interference (RNAi) (Kuwayama et al., 2006). However, less attention has been paid to the effects of genetic variation within *H. axyridis* elytra color patterns at the molecular level and information on the genes associated with these traits is scarce.

All organisms are subject to mutations as a result of normal cellular activity and/or interactions with the environment, leading to genetic variation (polymorphism) within species. At the DNA level, types of genetic variation include base substitutions(single nucleotide polymorphisms (SNPs)), insertions or deletions of nucleotide sequences within a locus, inversion of a segment of DNA within a locus and rearrangement of DNA segments around a locus of interest (Liu and Cordes, 2004). Many different instances of each type of mutation should accumulate in any given species, and the number and degree of the various types of mutations define the degree of genetic variation within a species. One can assume that each *H. axyridis* color pattern is controlled mainly by different loci, which have experienced each type of mutation. DNA marker technology can be applied to reveal these mutations. Different genomic DNA sequences from each type of mutation within each species cause shifts in the sizes of DNA fragments produced by digestion enzymes or with random amplification primers. Recently, DNA fingerprinting markers such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLPs) have been used for genetic diversity analysis, studying local marker saturation and gene mapping (Welsh and McClelland, 1990; Williams et al., 1990; Dodgson et al., 1997).

This study used AFLP analysis, which is one of the latest and most promising of the molecular fingerprinting markers (Vos et al., 1995) and combines the advantages of both RFLP and RAPD. Using AFLP analysis, many segregating markers were generated between the different color patterns of *H. axyridis*. These markers indicated the genetic diversity of *H. axyridis* and the causes of variations in the color patterns. Two AFLP markers linked to elytra color were also converted into a sequence-characterized amplified region (SCAR) marker. For this, two AFLP markers were reamplified and sequenced. These SCAR markers converted from AFLP markers increased the probability that loci linked to gene regions involved in color pattern evolution could be identified. This technique is likely to be of use in future understanding of the elytra color genetics of *H. axyridis*.

Materials and methods

Field Collection of H. Axyridis Populations

Overwintering *H. axyridis* adults were collected after they arrived at their aggregation sites (e.g., Majeon-ri, Gung-dong and Songgang-dong) in the Daejeon area in early November to late December in 2006 and 2007. After the specimens were collected, they were brought to the laboratory. All populations were placed in 15-cm diameter plastic Petri dishes with dried leaves. These were placed in wooden boxes and stored in an incubator at 10 °C until spring the following year. In March, all populations were removed from the wooden boxes and reared in a 15 °C incubator with ground chicken liver for food. Some spring populations of *H. axyridis* were collected in Gung-dong in June 2008. All spring populations were reared in a 15 °C incubator with ground chicken liver for food.

Insect Material

To identify unique AFLP bands specific to each color pattern, genomic DNA was extracted from four females of each of the four *H. axyridis* color types (Conspicua, Spectabilis, and Succinea 1 and 2). Each sample was selected from overwintering populations collected in Songgang-dong in 2006.

Six individuals of different sexes from each pattern were analyzed additionally to identify AFLP markers. Each sample was randomly selected from overwintering populations collected from three aggregation sites in 2006 and 2007. For SCAR amplification, four individuals of different sexes were analyzed. Each of these samples was selected randomly from overwintering populations collected from three aggregation sites in 2006 and 2007.

DNA Extraction

A key prerequisite for AFLP assay is the completeness of the digestion of the DNA by restriction endonucleases. An incomplete digestion is considered a major technical problem because even a small fraction of partially digested DNA fragments can result in detectable bands after amplification (Reineke et al., 1998). Partial digestion can result from a contamination of the DNA with inhibitors (e.g., negatively charged polysaccharides and phenols). This investigation performed DNA extraction using a modification of the CTAB method (Moeller et al., 1992), which provides DNA suitable for AFLP assays (Reineke et al., 1998).

Each insect body was frozen in liquid nitrogen and pulverized prior to the addition of 400 μl of extraction buffer (200 mM Tris–HCl pH 8.0, 200 mM NaCl, 30 mM EDTA, 0.5% SDS), 5 μl a proteinase K (30 mg/ml) and incubated for 60 min at 58 °C. Then 400 μl of 2 CTAB solution (100 mM Tris–HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) was added and incubated for 10 min at 65 °C. The homogenate was then extracted with 700 μl of phenol/chloroform/isoamylalcohol (25:24:1) and centrifuged at 16,000 × *g* for 15 min at 20 °C. The top aqueous phase was recovered and recentrifuged for 5 min with an additional 700 μl of phenol/chloroform/isoamylalcohol (25:24:1). The top aqueous phase was then precipitated with 0.7 volumes of isopropanol and washed with 70% ethanol, dried, and resuspended in 50 μl of distilled water (DW). Ribonuclease A (10 mg/ml) was added to the resuspended DNA for 60 min at 37 °C.

AFLP Analysis

AFLP analysis was performed using a method modified from that used by Vos et al. (1995). Adapter and primer sequences are given in Table 1. Aliquots of 200 ng of genomic DNA were digested with 7.5 U of EcoRI (New England Biolabs, Ipswich, MA, USA) in 0.5 μl of 10 NEBuffer EcoRI in a final volume of 25 μl for 12 h at 37 °C. The restricted DNA was washed and resuspended in 10 μl DW. DNA was again restricted with 10 U MseI (New England Biolabs) in 0.5 μl of 10 × NEB buffer # 2 in a final volume of 20 μl for 12 h at 37 °C. The restricted DNA was washed and resuspended in 10 μl DW. A solution containing 5 μl EcoRI adapter, 50 pmol MseI adapter, 175U T4 DNA ligase (Takara Bio Inc., Otsu, Japan), and 0.5 μl of 10 × T4 ligase buffer was added to resuspended DNA in a total volume of 20 μl and incubated for 12 h at 16 °C.

Preselective polymerase chain reaction (PCR) amplifications were then performed on 4 μl of ligated DNA in 50 μl volumes containing 5 μl of 10 Ex Taq buffer, 250 μM dNTP mixture, 25 pmol Eco + 0 primer, 25 pmol Mse + 0 primer, 5 μl of 0.01% bovine serum albumin (BSA) and 2.5 U Ex Taq (Takara). PCR conditions were 20 × (94 °C 30 s, 56 °C 1 min, 72 °C 1 min). After amplification, samples were diluted 1:5 with DW. Selective PCRs were performed in 25 μl containing 12.5 pmol Mse + 3 primer (see Table 1), 12.5 pmol labeled Eco + 3 primer, 125 μM dNTP, 1 μl preamplification product, 1.25U Ex Taq (Takara) and 2.5 μl of 10 Ex Taq buffer. Cycling conditions in the first cycle were 94 °C 30 s, 64 °C 30 s, 72 °C 1 min with the annealing temperature reduced by 0.7 °C over the next 12 cycles, then 23 (94 °C 30 s, 56 °C 30 s, 72 °C 1 min). The final PCR products were run on 6% denaturing polyacrylamide gels in 1 TBE buffer (pH 8.0) for 4 h at 2100 V. Analysis was carried out using silver staining kits (Bioneer, Daejeon, Korea), and gels were dried overnight before being photographed.