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Application of Restriction Fragment Length Polymorphism of the Region Encoded yba3 and yba4 of Buchnera aphidicola for Molecular Identification of Aphids【Research report】

以限制片段長度多型性選殖蚜蟲內共生菌Buchnera aphidicola yba3-yba4 DNA片段於蚜蟲分子鑑定之應用【研究報告】

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Abstract

A new molecular marker was developed for the identification of aphids. The regions encoding two species-specific orphan genes yba3 and yba4, from five Buchnera aphidicola strains associated with Aphis craccivora (BAc), Aphis rumicis (BAr), Aphis gossypii (BAg), Myzus persicae (BMp), and Uroleucon formosanum (BUf) were cloned and sequenced. The restriction fragment length polymorphism (RFLP) assay on the yba3-4 regions using the TaqI restriction enzyme produced distinguishable fragments in BAg, BAc, BMp, BUf, and a common B. aphidicola strain associated with Acyrthosiphon pisum (BAp). In view of the obligate symbiosis between B. aphidicola and its host, the RFLP pattern of the yba3-4 region may be a new and useful marker for identifying aphids belonging to different genera.

摘要

本研究目的在於發展出一種新的分子標記用來鑑定蚜蟲的種類。本試驗分別自五種蚜蟲：黑豆蚜Aphis craccivora、酸模蚜Aphis rumicis、棉蚜Aphis gossypii、桃蚜Myzus persicae及白尾紅蚜Uroleucon formosanum體內的共生菌Buchnera aphidicola中，選殖出一段含有B. aphidicola兩個特有的基因yba3及yba4的DNA片段。接著利用限制片段長度多型性 (RFLP) 的方法，對yba3-yba4 DNA片段進行限制內切酶剪切分析。試驗發現來自五種蚜蟲內共生菌的yba3-yba4 DNA片段，經由Taq I酵素剪切後，會產生不同大小的剪切片段。由於蚜蟲內共生菌與寄主蚜蟲有著絕對共生關係，因此RFLP分析yba3-yba4 DNA片段的結果，可作為一種分子標記，用以鑑定不同屬的蚜蟲。

Key words: Aphids molecular identification, Buchnera aphidicola, restriction fragment length polymorphism

關鍵詞: 蚜蟲分子鑑定、蚜蟲內共生菌、RFLP。

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Application of Restriction Fragment Length Polymorphism of the Region Encoded *yba3* and *yba4* of *Buchnera aphidicola* for Molecular Identification of Aphids

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ABSTRACT

A new molecular marker was developed for the identification of aphids. The regions encoding two species-specific orphan genes *yba3* and *yba4*, from five *Buchnera aphidicola* strains associated with *Aphis craccivora* (*BAc*), *Aphis rumicis* (*BAr*), *Aphis gossypii* (*BAg*), *Myzus persicae* (*BMp*), and *Uroleucon formosanum* (*BUf*) were cloned and sequenced. The restriction fragment length polymorphism (RFLP) assay on the *yba3-4* regions using the *Taq*I restriction enzyme produced distinguishable fragments in *BAg, BAc*, *BMp*, *BUf*, and a common *B. aphidicola* strain associated with *Acyrthosiphon pisum* (*BAp*). In view of the obligate symbiosis between *B. aphidicola* and its host, the RFLP pattern of the *yba3-4* region may be a new and useful marker for identifying aphids belonging to different genera.

Key words: Aphids molecular identification, *Buchnera aphidicola,* restriction fragment length polymorphism

Introduction

Aphids are plant-sap sucking insects which can cause serious damage to crops through direct feeding action. Using a needle-like stylet, aphids are capable of penetrating plant tissues and suck up phloem sap as their food source. This feeding behavior, however, not only results in leaf curling and growth stunting, but also transmits dozens of viruses harmful to crop growth (Syller, 2000). Although phloem sap contains mainly sugars with little amino acids, vitamins or lipids, the aphid population can still increase very rapidly (Douglas, 1996). This is mostly due to the nutrition support from its primary symbiotic bacteria, *Buchnera aphidicola*,

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which resides in specialized cells (bacteriocytes) at the abdomen of aphids and is transmitted vertically to eggs or young embryos (Baumann *et al.*, 1995). Studies have demonstrated that *B. aphidicola* can synthesize essential amino acids and riboflavin for its hosts (Douglas, 1998; Nakabachi and Ishikawa, 1999). In return, aphids offer *B. aphidicola* a safe and nutritionally rich environment. The endosymbiotic association between aphid and *B. aphidicola* presumably started 200-250 million years ago. This longtime association resulted in the co-evolution of both aphid and *B. aphidicola* (Moran *et al.*, 1993). Nowadays, this symbiosis has become obligate in nature, i.e., *B. aphidicola* cannot survive outside bacteriocytes, and at the same time, eliminating *B. aphidicola* by treatment with antibiotics significantly affects the reproduction and lifespan of aphids (Douglas and Prosser, 1992).

The genome sequence annotation of *B. aphidicola* strains has revealed three orphan genes, *yba2*, *yba3,* and *yba4*, with uncharacterized functions. The *yba2* gene was found in all three completely sequenced *B. aphidicola* strains associated with *Acyrthosiphon pisum* (*BAp*), *Schizaphis graminum* (*BSg*), and *Baizongia pistaciae* (*BBp*) (Shigenobu *et al.*, 2000; Tamas *et al.*, 2002; van Ham *et al.*, 2003). Nevertheless, it also shares a weak conserved domain with several functionally unknown proteins in *Xylella fastidiosa* and *Pseudomonas aeruginosa* (Shimomura *et al.*, 2002). However, *yba3* and *yba4* have only been identified in *B. aphidicola* strains and have not been reported in other microorganisms. The *yba3* gene was found in *BAp*, *BBp*, and a newly sequenced *B. aphidicola* strain associated with *Cinara cedri* (*BCc*) (Pérez-Brocal *et al.*, 2005). It is worth noting that *yba3* in *BSg* was annotated as a pseudogene which contains an authentic frameshift (Tamas *et al.*, 2002). The *yba4* gene, however, was only identified in *BAp* and *BSg*, and not in *BBp* or *BCc*.

In this study, we attempted to clone and sequence the *yba3* and *yba4* genes in *B. aphidicola* strains associated with *Aphis craccivora* (*BAc*), *Aphis rumicis* (*BAr*), *Aphis gossypii* (*BAg*), *Myzus persicae* (*BMp*), and *Uroleucon formosanum* (*BUf*), and apply this information in aphid identification.

Materials and Methods

Insect collection

Five species of Aphidinae, i.e., *Aphis craccivora*, *Aphis gossypii*, *Aphis rumicis*, *Myzus persicae*, and *Uroleucon formosanum,* used in this study were collected from PeiKou farm, Taichung, Taiwan between September and December in 2004 (Table 1).

Cloning and Sequencing

Total aphid DNA was prepared using the Gene-Spin™-V2 Genomic DNA Isolation Kit (Bio-Protech, Taiwan) according to the manufacturer's instructions. The purified DNA at a concentration of 20 ng/mL was stored at -20˚C. To clone full-length *yba3* and *yba4* genes, total aphid DNA was used as a template, and PCR was performed using BD Advantage™ 2 PCR Enzyme System (BD Biosciences). Since the *yba3-4* region is located between *kdtB* (phosphopantetheine adenylyltransferase) and *yhiQ* (hypothetical 26.9 kDa protein), the forward primer Y34F (5'-GCTATAAAT AAACAAATTTATCCTGATTTAG-3') corresponding to the 3' region of *kdtB* and the reverse primer Y34R (5'-GGATACATA GGATCTAAATAAATTACATC-3') corresponding to the 5' region of *yhiQ* were designed based on the genome sequence of *BAp* (Shigenobu *et al.*, 2000). The PCR condition was as follows: 95˚C for 1 min; 95˚C for 30 sec, 55˚C for 1 min, 68˚C for 3 min (35 cycles); and 70˚C for 10 min. Amplification products were visualized on 1% agarose gel by ethidium bromide staining. The pGEM-T Easy cloning kit (Promega) was used to clone PCR products

Table 1. Aphid species, symbiotic *Buchnera aphidicola* strains, host plants, and data on PCR products and genes in this study

Aphid species	*B. aphidicola* strain designation	Host plant	Length of PCR product [bp][a]	GC Content [mol %]	Encoded Genes	Reference
Acyrthosiphon pisum	*Buchnera* [*BAp*]	*Leguminosae: Pisum sativum*	2319	23.7	*yba3, yba4*	1,2[d]
Aphis craccivora	*Buchnera* [*BAc*]	Leguminosae: *Vigna sesquipedalis*	2491	21.5	*yba3, yba4*[c]	this study
Aphis gossypii	*Buchnera* [*BAg*]	Araceae: *Colocasia esculenta*	2491	21.7	*yba3*[b], *yba4*[c]	this study
Aphis rumicis	*Buchnera* [*BAr*]	Solanaceae: *Solannum nigrum*	2491	21.7	*yba3*[b], *yba4*[c]	this study
Myzus persicae	*Buchera* [*BMp*]	Cruciferae: *Brassica oleracea*	2376	20.6	*yba3*	this study
Uroleucon formosanum	*Buchnera* [*BUf*]	Campositae: *Lactuca indica*	2436	19.4	*yba3*	this study

[a] PCR was performed using primers annealing to *kdtB* and *yhiQ* genes [see Materials and Methods].
[b] Sequences of these two Yba3 proteins are identical.
[c] Sequences of these three Yba4 proteins are identical.
[d] Reference: 1, Shigenobu *et al*., 2000; 2, Shimomura *et al*., 2002.

according to the manufacturer's instructions. Clones successfully ligated with the inserts were sequenced by an automatic DNA sequencer. The GC contents of the PCR products were calculated using DNASTAR.

Gene annotation and multiple sequence alignment

Open reading frames were predicted using GeneMark, BlastX and BlastP search. Multiple sequence alignment and pair-wise comparison were carried out using the ClustalW program in DNASTAR. Protein sequences of Yba3 in *BAp* (accession number: BAB13273), *BBp* (accession number: AAO27231) and *BCc* (accession number: AAW72673), as well as Yba4 in *BAp* (accession number: BAB13274) and *BSg* (accession number: AAM68100) were obtained from the GenBank database.

Restriction Fragment Length Polymorphism (RFLP)

For the RFLP assay, approximately 0.4 μg of PCR amplification was digested with *Ssp*I or *Taq*I (Promega), separated by a 5% polyacrylamide gel, and visualized by ethidium bromide staining.

Results and Discussion

In our attempt to clone full-length *yba3* and *yba4* genes with primers designed to amplify the fragment between *kdtB* and *yhiQ*, the PCR products from all *B. aphidicola* strains were similar in size (Fig. 1), and comparable to the product of the same region for *BSg* (Tamas *et al.*, 2002). Sequences of 5' and 3' ends of these PCR products (data not shown) exhibited high homology to those of *kdtB* and *yhiQ*, indicating that all five *B. aphidicola* strains from aphids belonging to the Aphididae family in this study also contained these two genes.

PCR products containing *yba3* and *yba4* genes in *Buchenera* from the three aphids of *Aphis* genus, i.e., *BAc*, *BAr* and *BAg*, were the same in size (2491 bp), with

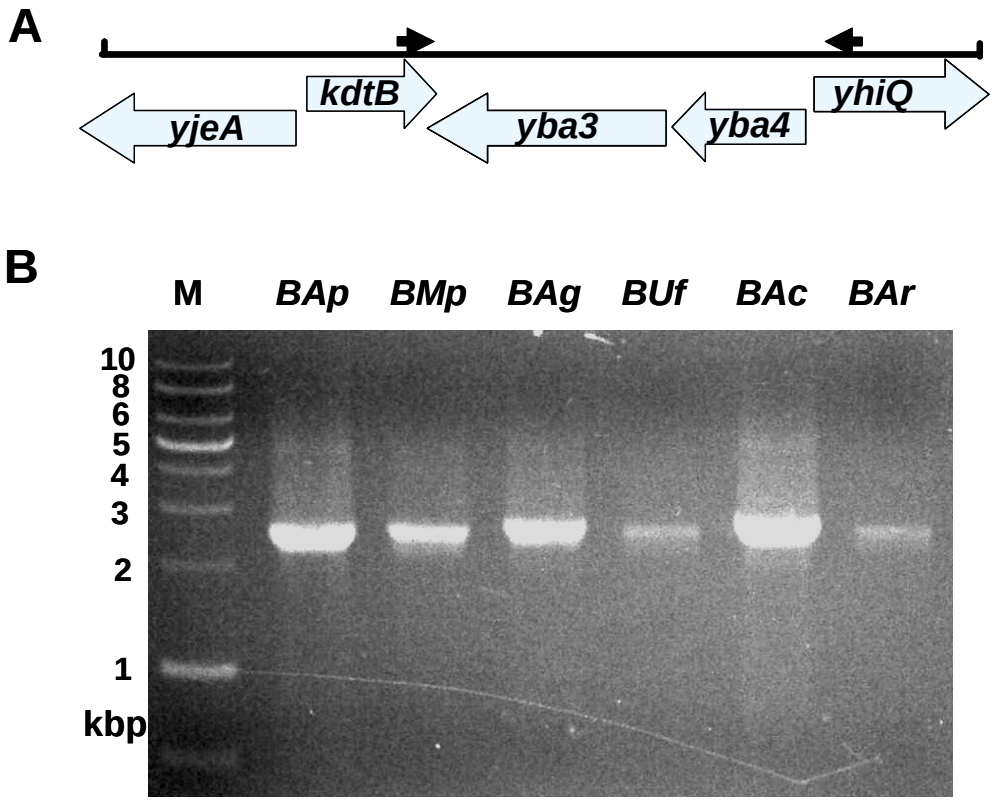

Fig. 1. Polymerase chain reaction (PCR) amplification of the region encoding *Buchnera aphidicola* orphan genes *yba3* and *yba4*. (A) A schematic representation of the gene order in *B. aphidicola* associated with *Ac. pisum*. The arrows indicate primer annealing sites. (B) The analysis of PCR products was conducted on a 1% agarose gel. The abbreviations of the names of *B. aphidicola* strains are shown in Table 1. M, molecular size standards.

nearly the same GC content (Table 1)**.** The lengths of the *yba3-4* regions of *BAp*, *BMp*, and *BUf* were slightly shorter (2319 bp, 2376 bp, and 2436 bp, respectively). Using GeneMake, BlastX and BlastP, it was found that the three aphids of *Aphis* genus contained both *yba3* and *yba4* genes (Table 1), as reported for *BAp* by Shigenobu *et al.* (2000) and Shimomura *et al.* (2002); and *BMp* and *BUf* contained only *yba3* (Table 1), as reported for *BBp* and *BCc* by van Ham *et al*. (2003) and Pérez-Brocal *et al.* (2005). Based on the present study and the studies cited above, *yba3* gene, found in practically all *B. aphidicola* strains, may play a critical role in the association of this endosymbiont with its host. On the other hand, *yba4* gene identified consistently in *BAc*, *BAr* and *BAg*, may have a specific function in aphids of the *Aphis* genus. Further work is needed to elucidate the functions of these two orphan genes in *B. aphidicola*.

The alignment of the protein sequences of Yba3 and Yba 4 in different *Buchnera* strains are shown in Fig. 2. No conserved regions were found in Yba3 proteins of *Buchnera* strains associated with aphids belonging to different families, i.e., Aphideae family (*BAr*, *BAg*, *BAc*, *BAp*), Pemphigidae (*BBp*) and Lachnidae (*BCc*). An alignment of Yba4 revealed only a few weakly conserved regions for *BAc*, *BAp* and *BSg* from aphids of different genera

Fig. 2. Alignment of (A) Yba3 or (B) Yba4 protein sequences was preformed using ClustalW program in DNASTAR. The abbreviations of the names of *Buchnera aphidicola* strains are shown in Table 1.

(Fig. 2B). The overall levels of identity of Yba3 and Yba4 among all strains of *B. aphidicola* differ extensively, varying from complete to nearly 10% identity (Tables 2 and 3).

The RFLP assay showed that the *Ssp*I-digesting patterns of the *yba3-4* regions of *BAc*, *BA*r and *BAg* were identical. However, one additional *Taq*I-digesting band (~250 bp) was observed in *BAc*, which, therefore, could be used to identify *BAc* from *BAr* and *BAg* (Fig. 3). The sequences of the *yba3-4* regions of *BAr* and *BAg* were almost the same (> 99% identity, data no shown). To-date, no commercial available restriction enzyme has been able to make distinguishable digesting patterns of the *yba3-4* regions of *BAr* and *BAg*. On the other hand, both the *Ssp*I and *Taq*I-digesting patterns of the *yba3-4* regions of *BAp*, *BMp*, and *BUf* were different (Fig. 3). Together, these results indicated that *Taq*I may be a suitable restriction enzyme for a RFLP assay.

In aphids, an identification system based

Table 2. Pair-wise comparison of Yba3 in *Buchnera aphidicola* strains between eight aphids

Identity (%)	*BAg*/*BAr*	*BAc*	*BAp*	*BMp*	*BUf*	*BBp*	*BCc*
BAg/*BAr*		99.7	36.8	38.6	34.4	12.0	10.2
BAc	99.7		37.1	38.9	34.4	12.3	10.2
BAp	36.8	37.1		67.3	47.7	15.0	16.8
BMp	38.6	38.8	67.3		53.1	14.2	14.2
BUf	34.4	34.4	47.7	53.1		15.4	21.1
BBp	12.0	12.3	15.0	14.2	15.4		14.2
BCc	10.2	10.2	16.8	14.2	21.1	14.2	

Table 3. Pair-wise comparison of Yba4 in three *Buchnera aphidicola* strains between five aphids

Identity (%)	*BAc*/*BAg*/*BAr*	*BAp*	*BSg*
BAc/*BAg*/*BAr*		18.8	39.3
BAp	18.8		18.8
BSg	39.3	18.8	

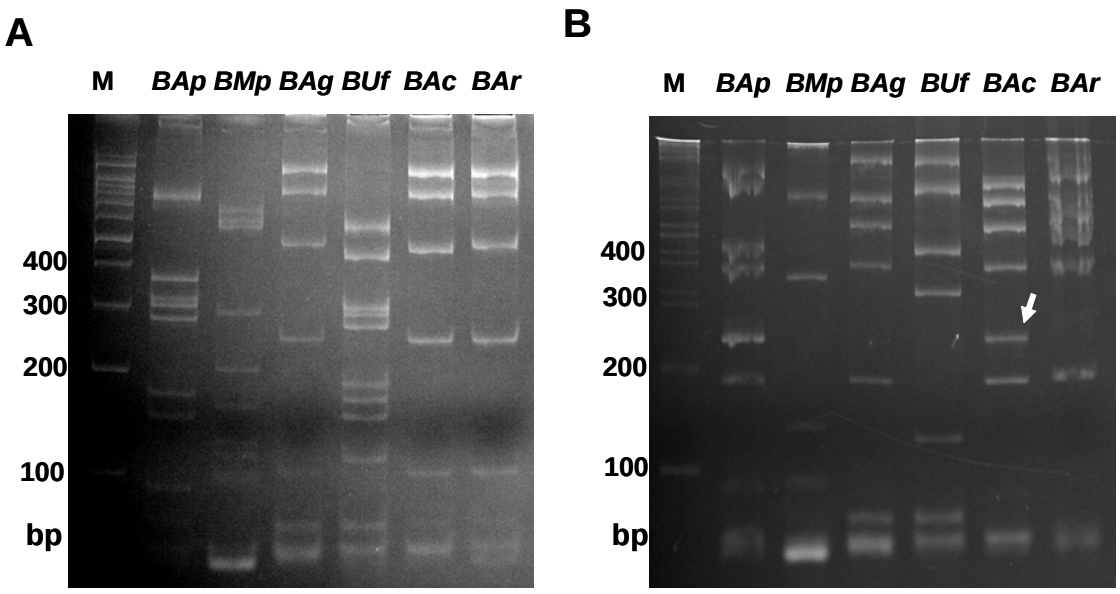

Fig. 3. Restriction fragment length polymorphism (RFLP) of the region encoding *yba3* and *yba4* genes. The PCR products were digested with restriction enzymes (A) *Ssp*I or (B) *Taq*I. The analysis of digested fragments was conducted on 5% polyacrylamide gels. The arrow indicates the digesting band which only presented in *BAc*, but not in *BAg* or *BAr*. The abbreviations of the names of *Buchnera aphidicola* strains are shown in Table 1. M, molecular size standards.

on adult morphology is difficult to apply to larvae or adults of a different morph because of their small size and extensive polymorphism. Therefore, molecular markers, such as microsatellite and ribosomal internal transcribed spacer (ITS) regions,

have become a useful tool to distinguish species with similar morphologies (Sunnucks *et al.*, 1997; Faten *et al.*, 2002). Since the symbiosis between *B. aphidicola* and its host is obligate, the RFLP pattern of *yba*3-4 region of *B. aphidicola* may be a new and useful marker for identification of aphids.

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以限制片段長度多型性選殖蚜蟲內共生菌*Buchnera aphidicola yba3-yba4* DNA片段於蚜蟲分子鑑定之應用

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摘　要

本研究目的在於發展出一種新的分子標記用來鑑定蚜蟲的種類。本試驗分別自五種蚜蟲：黑豆蚜 *Aphis craccivora*、酸模蚜 *Aphis rumicis*、棉蚜 *Aphis gossypii*、桃蚜 *Myzus persicae* 及白尾紅蚜 *Uroleucon formosanum* 體內的共生菌 *Buchnera aphidicola* 中，選殖出一段含有 *B. aphidicola* 兩個特有的基因 *yba3* 及 *yba4* 的 DNA 片段。接著利用限制片段長度多型性 (RFLP) 的方法，對 *yba3-yba4* DNA 片段進行限制內切酶剪切分析。試驗發現來自五種蚜蟲內共生菌的 *yba3-yba4* DNA 片段，經由 *Taq* I 酵素剪切後，會產生不同大小的剪切片段。由於蚜蟲內共生菌與寄主蚜蟲有著絕對共生關係，因此 RFLP 分析 *yba3-yba4* DNA 片段的結果，可作為一種分子標記，用以鑑定不同屬的蚜蟲。

關鍵詞：蚜蟲分子鑑定、蚜蟲內共生菌、RFLP。

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