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Use of molecular markers for entomological diversity assessment and their application in population study of aphids

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Molecular marker use is one of the most important tool in development in the field of population genetics from the last several years. These techniques are used to detect and exploit the DNA polymorphism among different individuals at species and/or population levels. Several molecular markers have been developed during the past few decades, and their various principles and procedures endow them different properties, according to which, some factors, such as time, cost, repeatability and difficulty, should be considered before selecting the most efficient molecular approach. We reviewed nine frequently-used molecular markers (barcoding, allozymes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellite DNA (SSR, STR), inter-simple sequence repeats (ISSRs), single nucleotide polymorphisms (SNPs), mitochondria DNA (mtDNA)) to be applied to population study of aphids as well as factors affecting genetic diversity of aphid. We aim at providing a basis for the selection of molecular markers in population genetics and obtaining a comprehensive acquaintance of genetic diversity study of aphids for tracing their evolutionary history implicating enlightenments for further aphid control.
Keywords: Molecular markers, aphid, genetic diversity, population, review.

L'utilisation de marqueurs moléculaires est l'un des outils les plus importants en développement dans le domaine de la génétique des populations. Ces éléments doivent être pris en considération avant de sélectionner l'approche moléculaire la plus efficace aux questions et modèles ciblés. Diverses approches en termes de marqueurs moléculaires sont décrites en relation avec l'étude des populations de pucerons : le bar-coding, les allozymes, le polymorphisme de longueurs de fragments de restriction (RFLP), le polymorphisme de longueur de fragment amplifié (AFLP), l'ADN polymorphe amplifié aléatoirement (RAPD), l'ADN microsatellite (SSR, STR), les répétitions de séquences inter-simples (ISSRs), le polymorphisme nucléotidique simple (SNP), l'ADN mitochondrial (ADNmt). La synthèse des applications de ces méthodes à l'étude de populations des pucerons est présentée ainsi que les principaux facteurs affectant la diversité génétique des pucerons. Cette démarche vise à fournir une base pour la sélection des marqueurs moléculaires dans la génétique des populations aphidiennes. La connaissance approfondie des approches appliquées permettra l'étude de la diversité génétique de pucerons encore non/peu étudiés pour investiguer les aspects évolutifs au sein de ces espèces entomologiques.

Mots-clés: Marqueurs moléculaires, pucerons, diversité génétique, population, révision.

1 INTRODUCTION

Phylogeography is a discipline about the historical evolution process of distribution patterns of different populations between and within species (Avise, 1996, 1998) while population genetics is a special microevolution research between populations. Molecular markers detect the genetic diversity of organisms based on the mutation of nuclear acids and related amino acid translation in proteins. These markers have become a powerful and comparatively efficient tool in genetic studies when morphological, cytological and biochemical markers do not allow insect discrimination at both inter- and intra-specific levels, allowing researchers to explore the track of speciation and evolution in many insect species such as *Ostrinia furnacalis* (Guenée 1854) (Li *et al.*, 2014), *Rhynchophorus ferrugineus* (Olivier 1790) (Wang *et al.*, 2015) and *Locusta migratoria manilensis* (Meyen 1835) (Zheng *et al.*, 2006) with various molecular markers.

For aphids, the taxonomic relationships between *Aphis gossypii* (Glover 1877) and related species have been investigated through mitochondrial DNA (mtDNA) marker (Lagos *et al.*, 2014; Lagos-Kutz *et al.*, 2014). *Diuraphis noxia* (Kurdjumov 1913) was another illustration for aphid model (Zhang *et al.*, 2014).

Learning the dynamic change in genome of the evolutionary history is essential to improve the integrated control effect of aphids. Despite the weak migrating ability, aphids may also migrate in some cases to maintain their continuation of species (Kieckhefer *et al.*, 1974), and the dispersal routes of a species can be found out through genetic diversity research by many genetic parameters such as heterozygosity, genetic equilibrium, allele frequencies and gene flow (Zhang *et al.*, 2014). Several population genetic studies have been performed through molecular markers for some other aphid species like *Myzus persicae* (Sulzer 1776), *Schizaphis graminum* (Rondani 1852), *Rhopalosiphum padi* (Linnaeus 1758) and *Sitobion avenae* (Fabricius 1775) (**Table 2**), whereas work for other aphid species such as *Rhopalosiphum maidis* (Fitch 1856) (Steiner *et al.*, 1985) almost remain lacking. In this article, we reviewed the basic principles, advantages and disadvantages of nine widely used molecular markers as well as their application in genetic differentiation study of aphids to provide basis for genetic diversity study of aphids subsequently.

2 FREQUENTLY-USED MOLECULAR MARKER METHODS

Population genetics mainly focuses on the dynamic changes within or between populations. Compared with traditional methods such as morphological markers, the emergence of molecular markers overcame the uncontrollable factors and has following advantages: 1) not affected by tissues and developmental stages; 2) not affected by environment; 3) abundant marker loci distributed in the genome; 4) high level of polymorphism; 5) the codominant markers can distinguish homozygous and heterozygous genotypes; 6) DNA can be stored for a long time. Hence molecular markers occupied the predominance and rapidly became main research approaches in population genetics. However, each of them has its own strong points as well as weaknesses (**Table 1**), which should be taken into consideration when choosing among these markers. In a word, we should carefully select the one that fulfill our requirements.

2.1 Barcoding

DNA barcodes is standardized, variable and short DNA fragments

Table 1: Advantages and limitations of the common molecular markers.

Molecular markers	Advantages	Limitations
DNA barcoding	Repeatable; Accurate, rapid and efficient; Universal	Introgression; Accuracy can be influenced by different evolutionary rate of genes; Pseudogenes phenomenon
Allozyme	Easy to analyze; Codominant inheritance; Low cost	Can only detect coding genes; Low polymorphism
RFLP	Alleles detection; Stable and repeatable; Homozygote and heterozygote can be distinguished	Expensive and laborious; Require high quantity and quality of DNA; Polymorphism is limited by the restriction enzyme sites
AFLP	Stable and efficient; Easier to perform than RFLP; High level of polymorphism; No need for DNA sequence information	No alleles detection; Require high purity of DNA; Analysis is complex; More expensive and laborious than RAPD; Dominant inheritance
RAPD	Easier to perform than AFLP; Time-saving; Labor-saving; Low cost; No need for DNA sequence information; High level of polymorphism	No alleles detection; Poor repeatability; Dominant inheritance; Homology of comigrating amplification products
SSR (STR)	Alleles detection; Wide distribution; Good repeatability; High level of polymorphism; Codominant inheritance	High cost; Development of microsatellite makers could be time-consuming
ISSRs	No need for DNA sequence information; Quick to perform; Low cost; High level of polymorphism	No alleles detection; Dominant inheritance; Homology of comigrating amplification products
SNPs	Wide distribution; Stable; Automatic analysis; Codominant inheritance	High cost; DNA secondary structure may interfere sequencing

mtDNA	Small genome; Easy to sequence and analyze; Quick evolution; No introns in genome; Maternal inheritance	May generate larger standard error than nuclear gene

This method was introduced by Hebert *et al.* (2003a) to identify species quickly and efficiently. They firstly selected cytochrome c oxidase subunit 1 (COI) as general marker to diagnose species across the animal kingdom (Hebert *et al.*, 2003b). This molecular marker is a kind of DNA taxonomy based on the specificity inside species and interspecific variability of the DNA sequences. A DNA barcode database "Barcode of Life Data Systems" (BOLD) was then set up as a DNA barcode reference library facilitating the information sharing (Ratnasingham & Hebert, 2007). Except for species identification, DNA barcoding can complement research areas, both in molecular phylogenetics and population genetics (Hajibabaei *et al.*, 2007; Silva-Brandao *et al.*, 2009; D'Acier *et al.*, 2014; Kinyanjui *et al.*, 2016).

To utilize this approach, we need to 1) extract the DNA; 2) design the primers and perform the PCR; 3) sequence the PCR product; 4) analyze and correct the result; 5) submit to DNA barcode database. The technique has been widely used because of several characteristics: 1) direct use of DNA sequence making the result repeatable; 2) accuracy, rapidity and efficiency with the establishment of DNA barcode database; 3) DNA barcoding is general with universal primers. Nevertheless, several limitations should be taken into consideration as listed in **Table 1**. For example, the different evolutionary rate of COI genes from variant organisms and the pseudogenes (i.e. Nuclear Mitochondrial DNAs - NUMTs) phenomena can affect the accuracy of COI-based DNA barcoding (Silva-Brandao *et al.*, 2009).

2.2 Allozymes

Allozymes are different forms of the same enzyme coded by alleles at the same genetic locus (Steiner & Joslyn, 1979). Allozyme analysis is based on the change of protein electrostatic charge when mutation previously occurred on DNA sequences. This marker is effective because of the correspondence between zymogram and alleles. Also, the expression of allozyme conforms to Mendel's law of inheritance, which makes the analysis easier. However, only coding genes can be detected and the variation of allozyme may not represent the diversity of the whole genome. Some species have little allozyme variation compared with other molecular maker techniques. For instance, allozymes showed low level of polymorphism in the genetic diversity study of *R. padi*, whereas microsatellite loci were highly polymorphic in aphid (Delmotte *et al.*, 2002).

2.3 Restriction fragment length polymorphism (RFLP*)*

RFLP was firstly used to identify sequence polymorphisms for genetic mapping of adeno-virus serotypes (Grodzicker *et al.*, 1974), and genetic linkage map was established on humans (Botstein *et al.*, 1980). It is a hybridization-based maker. The principle is to assess the variation of some sites on DNA chain resulting in changes of restriction enzyme sites and related fragments length between these loci.

The main procedures involve: 1) DNA extraction; 2) DNA digestion with restriction enzymes; 3) DNA fragment separation on agarose gel; 4) separated DNA fragment transfer from gel to membrane through Southern blotting; 5) fragment detection with probes by nucleic acid

hybridization and autoradiography (Terachi, 1993). The results of RFLP technique are stable and repeatable. Moreover, homozygote and heterozygote can be distinguished through this method. Nevertheless, RFLP require high quantity and quality of DNA, high workload and cost.

The level of polymorphism is limited by the restriction enzyme sites.

2.4 Amplified fragment length polymorphism (AFLP)

AFLP was invented by Zabeau & Vos (1993) to detect DNA polymorphism and was firstly called selective restriction fragment amplification (SRFA). It combines RFLP and PCR (Polymerase Chain Reaction) technologies, and use DNA fragments digested by restriction enzyme as PCR templates. Then, PCR products are separated by high resolution polyacrylamide gel electrophoresis (Vos *et al.*, 1995). AFLP is easier to perform compared with RFLP, and possesses the stability of RFLP and high efficiency of PCR. The limitation of this technique is high request of DNA purity, only dominant makers can be detected and the statistical analysis is difficult.

2.5 Random amplified polymorphic DNA (RAPD)

RAPD was firstly proposed by Williams & Welsh (1990) and developed in 20th century. The marker is based on PCR with about 10 bp length of single stranded random primer. The primer could combine with different sites on DNA template and produce fragments diverse in length which can be separated by agarose gel electrophoresis. RAPD technique is easy to perform, time- and labor-saving. Also, it needs little DNA sample and can be carried out regardless of the DNA sequence information. Nevertheless, this method has poor repeatability since the PCR results can be affected by many factors such as the concentration of DNA, reaction conditions and polymerase activity (Perez *et al.*, 1998).

2.6 Microsatellite DNA (SSR, STR)

Microsatellite DNA, discovered from the human genome library (Miesfeld *et al.*, 1981), is also called simple sequence repeats (SSR) or short repeats (STR). Tandem repeat sequences are composed by units of 1-6 nucleotides and distributed in the whole genome. Variation in numbers of tandem repeat sequences represents the polymorphism of the DNA sequences (Bell, 1996; Li *et al.*, 2002). Firstly, the development of microsatellite loci is essential for the establishment of this technique which includes several steps: 1) construction of microsatellite library; 2) screening for microsatellite loci; 3) design of primers; 4) PCR with designed primers; 5) evaluation of primers by agarose electrophoresis. Secondly, efficient microsatellite loci (primers with fluorescent probe) can be used for DNA samples via PCR. Thirdly, sequencing of PCR products by Genetic Analyzer is finally followed by data analysis. According to SSR results, different populations of *S. avenae* in Europe exhibited various genetic structures and reproductive patterns (Papura *et al.*, 2003) while *S. avenae* in Britain had high migratory potential (Llewellyn *et al.*, 2003). SSR loci are widely distributed, conform to Mendel's law of inheritance and have good repeatability, so the technique is frequently used to detect the population polymorphism (Balloux & Lugon-Moulin, 2002; Selkoe & Toonen, 2006). Nevertheless, expenditure should be taken into consideration since SSR is more expensive than AFLP and RAPD methods.

2.7 Inter-simple sequence repeats (ISSRs)

ISSRs technology is based on SSR method (Zietkiewicz *et al.*, 1994; Moreno *et al.*, 1998), and is easier than SSR since DNA sequencing is eliminated in this method. The SSR-anchored DNA is used as primer by adding 2-4 random nucleotides to 3' or 5' terminal of SSR sequences.

Table 2: Molecular markers used in genetic differentiation study of aphid populations.

Aphids	Molecular markers	References
Aphis fabae (Scopoli 1763)	SSR	(Sandrock *et al*., 2011)
	RAPD	(Béji *et al*., 2013)
	Barcoding	(Béji *et al*., 2015)
Aphis glycines (Matsumura 1917)	SSR	(Michel *et al*., 2009, 2010)
Aphis gossypii (Glover 1877)	mtDNA and nuclear genes	(Rebijith *et al.*, 2012)
	mtDNA	(Lokeshwari *et al.*, 2015)
	RAPD	(Martinez-Torres *et al.*, 1997a; Vanlerberghe-Masutti & Chavigny, 1998)
	Barcoding	(Kinyanjui *et al.*, 2016)
Acyrthosiphon pisum (Harris 1776)	mtDNA	(Barrette *et al.*, 1994)
	RFLP	(Birkle & Douglas, 1999)
	SSR	(Peccoud *et al.*, 2009)
	Barcoding	(Kinyanjui *et al.*, 2016)
Diuraphis noxia (Kurdjumov 1913)	SSR, mtDNA	(Zhang *et al.*, 2012, 2014)
	RAPD	(Puterka *et al.*, 1993)
Eriosoma lanigerum (Hausmann 1802)	ISSR	(Lavandero *et al.*, 2009)
	AFLP	(Timm *et al.*, 2005)
Macrosiphum euphorbiae (Thomas 1878)	RAPD	(Raboudi *et al.*, 2011)
Melaphis rhois (Fitch 1866)	Allozymes	(Hebert *et al.*, 1991)
Myzus persicae (Sulzer 1776)	SSR	(Guillemaud *et al.*, 2003; Vorburger, 2006; Fenton *et al.*, 2010)
	mtDNA and nuclear genes	(Rebijith *et al.*, 2012)
	RAPD	(Martinez-Torres *et al.*, 1997a)
	RAPD, mtDNA and nuclear genes	(Clements *et al.*, 2000)
	Barcoding	(Kinyanjui *et al.*, 2016)
Daktulosphaira vitifoliae (Fitch 1855)	SSR	(Vorwerk & Forneck, 2006)
Pterochloroides persicae (Cholodkovsky 1899)	mtDNA	(Lassaad *et al.*, 2013)
Rhopalosiphum maidis (Fitch 1856)	Allozymes	(Steiner *et al.*, 1985)
	Allozymes and mtDNA	(Simon *et al*., 1995)
Rhopalosiphum padi (L. 1758)	Allozymes	(Dedryver, 1996)
	RAPD	(Simon *et al.*, 1999; Bulman *et al.*, 2005; Tabikha & Adss, 2016)
	SSR and allozymes	(Delmotte *et al.*, 2002)
	mtDNA	(Martinez-Torres *et al.*, 1997b)
	SSR	(Gilabert *et al.*, 2015; Duan *et al.*, 2016)
	ISSRs	
	mtDNA and SSR	(Tabikha & Adss, 2016)

		(Valenzuela *et al.*, 2010)
Schizaphis graminum (Rondani 1852)	mtDNA	(Anstead *et al.*, 2002)
	Allozymes	(Shufran *et al.*, 1992)
	RAPD	(Lopes-da-Silva & Vieira, 2010; Kharrat *et al.*, 2012)
Sitobion avenae (Fabricius 1775)	SSR	(Llewellyn *et al.*, 2003; Figueroa *et al.*, 2005; Xin *et al.*, 2014)
	mtDNA	(Xu *et al.*, 2011)
	SSR and mtDNA	(Sunnucks *et al.*, 1997)
	RAPD	(De Barro *et al.*, 1995)

The results are obtained by agarose gel electrophoresis of PCR products. ISSRs can be carried out without any prior sequence information of target organism and is quicker than the other methods such as RAPD and RFLP (Godwin *et al.*, 1997). It needs low cost and technical requirements, does not require much DNA, and promotes the specificity of PCR. Therefore, this method is widely used for population genetics and systematics (Wu *et al.*, 1994; Weising *et al.*, 1995; Godwin *et al.*, 1997). Despite these advantages, some weaknesses such as the absence of allele detection, the dominant inheritance and homology of co-migrating amplification products may limit the application of ISSRs.

2.8 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms, as the name suggests, is the polymorphism caused by mutation such as transition, transversion, insertion and deletion of single nucleotide on DNA chain. SNPs are distributed in both coding region and non-coding region. This method detects the single nucleotide mutation through DNA sequencing or DNA chip technology. It has been used in fields like system evolution, medicolegal expertise, genetic linkage analysis of disease and receptor matching selection research since SNP technique is outstanding in aspects of wide distribution, stability and automatic analysis (Seddon *et al.*, 2005; Yue *et al.*, 2006). However, sufficient funds should be ensured if choosing this technique.

2.9 Mitochondria DNA (mtDNA)

Mitochondria, a kind of semi-autonomous organelle, exist in most eucaryon and are closely related with energy metabolism. Mitochondria genome is small and annular double-stranded with simple structure that contains 13 protein coding genes. Avise *et al.* (1987) firstly found that the variation in mtDNA sequence can offer the information of intraspecific evolution. Since mtDNA follows strict maternal inheritance and has no intron, and high level of mutation in genome within or between populations, this marker is widely used in insect such as the study of phylogenetics (Ortiz-Rivas & Martínez-Torres, 2010), systematics (Behere *et al.*, 2008), and genetic diversity (Anstead *et al.*, 2002; Lassaad *et al.*, 2013). Nevertheless, some mutations of mtDNA may not conform to selective neutrality and thus resulting in genetic hitchhiking (Kilpatrick & Rand, 1995). In addition, the assessment of mtDNA polymorphism may result in larger standard error than nuclear gene polymorphism (William *et al.*, 1995). Accordingly, combination of mtDNA and other molecular markers would be optimal choice.

3 GENETIC DIFFERENTIATION STUDY OF APHIDS

Aphids, like other organisms, maintain some genetic traits for continuation of species, meanwhile, new phenotypes and genotypes may emerge in order to adapt to changes in the environment in the long-range of evolution. To

date, more than 5000 aphid species have been described all over the world (Remaudiere & Remaudiere, 1997; Favret *et al.*, 2016). Many previous works demonstrated that genetic variation occurs within aphid species (i.e. among different populations) (Sunnucks *et al.*, 1997; Figueroa *et al.*, 2005; Sandrock *et al.*, 2011; Xin *et al.*, 2014). The genetic diversities of aphids have been repeatedly studied with various molecular markers (**Table 2**) and were affected by many factors such as life cycle (Simon *et al.*, 1996; Duan *et al.*, 2016), host plant (Hales *et al.*, 1997; Vorburger, 2006; Valenzuela *et al.*, 2010) and geography circumstance (Simon *et al.*, 1996; Martinez-Torres *et al.*, 1997b; Zhang *et al.*, 2014). These factors may force the occurrence of genetic diversity.

4 FACTORS AFFECTING THE GENETIC DIVERSITY OF APHIDS

4.1 Life cycle of aphids

The life history of aphids is complex, including both holocyclic life cycle with a cyclical sexual reproduction could happen under induced conditions and anholocyclic life cycle that shows continuous parthenogenesis with viviparous females (Duan *et al.*, 2016). Holocyclic life cycle combines the genetic materials of male and female. In this way, aphids can maintain species characteristics and obtain genetic heterogeneity simultaneously (Hales *et al.*, 1997). Anholocyclic life cycle was thought to have low genetic diversity and narrow adaptation because of the obligate parthenogenesis. Conversely, the facts prove that anholocyclic life cycle of aphid is ubiquitous and the parthenogenesis individuals have better adaptation than sexual ones under certain environmental conditions (Simon *et al.*, 1996). In one hand, an explanation could be that the genetic variation of parthenogenesis aphids mainly results from gene mutation, which is quick and abundant. On the other hand, parthenogenesis could be related to the selection of some environment more easily since gametogenesis may disassemble the adaptable genotype (Hales *et al.*, 1997).

4.2 Host plant

Aphids are closely associated with host plants during long-term coevolution, building up the phenomenon that the same species of aphids can exhibit variant morphological characteristics on different plants (Peccoud *et al.*, 2010). In addition, different developmental stages of the plant can have significant impacts on aphid behavior or reproduction (Karley *et al.*, 2004) and aphids feed on different tissues from the same plant appear to be polymorphic in mtDNA (Lassaad *et al.*, 2013). One aphid species may be highly specialized on a certain host among different host plants, consequently form the genetic variation even reproductive isolation. By example, genetic differentiation of *M. persicae* was found on different host plants (Takada, 1988) and two host biotypes of *A. pisum* on alfalfa and red clover were formed in north American (Caillaud & Via, 2000). Moreover, the different survival rates of aphids on resistant varieties and susceptible ones from the same plant species were demonstrated to be associated to aphid genotypes. The aphid resistance from host plant acts as driving force for the formation of new aphid genotypes such as *S. graminum* and aphid resistant varieties of wheat (Puterka & Burton, 1990).

4.3 Geography and climate conditions

Geography and climate conditions play significant roles during the evolution of insects especially those with poor migratory potential such as aphids. One species living in different regions would evolve in diverse directions under the natural selection pressure after geographic isolation for a certain period of time, which finally set the special genotypes and gene frequencies in these areas (Oldroyd & Fewell, 2007). Population differentiation of the same aphid species can occur after living in different environment (latitude, longitude, elevation and climate) for a long time.

For example, significant genetic diversity of *R.*

padi was found between populations from southern and western area in France (Martinez-Torres *et al.*, 1997b). Results also showed longitudinal clines in *R. padi*'s frequency distribution in maize fields in the northern half of France (Gilabert *et al.*, 2015). SSR data also showed that European *D. vitifoliae* lacks gene flow between different populations (Vorwerk & Forneck, 2006) whereas gene exchange among *M. persicae* populations in Britain is frequent (Fenton *et al.*, 2010). In addition, the genetic diversity of *S. avenae* (Huang *et al.*, 2013), *D. noxia* (Zhang *et al.*, 2014) and *A. gossypii* (Rebijith *et al.*, 2012) is influenced by geography and climate conditions.

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