# The application of methylation-sensitive amplified polymorphism (MSAP)in ecology

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**Abstract:** DNA methylation is one of the most important epigenetic modifications in living organisms and its application in ecology is becoming increasingly widespread. Based on the collection and review of relevant literature in ecological epigenetics, this paper introduces the principles, advantages, and limitations of Methylation-Sensitive Amplified Polymorphism (MSAP) technology, as well as its applications and prospects in ecology. MSAP has become a powerful tool for studying DNA methylation levels due to its wide application and ease of operation. It is particularly useful in exploring how organisms rapidly adapt to habitat changes and how invasive species overcome genetic bottlenecks. MSAP technology can effectively reveal epigenetic differences within or between biological populations, serving as a valuable complement to the study of genetic diversity and genetic variation.

**Keywords:** Methylation-Sensitive Amplified Polymorphism (MSAP), Ecology, Population Variation, Ecological Epigenetics

## 1. Introduction

With the advent of the genomic era and the increasing understanding of how organisms respond to the environment at the genetic level, the interaction between genotype and phenotype is becoming more evident. Although the complete genome sequences of multiple species have been published, progress in understanding how genomes function and adapt to complex environments has been relatively slow (Martin et al.,2011; Pigliucci,2010; Richards et al.,2009,2012a). Ecological epigenetics is the study of the relationship between epigenetic variation and ecological phenotypic variation. Changes in epigenetic mechanisms can alter gene expression and organismal function, leading to differences in morphological traits(Cubas et al.,1999;Kucharski et al.,2008;Manning et al.,2006;Morgan et al., 1999;Rakyan et al.,2003), without changing the DNA sequence(Richards,2006).Additionally, it has been found that some epigenetic marks can be stably inherited by the next generation(Jablonka&Raz,2009;Johannes et al.,2009;Verhoeven et al.,2010).However, there is considerable variation in epigenetic marks among individuals and populations (Herrera&Bazaga,2010, 2011; Herrera et al.,2012;Liu et al.,2012; Massicotte& Angers,2012;Massicotte et al.,2011;Richards et al.,2012b;Schrey et al.,2012).Therefore, epigenetic mechanisms are an important component of how organisms respond to their environment (Angers et al.,2010;Richards et al.,2010; Verhoeven et al.,2010). Understanding epigenetic variation will help explain scientific questions in ecology and evolution(Angers et al.,2010;Bossdorf et al.,2008;Richards et al.,2008,2010).

Epigenetic mechanisms (such as DNA methylation, chromatin remodeling, histone deacetylation, position effect, and small RNA interference) can influence gene expression, among which DNA methylation is the most well-studied epigenetic mechanism. DNA methylation typically refers to the attachment of a methyl group to cytosine, which is followed by a guanine in the DNA sequence (Bossdorf et al.,2010). DNA methylation has various effects on gene expression, usually manifested as a reduction in gene activity (Bossdorf et al.,2008; Jablonka&Lamb,2006). Currently, several techniques (such as HPLC, HPCE, bisulfite sequencing, and methylation fluorescence) are available to detect differences in DNA methylation, among which methylation-sensitive amplified polymorphism (MSAP)is the most commonly

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used method (Reyna-Lopez et al.,1997). This review summarizes the principles, advantages, and limitations of MSAP technology, as well as its applications and prospects in ecology.

## 2. Principle of MSAP

MSAP is a PCR-based technique for detecting genomic methylation variation, derived from the AFLP technology(Reyna-López et al.,1997).The technique employs two restriction enzymes, M s p I and H pa II, which have different sensitivities to methylated cytosines in their recognition sequence CCGG(Roberts et al.,2007).H pa II cannot cleave sites where both cytosines on the two strands are methylated or where either cytosine is methylated, meaning it cannot cleave sites containing m CCGG, Cm CGG, or m C m CGG. However, it can recognize sites where only one cytosine on one strand is methylated. In contrast, M s p I can cleave sites where the internal cytosine is methylated on either one or both strands, but it cannot cleave sites where the external cytosine is methylated, such as m CCGG(McClelland et al.,1994;Roberts et al.,2007).Based on these differences, the same DNA sequence can be amplified into distinct bands, which can be used to determine the methylation status and degree of cytosines at the 5'-CCGG sites(Vos et al.,1995).

## 3. Advantages of MSAP

The MSAP technique has several advantages(Schrey et al.,2013):(1)It can be used to study non-model organisms, including those lacking genome sequencing;(2)It is technically similar to AFLP, such as having the same experimental protocols, skills, and laboratory equipment;(3)It is cost-effective, scalable, and the same reagents can be used for different species;(4)It allows for high-throughput screening of a large number of individuals at multiple loci, thereby generating a large amount of data to detect mutations and differentiation occurring in populations and treatments;(5)It has high sensitivity for detecting environmentally induced DNA methylation variation. Therefore, despite certain limitations in interpreting the results, MSAP still holds great potential.

## 4. Applications of MSAP in ecology

A literature review reveals that over the past 12 years, more than 200 MSAP papers have been published in the field of ecology (Figure 1). The number of papers in this field has generally increased year by year, peaking in 2014 with 35 publications. Notably, in recent years, the number of papers focusing on species other than plants has also been increasing, such as the house sparrow Passer domesticus (L.) (Schrey et al.,2012), the yeast Metschnikowia reukaufii (Pitt &M. W. Mill) (Herrera et al.,2012), and the leaf-nosed bat Hipposideros armiger (Hodgson) (Liu et al.,2012). This trend indicates that the application scope of MSAP technology is continuously expanding.

### 4.1. MSAP and stress resistance research

Under adverse environmental conditions (such as herbivory, low temperature, salinity, and metal ions), the level and pattern of DNA methylation in organisms change. Herrera& Baza ga (2011) used wild populations of the violet Viola cazorlensis (Gand.) as their study subjects and investigated the genetic and epigenetic characteristics of natural populations under herbivory using AFLP and MSAP techniques. They found significant differences in DNA methylation among individuals, which were correlated with the degree of herbivory, and some methylation sites were associated with specific AFLP markers. This study highlighted the necessity of using MSAP analysis to investigate responses to specific external stimuli.

Xin et al. (2015) discovered that after 48 hours of low-temperature treatment, the cytosine methylation level in potatoes increased from 37.71%to 56.51%, with the changes in methylation patterns mainly originating from full methylation of CG sites on double-stranded D NA. Through sequencing of 78 polymorphic DNA fragments and semi-quantitative PCR detection, they found that genes related to cold stress could be regulated by changes in methylation or hemimethylation states.

Zeng et al. (2015) analyzed the methylation levels and patterns among Fraxinus mandschurica Rupr., Fraxinus velutina Torr., and their hybrid $F_1$ generation. The results showed that the hybrid $F_1$ seedlings exhibited better salt tolerance and growth advantages, but their DNA methylation patterns shifted to

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external cytosine methylation (7.34%). This finding suggested a correlation between salt tolerance and changes in DNA methylation.

Chen et al. (2015) examined the changes in methylation-sensitive amplified polymorphism (MSAP)in Arabidopsis thaliana seedlings under different concentrations of copper (Cu)treatment. They found that while the root growth rate and fresh weight of the seedlings did not significantly change under 0.25 and 1.0 $mg \cdot L^{-1}$ Cu treatments, the MSAP levels initially increased and then decreased with higher concentrations of Cu ions. MSAP was highly sensitive to low concentrations of Cu ions and could be used for early diagnosis and ecological safety assessment of Cu pollution.

Furthermore, the DNA methylation patterns of Metschnikowia reukaufii also changed in response to different environmental conditions (Herrera et al.,2012). The composition, concentration, and interactions of sugars in nectar significantly affected the probability of MSAP sites shifting from unmethylated to methylated states. Treatment with the methylation inhibitor 5-azacytidine(5-AZA) significantly inhibited the growth of Metschnikowia reukaufii communities. Changes in DNA methylation patterns were closely and positively correlated with the ability of Metschnikowia reukaufii to acquire resources from different habitats.

Therefore, MSAP is capable of effectively detecting changes in the level and pattern of DNA methylation in organisms under stress conditions, thereby providing a solid foundation for studying the epigenetic regulation of stress-related genes.

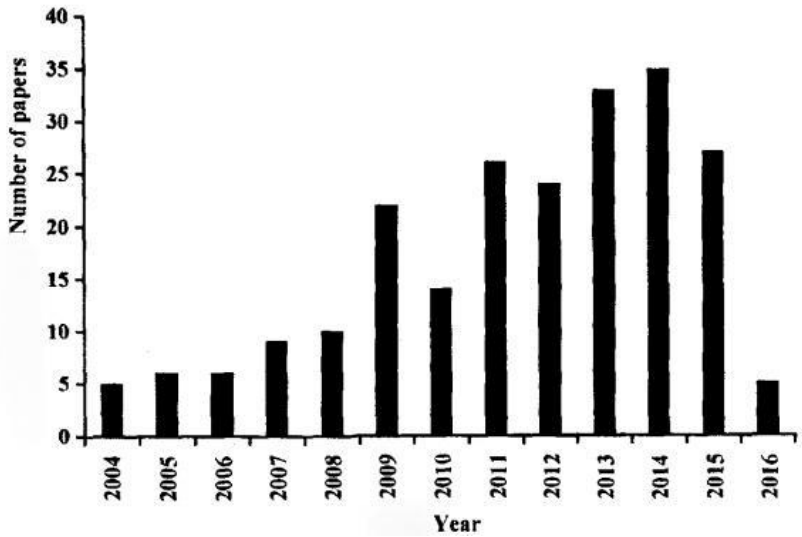


Figure 1. The number of MSAP-related papers published in the field of ecology from 2004 to 2016.

### 4.2. MSAP and genetic diversity research

MSAP is derived from AFLP technology. As a commonly used molecular marker method, AFLP has been widely applied in studies of germplasm resource diversity, genetic map construction, and gene localization. Therefore, MSAP can also be applied to research on genetic diversity. Massicotte et al. (2011) used MSAP technology to study the epigenetic variation in populations of the all-female clonal fish Chrosomus eos-neogaeus. This study identified 15 MSAP candidate loci and performed bisulfite sequencing to pinpoint the exact sites of DNA methylation differences. Among these, four candidate loci could not be identified, and at least 11 loci were inferred to have some similarity to the genome of zebrafish (Brachy danio rerio). In addition, sequencing revealed additional DNA methylation variation at adjacent sites, with some sites always being methylated or always unmethylated.

Roy et al.(2015)conducted genetic diversity analysis on three maize varieties, revealing that variety differentiation is related not only to genetic variation but also to epigenetic variation. Guarino et al. (2015) analyzed the genetic diversity of poplars on Sardinia and found that the genetic diversity level of poplars was very limited, while their epigenetic variation level was high. Environmental conditions significantly

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affected the hemimethylation of cytosines within the DNA strands. Clonal ramets from different geographical distributions exhibited different methylation patterns. This study indicated that plant diversity should not be limited to genetic aspects but should also consider epigenetic differences, especially in asexual reproduction.

Hong and Deng (2005) assessed the methylation patterns and levels of cytosines in the genomes of 24 navel orange varieties. The results showed that 4.7%to 15.0%of the CCGG sequences in the navel orange genome were methylated. Among the 18 pairs of amplification primers used,10 pairs exhibited polymorphism, yielding a total of 639 bands, of which 43 were methylated polymorphic bands, with an average methylation polymorphism(P)rate of 6.7%.DNA methylation occurred frequently in navel oranges, and there were significant differences in methylation patterns among varieties. In the CCGG sequences of navel oranges, external cytosine methylation(15.0%)was more common than internal methylation(4.7%).

The above studies have all demonstrated, to varying degrees, that DNA methylation is one of the important sources of random variation in natural populations, and MSAP technology is an important quantitative method for DNA methylation polymorphism.

### 4.3. Applications of MSAP in invasive species

The invasive plant Fallopia japonica (Houtt.), commonly known as Japanese knot weed, is an important material for studying the epigenetic adaptation of species to new environments. Japanese knotweed can invade a variety of habitats, and most of its traits show significant differences both within and among populations, despite its very low genetic diversity. Using AFLP and MSAP molecular markers, Richards et al.(2012b) investigated the differences in responses of Japanese knotweed to new habitats (beaches, salt marshes, and roadsides) and the correlation with epigenetic variation. The results showed that among 200 AFLP loci, only four loci exhibited polymorphism, and eight haplotypes were detected. However, there was considerable epigenetic variation among samples. Among 180 MSAP loci,19 polymorphic loci generated 128 epigenotypes, and some epigenetic loci varied depending on habitat conditions.

Alternanthera philoxeroides (Mart.) Griseb., commonly known as alligator weed, is an amphibious invasive plant that exhibits different phenotypes in different habitats but has very low genetic variation. Gao et al. (2010) used MSAP to analyze the genetic and epigenetic variation of alligator weed from different sources. The study found that 78.2%of methylation changes were associated with different water sources, indicating that the epigenetic regulatory system is highly sensitive and flexible to environmental conditions. This research described the mechanism of alligator weed's invasion into different habitats at the molecular level. Therefore, MSAP plays an important role in elucidating the molecular mechanisms by which invasive species adapt to new environments in a short period of time.

## 5. Limitations of MSAP

MSAP technology has two main limitations(Schrey et al.,2013):(1)When the restriction enzymes M s p I and H pa II fail to digest the DNA(Salmon et al.,2008),the observed banding patterns in the experiment can be caused by both genetic factors(mutations at restriction sites, changes in adjacent restriction sites)and epigenetic factors(hypermethylation, methylation of all cytosines at restriction sites).This can lead to the omission of some methylation states.(2)The scoring method for AFLP-type banding patterns needs further discussion. Due to differences in PCR, it is difficult to establish a unified standard across laboratories. Moreover, each sample must be compared between two reactions. In some cases, differences between M s p I and H pa II digestion reactions may be caused by inconsistent restriction enzyme digestion or differences in PCR reactions, rather than by methylation differences.

Additionally, MSAP can only infer genome-wide epigenetic variation based on differences in fragment lengths, with the adjacent sequences of each site being unknown. Therefore, it is necessary to rely on extraction and fragment sequencing to identify homologous sequences in databases (Massicotte et al.,2011; Salmon et al.,2005). Furthermore, MSAP cannot distinguish epigenetic differences between heterozygotes.

## 6. Future development of MSAP

Due to the limitations of MSAP technology, the following issues should be considered in future research. First, genetic diversity and DNA methylation polymorphism data should be collected simultaneously. These

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data are crucial for understanding the interactions between epigenetic and genetic factors in the response of organisms to environmental stimuli (Herrera& Baza ga, 2011; Richards et al.,2012b). Second, the use of techniques other than MSAP to identify methylation targets can significantly increase the reliability of experimental results. Extracting MSAP bands for sequencing and performing bisulfite sequencing (Massicotte et al.,2011) will be the first steps in addressing the limitations of MSAP technology. With the development of next-generation sequencing (NGS)technologies, NGS has been applied to epigenetic ecology (Platt et al.,2015; Robertson&Richards,2015).

Third, experimental methylation inhibition using drugs such as 5-azacytidine can be employed if necessary, to better understand the impact of DNA methylation on phenotypes (Bossdorf, 2010; Herrera et al., 2012). Fourth, common garden experiments should be incorporated into studies of natural variation (Richards et al.,2012b). In addition, samples should be processed uniformly in the laboratory (Herrera et al.,2012), invasive species should be investigated (Richards et al., 2012b), environmental stressors should be clarified (Herrera&Bazaga,2011), and habitat conditions should be distinguished (Herrera et al.,2012; Richards et al.,2012b).

## 7. Conclusion

Additionally, numerous studies have demonstrated that epigenetic mechanisms are a crucial mechanism for population expansion within new habitats during biological invasions (Chwedorzewska &Bednarek, 2012; Richards et al.,2012b; Schrey et al.,2012). DNA methylation will play an increasingly important role in the response and rapid adaptation of biological populations to environmental changes triggered by global climate change. It also provides valuable insights into a range of biological issues, including responses to the environment, global change, and human health. By analyzing differences across multiple populations in various environments, MSAP technology can identify specific populations (or species) with the greatest capacity to adapt to environmental changes. This will reveal which populations are at risk of decline due to a lack of necessary diversity. Ultimately, these questions all boil down to the decryption of how genotype is translated into phenotype. MSAP technology can be used in conjunction with target-specific techniques to elucidate the role of DNA methylation in individual responses to environmental changes.

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