Discrimination of the Anopheles Sp. Using Different Molecular Techniques

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Abstract- ***Malaria is one of the most common infectious diseases and public health problems around the globe. Malaria parasite is transmitted by Anopheles mosquitoes classified under Family Culicidae of Order Diptera and. The Anopheles complex has been particularly incriminated as one of the important malaria vectors in Sri Lanka and other part of the island. The present review provides basic aspects to differentiate morphologically identified sibling species of the Anopheles complex by different DNA based molecular techniques such as random amplified polymorphic DNA, restriction fragment length polymorphisms. DNA barcoding and Polymerase chain reaction. Moreover, it is particularly useful when using small amount of starting materials from particular specimen or from immature stages.***

Molecular technique provides a powerful tool for effective examination on the population dynamics of mosquitoes. It enables more detailed understanding on the relationships between the vectorial capacity, genetic makeup and geographical origin for a particular species of Anopheles, more detailed and precise taxonomy, as well as evolutionary studies.

Indexed Terms- Anopheles, Malaria, Mosquitoes, Molecular techniques, DNA

I. INTRODUCTION

In worldwide, around a billion people become infected with vector-borne diseases such as malaria, dengue fever, yellow fever and West Nile virus in every year. (WHO, 2017). Malaria is one of the most common infectious diseases and a great public health problem around the world, of which more than 91 countries were declared as an epidemic regions (CDC, 2017). Malaria threat has been reduced significantly during the last few years in Sri Lanka and declared as malaria free Sri Lanka in 2016. (World Health Organization, 2016).

Human malaria is exclusively transmitted by *Anopheles* spp. mosquitoes in Sri Lanka and other part of the island. Mostly all key malaria mosquito vectors are members of complexes or groups comprising morphologically indistinguishable sibling species. Mostly identification based on the morphological criteria fails when sibling species and species with overlapping morphological characters are involved.

The correct identification of disease vectors is the first step towards implementing an effective control programme. Traditionally, malaria control was based on the morphological differences observed in adults and larvae between different mosquito species. However, the discovery of species complexes meant that genetic tools were needed to separate the sibling species and now a day there are standard molecular techniques that are used to identify the two major malaria vector groups of mosquitoes.

Advanced DNA based molecular technology have facilitated the development of simple and rapid molecular tools for the identification of sibling species. Various genetic techniques are available for identification of isomorphic species (Besansk et al.,1992, Hill &Crampton 1994) like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP). DNA barcoding, Polymerase chain reaction or sequencing method have revolutionized population genetics and insect systematics.

II. JUSTIFICATION

Anopheles species has been particularly incriminated as one of the malaria vectors in island wide. All the members of the complex should be studied in detail to

formulate effective vector control strategies. Morphological properties are widely used to identify and classify mosquito species without the need for specialized and expensive equipment in field studies. Since reported morphological characteristics are not useful, a simple DNA based technique is more suitable to identify the members of Anopheles complex. In this background this study suggests that various molecular techniques for identification of Anopheles sp

III. POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) based assay which was developed in 1985 by Saiki et al., (1985) become popular for species identification as the technology is vastly improved and reagent cost is also reduced. The PCR based assay essentially requires variation in nucleotide sequence across species. Information gathered has revealed that two features of the ITS2 region of Anopheles species that are diverse region for developing PCR- based species diagnostic assays. First, these regions are relatively short (generally less than 1 kb), making PCR amplification of the intervening ITS2 from flanking conserved primers relatively simple. Second, intra species variation in ITS2 sequences is lower than interspecies variation, thus permitting the development of PCR diagnostic assays (Wesson et al, 1992). The use of diagnostic length for the identification of diagnostic sequences for cryptic Anopheles species complexes is a valuable technique.

Erlank et al (2018) described the diagnostic of three species (Anopheles pretoriensis, A rufipes and A rhodesiensis) amplified members of the An. funestus group and four species (An. pretoriensis, An. rufipes, Anopheles listeri and Anopheles squamosus) amplified members of the An. gambiae complex by Standard PCR assay.

Gunathilaka et al (2015) performed the PCR assay to distinguish the members of Anopheles culicifacies sibling species A, B, C, D and E and following band sizes were obtained 359 bp, 248 bp, 95 + 248 bp, 166 + 359 bp and 178 + 248 bp to compare the sibling species of A, B, C, D and E respectively. Fettene et al (2002) distinguished the sibling species A and B of An.quadriannulatus s.l as well as other members of An.gambiae complex by modified PCR assay.

Scott et al (1993) identified the An. gambiae, An. Arabiensis, An. quadriannnulatus and either An.melas in western Africa or An. melas in eastern and southern Africa by the ribosomal DNA polymerase chain reaction. Dusfour et al. (2007) developed a multiplex polymerase chain reaction assay based on the two mitochondrial DNA markers to clearly identify the members of An. sundaicus s.s, An. epiroticus and An. sundaicus.

H.K.Phuch et al. (2003) described the development of single multiplex PCR for identifying An.minimus species A and C and distinguishing the four related species, An.aconitus, An.jeyporiensis, An.varuna and An.pampanai. The rDNA ITS2 region was amplified using conserved 5.8s and 28s primers modified from those used by Paskewitz &Collins (1990). Based on the species-specific differences, six primers were designed as reverse primers. These reverse primers were mixed with 5.8S forward primer to amplify the whole ITS2 region (Manonmani et al., 2001). This diagnostic produced a 184 bp PCR product with template DNA from An. minimus A, a 252 bp band for An. varuna, a 306 bp band for An. aconitus, a 346 bp band for An. jeyporiensis, a 452 bp band for An. pampanai and a 509 bp band for An. minimus C. This species- specific diagnostic assay produced significantly different size of products.

Two multiplex PCR was developed to identify the two sets of morphologically similar species of malaria mosquitoes incorporating species- specific primers derived from direct sequencing of the ITS2 region. One PCR reaction identified Anopheles oswaldoi and an undescribed species (species C) and second PCR reaction identified mosquitoes as belonging to a complex of four species, which includes An.deanorum, An.albitarsis A, An.albitarsis B and An.marajoara. (Corey and Brelsfoard et al., 2005)

The PCR-SSCP assay diffrentiate A. rivulorum, A. funestus, A. vaneedeni, and A. leesoni from each others (Hackett et al., 2000). However, this method fails to distinguish between A. vaneedeni and A. parensis. These two species are morphologically similar to A. funestus (Gillies et al., 1987). Hackett and others used the ITS2 PCR to distinguish A. funestus and A. rivulorum only.

Koekemoer et al (2002) identified five of most commonly found members of An.funestus group. such as A. funestus, A. vaneedeni, A.parensis, A.leesoni and A.rivulorum. Primers were designed using sequence of ITS2 fragments. After PCR production lengths of amplified species – specific products were verified such as A. leesoni ≈146 bp, A. funestus ≈505 bp, A. vaneedeni ≈587 bp, A. parensis ≈252 bp, and A. rivulorum ≈411 bp. Here, all six primers can be included in a cocktail PCR reaction mix simultaneously without any effect on amplification. Each unknown specimen can be identified without performing five separate PCR reactions.

rDNA-ITS2 PCR assay was developed to group the five species of Anopheles culicifacies complex into the two categories (A and D and B, C and E) without the need of an intermediate RFLP assay, thus reducing the number of steps involved in the available identification assay and significantly cutting down the time and cost. (Manonmani et al.,2007)

Anopheles gambiae sensu lato (s.l) species complex contains the most important mosquito vector of malaria in Africa. Fast identification of the two main species which vector malaria, Anopheles arabiensis and An. gambiae sensu stricto, from the non-vector species Anopheles quadriannulatus is frequently required as part of vector control programme. Taq-Man assay was developed that distinguishes between the main malarial vectors and non-vector. This method describes the rapid and sensitive assay that very effectively identifies the two main malaria vectors from the non-vector sibling species (Chris Bass et al. 2007). Moreover, Taq Man single nucleotide polymorphism genotyping proved for the identification of An, gambiae s.l and An.arabiensis with high success rate specific results (Edward D Walker et al, 2007)

Bass et al (2008) developed a new multiplex real time PCR assay for identifying members of An.gambiae complex. It uses three probes labelled with fluorophores with distinct emission and excitation spectra, allowing simultaneous detection of two main malaria vectors from the non- vector sibling species.
The TaqMan assay proved to be the most sensitive and specific for the identification of Anopheles parensis, Anopheles leesoni, Anopheles vaneedeni, Anopheles rivulorum and Anopheles funestus s.s. This assay very effectively identified all five members of the An.funestus group (Vezenegho et al, 2009).

IV. ALLELE- SPECIFIC PCR ASSAY (AS-PCR)

The generalization of partial or complete sequencing of many genomes allowed the development of identification assays based on a single step easier to implement. These assay named as allele- specific (AS-PCR) or PCR amplification of specific alleles (PASA) Anopheles fluviatilis, one of the major vectors of malaria in India, is a complex of at least three cryptic species provisionally designated as species S, T, and U. Species S, T, and U are morphologically indistinguishable at any stage of their life cycle and can be identified only by the examination of species-specific fixed inversions in the polytene chromosomes. An. fluviatilis complex were differentiated by an allele-specific polymerase chain reaction assay, which is based on differences in nucleotide sequences in D3 domain of 28S ribosomal DNA (Singh et. al., 2004 a). In addition, Singh et al. (2004b) developed an allele-specific polymerase chain reaction to the D3 domain of 28S rDNA that discriminated Anopheles culicifacies species complex. The assay was further validated using chromosomally identified specimens of Anopheles culicifacies from different geographical regions of India representing different sympatric associations.

Goswami et al. (2005) developed PCR-RFLP of mitochondrial COII and ITS2 of rDNA markers for the identification of members of Anopheles culicifacies complex. Goswami et al. (2006) conducted a study to identify all members of Anopheles culicifacies complex using allele specific polymerase chain reaction assays. The PCR assays developed from the D3 and ITS2 regions of rDNA failed to identify all the members of the Anopheles culicifacies complex while COII sequences showed single bp difference that have been used to differentiate all the members of this complex8

Anopheles culicifacies, a complex of five isomorphic sibling species, is a major vector of malaria in India and neighboring countries. The five species are provisionally designated as species A, B, C, D and E. Polymerase chain reaction & polymerase chain

reaction-restriction fragment length polymorphism can differentiate the species A and D from species B, C, and E. They reported allele-specific PCR assays is possible to identify individual specimens of any of the species of this complex (Geeta et al., 2006).

Distinction between members of An.nili group is difficult, mainly based on morphological characters. In order to achieve a quick and inexpensive tool for rapid identification of An.nili group is an allele specific PCR. That combine five primers were developed. ITS2 region was used as template to design diagnostic PCR. Resulting PCR products differ from one another by at least 30 bp. So that they easily separated on agarose gel. The size of the diagnostic band is 188 bp for the typical An.nili, 357bp for An.nili Oveng, 408 bp for An.carnevalei and 329 bp for An.somalicus (Kengne et al, 2003). Moreover, Anopheles moucheti (Evans) s.l is a major malaria vector in Africa. To differenciate the species, ITS2 of all An.moucheti s.l specimen was designed together with three reverse primers AMou, Aber and ANig respectively, specific to An.m.moucheti, An.m.bervoetsi and An.m.nigeriensis respectively. The size of the diagnostic band was 378 bp for An.m.bervoetsi, 312 bp for An.m.moucheti and 249 bp for An.m.nigeriensis based on this assay each subspecies within An.moucheti were identified (Kengne et al., 2007).

Sharpe et al. (1999) demonstrated that allele-specific amplification of the D3 variable region of the 28S rDNA gene distinguished An. minimus A rom C and single-strand conformation polymorphism (SSCP) of the D3 amplified region discriminates four species, An. varuna, An. aconitus, An. minimus A and C. Unfortunately, this method is more time consuming and the results less easy to interpret than those from conventional polymerase chain reaction. An alternative approach was adopted by Van Bortel et al. (2000) who used PCR amplification of the rDNA internal transcriber spacer 2 (ITS2) region followed by BsiZI restriction enzyme digestion to distinguish An. aconitus, An. jeyporiensis, An. minimus A and C, An. pampanai, An. varuna and subsequently An. culicifacies (Van Bortel et al., 2002).

Walton et al (1999) developed AS-PCR to identify the members of Anopheles dirus complex which is most important vectors of malaria. It identified as species A, B, C, D by the AS – PCR method.

V. SPECIES- SPECIFIC –PCR

Sibling species under A. gambiae complex were characterized by polymerase chain reaction using species specific single nucleotide polymorphism in the intergenic spacer region with primers specific for An. gambiae s.s., An. arabiensis, An. melas, An. merus and Anopheles quadriannulatus (Kabbale, 2016)

Six sibling species of the Anopheles maculipennis identified based on the second internal transcribed spacer of the ribosomal DNA. It was amplified and sequenced for all six species. Based on differences in the nucleotide sequences, species specific primers were constructed for PCR amplification of mosquito DNA that in combination with a universal primer generate amplification products of different length, each unique for one species (Proft et al., 1999).

Cohuet et. al (2003) differentiated the five sibling species of Anopheles funestus such as An. funestus, An. leesoni, An. parensis, An. vaneedenip and An. rivulorum. They used the species - specific PCR assay which is supplemented by a primer specific to An. rivulorum and thus makes it possible to differentiate the five species of the An. funestus group.

Anopheles funestus is a major vector of malaria in Africa. It belongs to a group of sibling species that can be identified morphologically only at certain stages of their development. A diagnostic polymerase chain reaction based tool made it possible to differentiate five species of the group. This assay seems to be relevant over all their distribution area for four of these species: An. funestus, An. leesoni, An. parensis, and An. vaneedenip. The fifth species, An. rivulorum, is the second most abundant species of the group. The species-specific PCR assay is supplemented by a primer specific to An. rivulorum-like and thus makes it possible to differentiate the five species of the An. funestus group and the newly defined taxon (Claire Garros et al., 2004).

A polymerase chain reaction based diagnostic assay was developed to differentiates the sibling species of Anopheles claviger complex, An. claviger s.s. and An.

petragnani. The assay facilitates differences in the internal transcribed spacer 2 ribosomal DNA sequences to generate PCR products of specific length for each of the two species. Based on the ITS2 sequences, species-specific primers were selected and it produced DNA fragments of 269 base pairs for An. claviger s.s. and 367 base pairs for An. petragnani when used in combination with the 5.8S primer. Helge kampen et.al., (2003) designed specific primers for two sibling species and also they tested PCR conditions for yield of specific DNA fragments.

Manonmani et al. (2001) used species-specific differences in the nucleotide sequences of rDNA ITS2 region to develop a diagnostic PCR assay for two sibling species of the Anopheles fluviatilis complex, members of which are major vectors of malaria in Central and Northern parts of India.

VI. RFLP – PCR ASSAY DIAGNOSTIC

Diverse regions can be used to devise DNA hybridization or restriction fragment length polymorphism (RFLP) assays (Collins et al, 1987, Beebe & Saul, 1995). In molecular biology, RFLP is a technique that exploits variations in homologous DNA sequences. It refers to any variation in DNA between individuals revealed by restriction enzymes of lengths in consequence of such variations. In RFLP analysis, DNA sample is digested into pieces by restriction enzymes and resulting restriction fragments are separated according to their lengths by gel electrophoresis. RFLP analysis is also an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease and paternity testing

Sibling species of the Anopheles subpictus complex in Sri Lanka were identified by using a Restriction Fragment Length Polymorphism – Polymerase Chain Reaction assay based on the variations in the Internal Transcribed Spacer (ITS2) region. PCR assay was done with the available common forward primer ITS-2A (5' - TGT GAA CTG CAG GAC ACA T- 3') and species-specific reverse primers ITS-2B (5'- TAT GCT TAA ATT CAG GGG GT - 3') in order to amplify the ITS2 region**.** An analytical restriction digestion was performed by using NCo 1 restriction digestion enzyme. The RFLP – PCR assay define two different molecular forms among the complex. One; species A, form comprise of morphologically different species A, B and C and the other; species B, consists of morphologically different species D. (Kothai, 2016)

Morphological and molecular identification was found for species An. albimanus, An. aquasalis, An. darlingi and An. triannulatuss.l. However, disagreement was found for species An. nuneztovaris.l, An. neomaculipalpus, An. apicimacula and An. punctimacula. The ITS2-PCR-RFLP assay proved valuable for discriminating species of northern and western Colombia, especially those with overlapping morphology in the Oswaldoi Group (Cienfuegos et al., 2011).

Anopheles longipalpis is morphologically similar to the major African malaria vector Anopheles funestus at the adult stage although it is very different at the larval stage. Six species (An. funestus, An. funestus-like, An. parensis, Anopheles rivulorum, An. vaneedeni and An. leesoni) in An. funestus group and An. longipalpis type C were subjected to RFLP assay. The enzyme, EcoRI digested only An. longipalpis type C and An. funestus-like after species-specific An. funestus group PCR assay. The An. longipalpis and An. funestus-like digestion profiles were characterized by three fragments, 376 bp, 252 bp and 211 bp for An. longipalpis type C and two fragments, 375 bp and 15 bp for An. funestus-like. (Choi et al., 2010)

The Anopheles annular is a group of mosquitoes distributed in Southeast Asia. Four members of this group are morphologically very similar and often difficult to distinguish. Comparison of the D3 sequences of the four species revealed two SmaI restriction sites in A. nivipes, but only one site in A. philippinensis, A. annularis and A. pallidus. The ApaI site was present in both A. philippinensis and A. pallidus, while an NcoI site was present in A. pallidus only. Restriction digestion of PCR products of D3 fragment individually with SmaI, ApaI and NcoI produced a distinctive pattern for all four species. They presented, a PCR-RFLP method to distinguish the four members of the A. annularis group of mosquitoes (Mohammad Tauqeer Alam et al., 2007a).

Anopheles minimus s.l., one of the most widespread malaria vectors in South East Asia. It is a complex of

at least two isomorphic species and based on morphology, members of the complex are difficult to distinguish from closely related species. An identification method was developed for An. minimus species A and C and four related species, An. aconitus, An. pampanai, An. varuna and An. jeyporiensis. The entire ITS2 rDNA fragments, including partial sequence of 5.8S and 28S of six identified species were sequenced. The fragments exhibited distinct differences between species and based on these sequences the restriction endonuclease BsiZI was used and it distinguishes the all six species. However, it did not give proper results for An. pampanai. Moreover, MspI also used for identification of An. aconitus, An. varuna and An. Jeyporiensis. But it falis to discriminate the members of the An. minimus species complex (Van Bortel et.al., 2000).

PCR-RFLP-ITS2 assay performed with AluI, FspI or DraIII is useful for identification of species belonging to the Oswaldoi Group of Anopheles subgenus. As such, amplified ITS2 products were selected for the enzyme restriction analysis and digested. In addition to single restriction digests, double restriction digests with AluI/FspI were used to discriminate An. nuneztovari s.l. from An. benarrochi s.l. (Cienfuegos et al., 2011, Matson et al., 2008).

VII. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

The randomly amplified polymorphic DNA- PCR (RAPD-PCR) which was developed by Williams et al. in 1990 belongs to the first category. Here, arbitrary regions of the genome are amplified using a single decider primer. The assay based on species specific differences in hyper variable regions of the genome in all species.

Favia et al (1994) examined RAPD which is rapid and reliable tool for genome analysis in the malaria vector of Anopheles gambiae. This technique identified the different mosquito strains of An. gambiae s.s. and An. arabiensis.

Anopheles culicifacies is a complex of 5 isomorphic types A, B, C, D and E in India with varying biological characteristics. Varun et al developed RAPD markers that could differentiate all five sibling species of An. culicifacies in India. A number of RAPD primers were screened and genetic fingerprints for all 5 sibling species of An. culicifacies were developed using a selected primer. A total of 34 loci representing these sibling species using selected primer were polymorphic and able to differentiate the sibling species and showed 100% polymorphism (Varun et al., 2015)

Wilkerson et al. (1993) differentiated the two morphologically indistinguishable taxa within Anopheles gambiae complex, An.gambiae Giles and An.arabiensis Patton. Formerly, these two taxa have been distinguished by polythene chromosome banding. But RAPD analysis proved as more efficient tool in discrimination of two species of An.gambiae and An.arabiensis.

Kengne et al. (2001) described use of random amplified polymorphic DNA markers for the identification of species in the An. minimus group. RAPD fragments specific for species A and C were cloned and sequenced. From these sequences, specific primer pairs were designed to carry out multiplex PCR able to identify five species, An.minimus A and C, An. aconitus, An. pampanai and An. varuna. In this assay, PCR products from hybrids of An. minimus A and C were different from both parents.

VIII. DNA – BARCODING

DNA barcoding is a molecular method that is increasingly become a popular for the identification of animal species based on the partial mitochondrial DNA sequences (Hebert et al., 2003).

Weeraratne et al. (2017) described the identification of Anopheleline species mosquitoes based on the DNA barcoding technique which are major mosquitoes group of interest in Sri Lanka. 15 species of mosquitoes species were collected such as Anopheles aconitus, Anopheles annularis, Anopheles barbirostris, Anopheles culicifacies, Anopheles jamesii, Anopheles karwari, Anopheles maculatus, Anopheles nigerrimus, Anopheles pallidus, Anopheles peditaeniatus, Anopheles pseudojamesi, Anopheles subpictus, Anopheles tessellatus, Anopheles vagus, and Anopheles varuna. This study reflects the

importance and feasibility of COI and ITS2 genetic markers in identifying anophelines and their sibling species.

Rubio et al. (2016) distinguished the An.neivai from sympatric species and indicates genetic variability within the species through DNA barcoding techniques.

In Abigal chan et al (2014) they explored the applicability of mitochondrial cytochrome C oxidase subunit I (COI) gene based DNA barcoding as an alternative tool to identify the mosquito species. COI based DNA barcoding achieved a 100% of success rate in identifying the mosquito species of Aedes aegypti, Anopheles sinensis, Culex vishnui and Culex mimulus. COI based DNA barcoding is a useful tool for identification of mosquito species.

Prakash et al. (2006) identified members of the Anopheles minimus and Anopheles dirus complexes from north eastern region of India using sequences for the ITS2 of rDNA. They differentiated Anopheles minimus (species A) of the Anopheles minimus complex and Anopheles baimaii (species D) of the Anopheles dirus complex from Arunachal Pradesh, Assam, Meghalaya and Nagaland. Alam et al. (2006) reported sequence analysis of rDNA ITS2 and D3 regions of the four members of Anopheles annularis group- Anopheles nivipes, Anopheles philippinensis, Anopheles annularis and Anopheles pallidus for their molecular identification from Andaman and Nicobar Islands. They found that there was no intraspecific sequence variation among the specimens and interspecific sequence differences were greater for ITS2 than the D3 regions

Singh et al. (2006) examined the conspecificity of Anopheles fluviatilis S and Anopheles minimus C by analyzing the DNA sequences of nuclear ribosomal ITS2 and D2-D3 domain of 28S rDNA.

Kumar et al. (2007) studied DNA barcodes for several species of mosquitoes belonging to 15 genera, prevalent in India which included major vector species. However, two closely related species, Ochlerotatus portonovoensis and Ochlerotatus wardi could not be identified as separate species based on DNA barcode approach as their lineages indicated negligible genetic divergence. Alam et al. (2007a) reported the sequence analysis and a method for their molecular identification of the rDNA ITS2 and D3 regions of the four members of the Anopheles annularis group- Anopheles nivipes, Anopheles philippinensis, Anopheles annularis and Anopheles pallidus from Assam and Andaman and Nicobar Islands. Alam et al. (2007b) analyzed the distribution of Anopheles annularis complex collected from Sonapur (Assam), Jabalpur (Madhya Pradesh), Ranchi (Jharkhand), and Ghaziabad (Uttar Pradesh) and developed a molecular method using the rDNA ITS2 and Domain 3 for identification purposes.

Alam et al. (2008) described the sequencing of rDNA ITS2 and D3 loci of Anopheles stephensi stephensi (type form) and Anopheles stephensi mysorensis. They also revealed that these populations showed identical sequences at both rDNA loci.

CONCLUSION

The study of Anopheles taxonomy is greatly benefits from the powerful information provided by DNA sequences. The identification and detection of Anopheles species specially sibling species are readily discriminated by use of molecular identification assays. All species of Anopheles mosquitoes have examined having species-specific sequences that can form the basis of the diagnostic assays.

The present review reveals that morphologically identified Anopheles complex could be distinguished easily by different molecular techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP). DNA barcoding, Polymerase chain reaction, Allele-Specific PCR and DNA – Barcoding method. And also it minimizes the time and money in the laboratory and also ensure the data reliability. In addition, it minimizes time and ensuring more reliability in molecular characterization.

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