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Restriction Site Polymorphism of mtDNA for Differentiating *Anopheles quadrimaculatus* (Say) Sibling Species

미토콘드리아 DNA 제한효소 절단부위 변이에 의한 *Anopheles quadrimaculatus* (Say) 모기의 자매종 구별

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ABSTRACT Three mitochondrial cDNA probes from *Aedes albopictus* were used to demonstrate restriction site polymorphism in mtDNA of three sibling species of *Anopheles quadrimaculatus*(Say). It was shown by DNA hybridization to have substantial sequence homology between the mtDNA of different genus. The probes revealed local restriction site variation between members of the *Anopheles quadrimaculatus* sibling species complex. Mitochondrial DNA (mtDNA), isolated from individual mosquitoes was digested by type Ⅱ restriction enzymes and four enzymes were found to be useful for the purpose. Hind Ⅲ alone could be used to obtain a diagnostic restriction pattern.

KEY WORDS *Anopheles quadrimaculatus*, mtDNA, sibling species, restriction site polymorphism

초 록 *Anopheles quadrimaculatus*(Say) 姉妹種 間의 미토콘드리아 DNA의 제한효소 절단부위변이를 *Aedes albopictus*의 미토콘드리아 cDNA를 probe로 이용하여 조사하였다. DNA hybridization에 의해 두 屬間에는 mtDNA 염기서열의 상당한 相同性이 있음을 알 수 있었다. 개체 모기로부터 분리한 DNA를 제한효소를 사용하여 절단한 결과 자매종간에 다른 양상을 볼 수 있었으며 Hind Ⅲ에 의한 mtDNA 절편만으로도 자매종들을 동정할 수 있었다.

검 색 어 *Anopheles quadrimaculatus*, 미도콘드리아 DNA, 자매종, 제한요소 절편

Anopheles quadrimaculatus (Say) is widely distributed over the eastern United States. Cytogenetic and hybridization studies, so far, revealed the presence of three sympatric sibling species (Kaiser et al. 1988, Lanzaro et al. 1988). Cytological analysis of hybrids revealed fixed and floating inversions which could be used as a diagnostic tool for differentiating species A and B. A third sibling species, C, was identified in sympatry with species A. Cross matings between this species with A and with B resulted in either sterile hybrids or sterile backcross progeny (Kaiser et al. 1989). The ovarian polytene chromosomes of species C had indistinct, diffuse bands unsuitable for comparison with species A and B. Narang et al. (1989) compared genetic differentiation among the sibling species and identified them by an electrophoretic taxonomic key. However, these methods are time consuming and requires much work and experience. Meanwhile, polymorphism of cleavage sites in mitochondrial DNA has

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Table 1. Restriction fragment profiles for mtDNA or *Anopheles quadrimaculatus* sibling species probe hybridized with mitochondrial cDNA from *Aedes albopictus*

Enzymes	Fragment size(kbp)		
	Species A	Species B	Species C
Hindi Ⅲ	6.1, 2.6, 2.1	6.1, 2.2, 2.1	4.5, 2.6, 2.1
EcoR I	6.1, 3.4	6.1, 3.4	6.1, 4.7
Xho I	13.8, 3.3, 2.5	13.8, 3.3	−
Bgl Ⅱ	14.3, 2.6	14.3	−

been recognized as a powerful tool in insect systematics especially for high−resolution analysis of evolutionary process (Shah & Langley 1979, Latorre et al. 1986, Hale & Singh 1986, Harrison et al. 1985, Trick & Dover 1984, Moritz et al. 1986). In this paper, we report mtDNA restriction site polymorphism in the three sibling species of *An. quadrimaculatus* which can be used in differentiating them.

MATERIALS AND METHODS

Sibling Species Cytogentic and hybridization studies of isofemale lines established from the progeny of wild females led to the identification of sibling species A, B, and C (Kaiser et al. 1988, Kaiser et al. 1989). Adults of both sexes were frozen at −70℃ until used for total DNA preparation. Species A and B were originated from Kanapaha Botanical Garden, Alachua County and species C was from Shell Mound, Levy County, Florida.

DNA Sample from Individual Mosquito Single mosquito in an eppendorf tube was homogenized in 60 μℓ homo. buffer (0.1M NaCl, 0.2M sucrose, 0.01 M EDTA, 0.03 M Tris−HCl, pH 8.0) with a motor driven pestle to which 30 μℓ lysis buffer (0.25M EDTA, 2.5%(w/w) SDS, 0.5 M Tris−HCl, pH 9.2) was added after homogenization. Incubation was done for 40 minutes at 55℃. The tube was placed on ice for 1 hour after adding 50 μℓ KAc(8M) to the homogenate and then was centrifuged at 4℃ at 12,000 × g for 15 minutes. The supernate was transferred into a new tube and total DNA was ethanol precipitated.

Mitochondrial DNA Isolation Mitochondrial DNA was isolated from approximately 15 g of sibling species A pupae. Anopheline pupae have much less anthraquinone pigment than adults and give cleaner preparations. The pupae were homogenized in a Dounce tissue homogenizer for a few strokes in 38 mℓ cold MIM buffer (0.01M EDTA, 0.25 M sucrose, 0.03 M Tris−HCl pH 6.0). The homogenate was centrifuged at 1,200 × g for 15minutes at 4℃, and the supernate was then contrifuged at 20,000 × g for

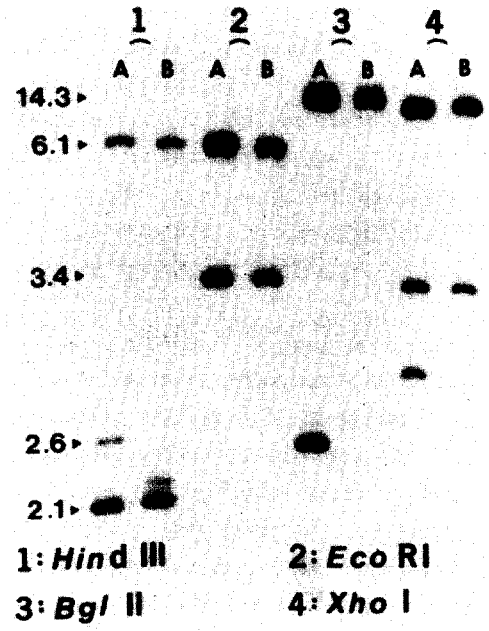

Fig. 1. Autoradiograph of restriction enzyme digested mtDNA of *Anopheles quadrimaculatus* sibling species A and B, fractionated on 0.8% agarose, transferred to nitrocellulose, and hybridized with mitochondrial cDNA probes from *Aedes albopictus*.

20 minutes. The pellet was resuspended to a total volume of 2.5 mℓ STE(0.1M NaCl, 0.01 M EDTA, 0.05M Tris pH 8.5) and mixed with an equal volume of 20% sucrose. This was layered on top of a step gradient made up of 15 mℓ 1.5M sucrose, 0.01M Tris(pH 7.5), and 0.005 M EDTA for the bottom, and 15mℓ 1.0M sucrose, 0.01M Tris(pH 7.5), and 0.005M EDTA for the top. The gradient was spun at 100,000 × g for 20 minutes at 4℃. Mitochondria were isolated from the interface, diluted three-fold with 0.01 M Tris(pH 8.0), and 0.001M EDTA, and centrifuged at 20,000 × g for 20 minutes. The pel-let was resuspended and brought up to 12.5 mℓ with STE,. 15.2 g CsCl and 0.4 mℓ (10 mg /mℓ) ethidium bromide were added, and the mixture was centrifuged to equilibrium (40h; 45,000 rpm;Ti 70.1 rotor) in a Beckman L8-70M ultracentrifuge. Supercoiled mtDNA band was visualized by fluorescence under UV light at 305 nm and removed with a needle. Ethidium bromide was removed by repeated extractions with 1-butanol and CsCl by dialysis against TE. The DNA was precipitated with 1/2 volume of 7.5M ammonium acetate and a 2-fold volume of 100% ethanol, and pelleted. The pellet was vacuum dried and resuspended in TE.

Probe hybridization mtDNAs from species A and B and total DNAs from individual mosquitoes were limit-digested with restriction enzymes purchased from commercial suppliers and used in accordance with the manufacturer's recommendations. Digests were electrophoresed on horizontal agarose gels submerged in 1X TBE buffer and transferred to nitrocellulose filters according to Southern(1975) after an initial depurination step. Mitochondrial cDNA probes from *Aedes albopictus* genome were kind gifts from Dr. D. T. Dubin;K8, -2.8 kbp;K14, -1.9kbp;K50, -2.5 kbp. Plasmids were pooled and nick-translated with [^{32}P]dATP. Hybridizations were conducted at 68℃ overnight, with approximately 10^7 dpm activity of probe in 30% formamide, 5X SSPE, and 2% SDS, Filters were washed four times at room temperature in 2X SSPE with 0.1% SDS for 15 min., dried, and autoradiographed at -70℃ with Kodak X-Omat R film using intensifying screens.

RESULTS AND DISCUSSION

It proved possible to demonstrate unique restriction patterns of mtDNA by hybridizing purified mtDNA digests of species A and B with cloned probes of closely related *Ae. albopictus* mtDNA(Fig. 1). Among the restriction endonucleases, Hind Ⅲ, Xho Ⅰ, and Bgl Ⅱ produced unique fragment patterns. Although the probes were not from any of the 3 sibling species and did not cover the whole mtDNA genome, the total size of the hybridized fragments resulted from each endonuclease digests was big enough to show the feasibility of using those probes for establishing restriction polymorphisms without a laborious cloning procedure of mtDNA of interest. This indicates a relative homology between mtDNA genomes of *Anopheles and Aedes*. It is the number of restriction sites in homologous regions that concerns-rather than the size of probes itself. Trick and Dover (1984) used an approximately 750bp-long Mbo Ⅰ fragment of *Glossina morsitans morsitans* DNA for demonstrating Hinf Ⅰ restriction polymorphism of several *G. morsitans* subspecies mtDNA.

Using the above three restriction enzymes and EcoR Ⅰ, significant differences in probe-hybridized fragment patterns among the three species were observed. All four enzymes were useful for differentiating the sibling species, however, Hind Ⅲ could alone produce autoradiographic banding patterns diagnostic to each sibling species (Table 1). The nature of

the probe used in this study and the sample size were not sufficient to describe and analyze the full genetic variability among the sibling species. However, our results showed large genetic variability which was unlikely, considering that the three species were sympatric and that the relative size of the probes.

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