in Amazon, it was reported that the highly dissected terrain results in restricted gene flow over short distances, whereas dissection of the terrain[6] and elevation gradient together lead to variable climatic patterns resulting in different selective regimes[4].

1. Royle, J. F., *Illustrations of Botany and other Branches of the Natural History of the Himalayan Mountains and of the Flora of Cashmere. Vol. 1*, Todays and Tomorrows Printers and Publishers, Delhi, 1839 (repr. 1990), pp. 331–333.
2. Hooker, J. D., *The Flora of British India*, Todays and Tomorrows Printers and Publishers, New Delhi, 1886.
3. Purseglove, J. W., Brown, E. G., Green, C. L. and Robbins, S. R. J., *Spices*, Longman, London, 1981, vol. 1.
4. Gentry, A. H., Floristic similarities and differences between southern Central America and upper Central Amazonia. In *Four Neotropical Rainforests* (ed. Gentry, A. H.), Yale University Press, New Haven, Connecticut, 1990, pp. 141–157.
5. Gamble, J. S., *Piperaceae*. In *Flora of the Presidency of Madras*, London, 1925, pp. 1202–1210.
6. Marquis, R. J., Biogeography of neotropical *Piper*. In *Piper: A Model Genus for Studies of Phytochemistry, Ecology and Evolution* (eds Dyer, L. A. and Palmer, A. D. N.), Kluwer Academic/Plenum Publishers, New York, 2004, pp. 78–96.
7. Ravindran, P. N., Balakrishnan, R. and Nirmal Babu, K., Morphometrical studies on black pepper (*Piper nigrum* L.) II. Principal component analysis of black pepper cultivars. *J. Spices Aromat. Crops*, 1997, **6**, 21–29.
8. Mathew, P. J., Mathew, P. M. and Kumar, V., Graph clustering of *Piper nigrum* L. (black pepper). *Euphytica*, 2001, **118**, 257–264.
9. Mathew, P. J., Jose, J. C., Nair, G. M., Mathew, P. M. and Kumar, V., Assessment and conservation of intraspecific variability in *Piper nigrum* (black pepper) occurring in the Western Ghats of Indian Peninsula. *Acta Hortic.*, 2005, **676**, 119–126.
10. Hijmans, R. J. and Spooner, D. M., Geographic distribution of wild potato species. *Am. J. Bot.*, 2001, **88**, 2101–2112.
11. Ganeshaiah, K. N., Narayani Barve, Nilim Nath, Chandrashekara, K., Swamy, M. and Uma Shaanker, R., Predicting the potential geographical distribution of the sugarcane woolly aphid using GARP and DIVA-GIS. *Curr. Sci.*, 2003, **85**, 1526–1528.
12. Hijmans, R. J., Guarino, L., Cruz, M., Jarvis, A., O'Brien, R., Bussink, C. and Mathur, P., DIVA-GIS version 5.1. A geographic information system for the management and analysis of genetic resources data. Manual, International Potato Center and International Plant Genetic Resources Research Institute, Lima, Peru. 2005.
13. Jaramillo, M. J. and Callejas, R., Current perspectives on the classification and phylogenetics of the genus *Piper* L. In *Piper: A Model Genus for Studies of Phytochemistry, Ecology and Evolution* (eds Dyer, L. A. and Palmer, A. D. N.), Kluwer Academic/Plenum Publishers, New York, 2004, pp. 179–198.
14. Ravindran, P. N., Balakrishnan, R. and Nirmal Babu, K., Numerical taxonomy of South Indian *Piper* L. (*Piperaceae*) I. Cluster analysis. *Rheedea*, 1992, **2**, 55–61.
15. Murthy, Y. S., Studies in the order Piperales IV. A. Contributions to the study of vegetative anatomy of three species of *Piper*. *Proc. Natl. Inst. Sci. India*, 1973, **25**, 31–38.
16. Rahiman, B. A. and Bhagawan, S., Analysis of divergence in eight species of *Piper* using D^2-statistics. *Bot. Bull. Acad. Sin.*, 1995, **26**, 39–45.
17. Ravindran, P. N., Asokan Nair, R., Nirmal Babu, K., Chandran, K. and Nair, M. K., Ecological and morphological notes on *Piper* species from the Silent valley forest, Kerala. *J. Bombay Nat. Hist. Soc.*, 1990, **87**, 421–426.
18. Gaston, K. J., Species richness: measure and measurement. In *Biodiversity, a Biology of Numbers and Difference* (ed. Gaston, K. J.), Blackwell Science, London, UK, 1996, pp. 202–229.
19. Hijmans, R. J., Garret, K. A., Huaman, Z., Zhang, D. P., Schreuder, M. and Bonierbale, M., Assessing the geographic representativeness of genebank collections: the case of Bolivian wild potatoes. *Conserv. Biol.*, 2000, **14**, 1755–1765.
20. Ravindran, P. N., Balakrishnan, R. and Nirmal Babu, K., Morphometrical studies on black pepper (*Piper nigrum* L.). Cluster analysis of black pepper cultivars. *J. Spices Aromat. Crops*, 1997, **6**, 9–20.

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kdr allele-based PCR assay for detection of resistance to DDT in *Anopheles culicifacies* sensu lato Giles population from Malkangiri District, Orissa, India

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Knock-down resistance in *Anopheles culicifacies* based on region II of the para-type sodium channel gene was detected by PCR assay. Diagnostic PCR assay developed in *Anopheles gambiae* based on region II of the para-type sodium channel for detecting *kdr* resistance was optimized and tested for its utility in detecting DDT susceptible, heterozygous and resistant *An. culicifacies* mosquito population from Malkangiri area in Orissa, India. Four primers were utilized, among which two (Agd1 and Agd2) were basically designed from region II of the para-type sodium channel gene of *An. gambiae* and the other two (AcAgd3 and AcAgd4) redesigned from the same region of *An. culicifacies*. The primer sets could amplify the fragments of 137 and 196 bp respectively, for homozygous susceptible and resistant genotypes and also heterozygous genotypes in *An. culicifacies*. Among *An. culicifacies* that survived DDT exposure, the homozygous and heterozygous resistance allele frequencies were 0.8 and 0.03 respectively. The result of the present study thus showed that this assay was capable of determining *kdr* genotypes in a single mosquito at this locus and has potential in detecting *kdr* resistance among *An. culicifacies* populations.

Keywords: *Anopheles culicifacies*, knock-down resistance, para-type sodium channel gene, PCR assay.

THE problem of insecticide resistance is the result of excessive and indiscriminate use of several insecticides, includ-

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ing DDT for vector control during the Second World War and thereafter. Extension of resistance from DDT to other chlorinated hydrocarbons rendered the newer insecticides ineffective for vector control. Pyrethroid insecticides are now being used on a larger scale in agriculture and for impregnating bed nets for malaria control, as they act on the nervous system by modifying the gating kinetics of voltage-sensitive sodium channels[1]. In recent times, control of resurgent malaria has become a formidable task in view of the factors considered to be impeding malaria control efforts in India, which include vector resistance to insecticides, particularly pyrethroids. Pyrethroid resistance is characterized by a marked reduction in the intrinsic sensitivity of the insect nervous system to these insecticides and is associated with the mutation with a para-type gene of *Anopheles gambiae*[2]. It confers resistance not only to pyrethroids but also to DDT, which shares a similar mode of action[3]. Permethrin/DDT resistance has been documented for several malaria vector mosquitoes, viz. species of the *An. gambiae* Giles complex and *An. funestus* in African countries; *An. albimanus* in Central America, *An. Sacharovi* in Turkey and *An. culicifacies, An. stephensi, An. annularis, An. subpictus* in India[4,5].

An. culicifacies sensu lato Giles is the principal vector of malaria in vast rural areas of the Indian subcontinent and about 70% of the Indian malaria cases are attributed to transmission by this taxon[6]. Hence, control of *An. culicifacies* has been the major concern of the National Anti-Malaria Programme. Resistance of *An. culicifacies* to DDT and its analogues has been reported from Delhi[7], Maharashtra[8], Uttar Pradesh[9], Bihar[10], Rajasthan[11], Haryana[12], Orissa[13] Gujarat[4] and many other parts of the country. Monitoring DDT/pyrethroid resistance needs to be strengthened as an integral component in vector control. Early detection of resistance is important to formulate a suitable management strategy that can be introduced at the appropriate time.

More recently, biochemical and molecular biological assays have been developed to detect resistance mechanisms in individual mosquitos[14]. However, biochemical assays have limitations such as the use of fresh samples and the need for a cold chain to transport samples to the Central laboratories. Polymerase chain reaction (PCR) assays offer solutions to these problems, apart from being highly sensitive and specific. Mosquito samples for the PCR assay can be dried and sent to any laboratory through regular mail from distant places[15]. A PCR assay was recently developed in *An. gambiae* to detect the susceptibility and resistance alleles mediated by a mutation in region II of the para-type sodium channel gene, which showed that resistance is conferred by DDT/pyrethroid target insensitivity[2]. This assay has now been validated on a few mosquito species in South Africa and elsewhere[16]. In the present study, we have optimized this PCR assay by redesigning the primers specific to *An. culicifacies* and evaluated its potential in detecting DDT susceptible, resistant and heterozygous *An. culicifacies* population from villages of Malkangiri District, Orissa, India.

Indoor resting collections were made in human dwellings and cattle sheds in Totaguda, Hatiamba and Mantriamba villages of Malkangiri District, between 6.00 and 8.00 h using aspirator and torches, and from the total anophelines, *An. culicifacies* were separated. Fully fed *An. culicifacies* females were used for the test. The insecticide susceptibility tests were carried out following the standard procedure[17] against the diagnostic dose of DDT (4%). Insecticide-impregnated papers used in this study were prepared at VCRC, Pondicherry following the standard procedure[18]. Ten to twenty *An. culicifacies* were exposed with triplicates for 1 h to the diagnostic dose of DDT. Controls in duplicates were exposed to olive-oil paper simultaneously. The proportion of mosquitoes dead/knocked-down and those that survived within 24 h of post exposure holding period was taken as indicator of susceptible and resistant mosquitoes respectively[17]. The dead and live mosquitoes were collected and dried in 1.5 ml microfuge tube at 95°C in a dry bath for 3 h and utilized individually for the PCR assay.

Extraction of genomic DNA from a single mosquito and subsequent diagnostic PCR assay were carried out using the method of Martinez-Torres *et al.*[2] with some modifications. Among the four primers Agd1, Agd2, Agd3 and Agd4, two primers (Agd1 and Agd2) that asymmetrically flank the region of the mutation were used as such. While comparing the sequences of the region of the sodium channel of *An. gambiae* with *An. culicifacies*, mismatches were observed in the sequences of the primers Agd3 and Agd4 regions (Figure 1). Therefore, primers Agd3 and Agd4 were redesigned based on the sequences that have been determined for *An. culicifacies* and deposited in GenBank (accession no. AY342398) and designated as AcAgd3 and AcAgd4. The nucleotide sequence of each primer is as follows:

An. gambiae:
5′-AAT-TTGCATTACTTACGACA-3′...**Agd3**
An. culicifacies:
5′-ACT**G**CTA**GG**TTACTTACGACA-3′...**AcAgd3**
An. gambiae:
5′-C**T**GTAGTGATAGGAAATTTA-3′...**Agd4**
An. culicifacies:
5′-CA**G**TAGTGATAGGAAATTTA-3′...**AcAgd4**

Two microlitres of extracted DNA was added to the master mix containing 3.0 µl 10X buffer, 1.5 µl of 1.0 mM $MgCl_2$, 2 µl to 10 mM dNTPS, 1.0 µl of 0.3 pM each of Agd1, Agd2 (ref. 2) AcAgd3 and AcAgd4 (redesigned) and 4 units of Taq polymerase (Finnzymes, Finland) and the volume of the reaction mix was made up to 30 µl by adding double-distilled water. The PCR reaction conditions were standardized at 94°C for 5 min followed by 94°C for 1 min, 48°C for 2 min, 72°C for 1.5 min for 30 cycles with the final extension of 72°C for 10 min. The amplified fragments

were analysed using a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. Primers Agd1 and Agd2 amplify a 293 bp fragment common to all genotypes as internal control. Within this fragment are the 196 bp fragments amplified by Agd1 and AcAgd3 in resistant insects and the 137 bp fragment amplified by Agd2 and AcAgd4 in susceptible insects. Presence of all these three bands indicated heterozygote.

The response of the mosquitoes (knock-down/mortality) to DDT (4%) exposure obtained using the WHO susceptibility test (bioassay) was grouped and analysed under two categories. Category I included mosquitoes that were knocked-down/dead within 24 h post-exposure holding period, and category II comprised of mosquitoes that survived beyond 24 h post-exposure (Table 1). Based on the knock-down and mortality observed by the conventional method, 12 mosquitoes under category I were considered to be susceptible whereas 76 under category II were resistant.

Genomic DNA of every individual mosquito that was knocked-down/dead as well as those alive after 24 h in the bioassay was extracted and subjected to PCR assay. Of the 12 mosquito samples under category I, seven were homozygous susceptible genotypes showing amplification of 137 bp (for susceptibility) with Agd2 and AcAgd4 primers as also the control band of 293 bp. Under category II, 53 mosquitoes showed amplification of 196 bp fragment with primers Agd1 and AcAgd3, indicating a high frequency (0.8) of homozygous resistant mosquitoes. All possible genotypes visualized electrophoretically are shown in Figure 2. Further analysis of the overall results showed that of the total 88 mosquitoes exposed to bioassay test, as many as 76 survived exposure to DDT indicating resistance. This is also reflected in the PCR assay, wherein higher frequency of *kdr* alleles (0.8) was obtained. However, some bioassay survivors at a frequency of 0.17 showed amplification of 137 bp fragment, indicative of homozygous susceptible genotype. Similar observation upon PCR analysis has also been reported in *An. gambiae* (25% in CIG strain) by Brooke *et al.*[16], who have suggested that some other mechanism of resistance other than *kdr* may also be operational. In this study some non-specific bands of higher molecular weight were obtained (Figure 2) and such non-specific amplification has also been documented in other mosquitoes[16]. This PCR assay detected two heterozygous-resistant mosquitoes, which is an additional advantageous feature of molecular tools over conventional bioassay tests. Molecular tools are, therefore, efficient in picking up very low frequencies of resistant genes, which are mainly present in heterozygous form and resistance management can be implemented at an early stage, if such genotypes are detected. However, some 14% samples did not show any meaningful amplification, which could be due to the poor quality of mosquitoes (such as

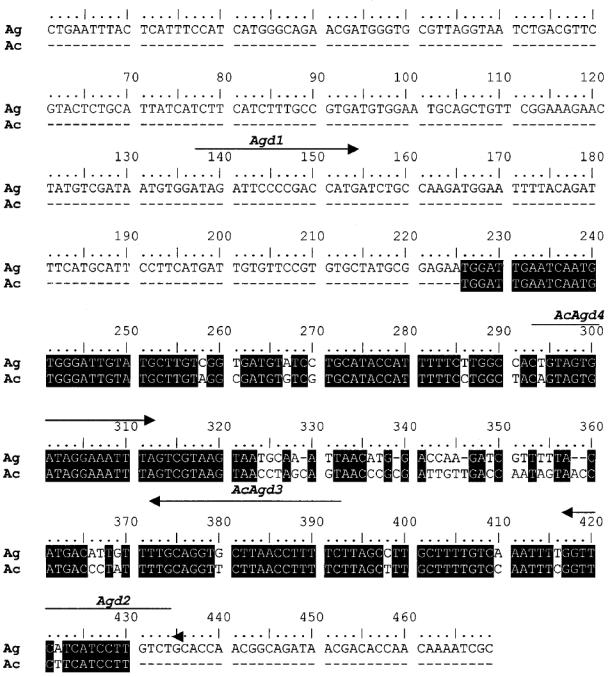

Figure 1. ClustalW alignment of nucleotide sequence of *Anopheles gambiae* Giles (accession no. Y13592) and *An. culicifacies* (accession. no. AY 342398) retrieved from GenBank. The box shade format indicates identical residues with black background. Location of primers sequences Agd1, Agd2, AcAgd3 and AcAgd4 is underlined. Ag, *An. gambiae*; Ac, *An. culicifacies.*

Table 1. Phenotype data using WHO susceptibility test and genotypes as assessed by PCR assay

	PCR assay				
WHO bioassay test	Total	SS	SR	RR	Neg
Dead/knocked-down	12	7	1	1	3
Survivors	76	12	2	53	9

Genotypes: SS, Homozygous susceptible; RR, Homozygous resistant; SR, heterozygous and Neg, PCR negative without amplification of diagnostic bands.

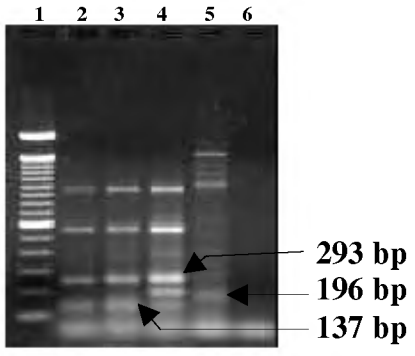

Figure 2. Amplification of diagnostic fragments of three *kdr* genotypes in *An. culicifacies* exposed to DDT (4%). Lane 1, 100 bp molecular weight marker; lanes 2, 3, Homozygous susceptible; lane 4, Heterozygous; lane 5, Homozygous resistant and lane 6, Negative control.

decayed and fungus-rotten) or other reasons that need to be investigated further.

In the present study, we have optimized reaction conditions through few modifications, including redesigning the primers (Agd3 and Agd4) used in the diagnostic PCR assay of Martinez-Torres *et al.*[2]. Primers specific to *An. culicifacies* were designed (AcAgd3 and AcAgd4) based on the mismatches observed in the Agd3 and Agd4 region of the para-type sodium channel gene sequence of *An. culicifacies* in comparison with that of *An. gambiae*. This PCR assay has potential in the detection of *kdr* resistance among *An. culicifacies* populations in India, but it needs to be further tested on a larger number of samples against each species of *Anopheles*. Since *kdr* mechanism of DDT resistance appears to be operating only among a part of the population, there is a need to understand other mechanisms conferring pyrethroid/DDT resistance among them and develop multiplex PCR assays.

1. Lund, A. E. and Narahashi, T., Kinetics of sodium channel modification as the basis for the variation in the the nerve membrane effects of pyrethroid and DDT analogs. *Pestic. Biochem. Physiol.*, **20**, 203–216.
2. Martinez-Torres *et al.*, Molecular characterization of pyrethroid knock down resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. *Insect Mol. Biol.*, 1998, **7**, 179–184.
3. Prasittisuk, C. and Curtis, C. F., Further study of DDT resistance in *Anopheles gambiae* Giles and a cage test of elimination of resistance from a population by male release. *Bull. Entomol. Res.*, 1982, **72**, 335–344.
4. Singh, O. P., Raghavendra, K., Nanda, N., Mittal, P. K. and Subbarao, S. K., Pyrethroid resistance in *An. culicifacies* in Surat district, Gujarat, West India. *Curr. Sci.*, 2002, **82**, 547–550.
5. Pillai, M. K. K., Vector resistance to insecticides. *Proc. Natl. Acad. Sci., India*, 1996, **66**, 96.
6. Parida, S. K., Gunasekharan, K., Sadanandane, C., Patra, K. P., Sahu, S. S. and Jambulingam, P., Infection rate and vectorial capacity of malaria vectors in Jeypore hill tract. *Indian J. Malariol.*, 1991, **28**, 207–209.
7. Ansari, M. A., Sharma, Y. D., Roy, A., Biswas, S. and Sharma, P. K., Epidemiologic investigations of a malaria outbreak in northern Delhi area. *Am. J. Mosq. Control Assoc.*, 2001, **17**, 216–220.
8. Deobanker, R. B. and Palkar, N. D., Magnitude of DDT resistance in *Anopheles culicifacies* in Maharashtra state. *J. Commun. Discord.*, 1990, **22**, 77–79.
9. Prasad, R. N., Virk, K. G., Sharma, T. and Dutta, G. D. P., Malaria epidemic in Baniyani village into the recent malaria outbreak in district Farrukhabad (U.P.). *Indian J. Malariol.*, 1992, **29**, 219–224.
10. Sharma, S. N., Preliminary observations on the susceptibility of the anopheline species to insecticides in Bishnugarh District, Hazaribagh, Bihar. *J. Commun. Disord.*, 1993, **25**, 36–37.
11. Bansal, S. K. and Singh, K. V., Insecticide susceptibility status of some anophelines in the district Bikaner, Rajasthan. *Indian J. Malariol.*, 1996, **33**, 1–6.
12. Raghavendra, K., Subbarao, S. K. and Sharma, V. P., An investigation into the recent malaria outbreak in district Gurgaon, Haryana, India. *Curr. Sci.*, 1997, **73**, 766–770.
13. Sharma, S. K., Upadhyay, A. K., Haque, M. A., Singh, O. P., Adak, T. and Subbarao, S. K., Insecticide susceptibility status of malaria vectors in some hyperendemic tribal districts of Orissa. *Curr. Sci.*, 2004, **87**, 1722–1726.
14. Brogdon, W. G. and McAllister, J. C., Insecticide resistance and vector control. *Emerg. Inf. Dis.*, 1998, **4**, 1–14.
15. Vasuki, V., Hoti, S. L., Sadanandane, C. and Jambulingam, P. A., Simple and rapid DNA extraction method for the detection of *Wuchereria bancrofti* infection in the vector mosquito, *Culex quinquefasciatus* by Ssp I PCR assay. *Acta Trop.*, 2003, **86**, 109–114.
16. Brooke, B. D., Hunt, R. H., Koekemoer, L. L., Dossou-Yovo, J. and Coetzee, M., Evaluation of a polymerase chain reaction assay for detection of pyrethroid insecticide resistance in the malaria vector species of the *Anopheles gambiae* complex. *J. Am. Mosq. Control Assoc.*, 1999, **15**, 565–568.
17. World Health Organization, *Manual on Practical Entomology in Malaria. Part II – Methods and Techniques*, World Health Organization, Geneva, 1975, pp. 141–147.
18. World Health Organization, pesticide evaluation scheme. WHO/VBC/82, 1982 846, vol. 20 (mimeographed document).

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Pathogenic and molecular characterization of Indian isolates of *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt

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Wilt (*Fusarium oxysporum* f. sp. *ciceris*) is considered as one of the major factors for low productivity of chickpea (*Cicer arietinum*). The present study was conducted to determine pathogenic and genetic variability of isolates of *F. oxysporum* f. sp. *ciceris* collected from different parts of India. Pathogenic virulence study of 25 isolates of the pathogen on international set of differential cultivars revealed the existence of three new races of the pathogen in India, besides characterization of new isolates in the known four races of the pathogen. Genetic variability within 24 isolates representing seven races of *F. oxysporum* f. sp. *ciceris* was assessed by RAPD, with a set of 40 ten-mer primers. UPGMA cluster analysis divided the isolates into seven distinct clusters at 0.55 genetic similarities. The most virulent isolate obtained from wilt sick field of IARI (MB-4C), New Delhi was distinct from others.

Keywords: Chickpea, Indian isolates, pathogens, wilt.

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