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Molecular characterization of *Anopheles maculipennis* complex (Diptera: Culicidae) in Northern Morocco

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Le complexe *Anopheles maculipennis* (Meigen 1818) est composé d'espèces morphologiquement semblables qui ne peuvent être identifiées que par des techniques moléculaires. Dans ce cadre, les moustiques du complexe *A. maculipennis* ont été collectés entre 2007 et 2009 dans 8 provinces du Nord-Ouest du Maroc, où le paludisme est endémique durant ces dernières années. Au total, 407 moustiques incluant 185 adultes et 222 larves appartenant aux stades III et IV ont été identifiés par PCR multiplex. La seule espèce du complexe dans toute la région d'étude était *Anopheles labranchiae* Falleroni 1926, le principal vecteur du paludisme au Maroc. Ces résultats sont cohérents avec une étude précédente menée dans les provinces de la même zone.
Mots-clés: PCR Multiplex, complexe d'*Anopheles maculipennis, Anopheles labranchiae,* Nord du Maroc.

Anopheles maculipennis (Meigen 1818) complex is composed of morphologically similar species that can only be distinguished by molecular techniques. In this framework, mosquitoes of the *A. maculipennis* complex were collected in 2007 and 2009 in 8 provinces from North West of Morocco, where malaria was endemic until recent years. Totally, 407 mosquitoes including 185 adults and 222 III and IV instar larvae were identified by multiplex PCR assays. The only species of the complex recorded in all the study sites was *Anopheles labranchiae* Falleroni 1926, the main malaria vector in Morocco. These results are consistent with previous study carried out in the provinces of the same area.
Keywords: PCR Multiplex, *Anopheles maculipennis* complex, *Anopheles labranchiae*, North Morocco.

1. INTRODUCTION

Malaria remains the most important vector born disease in the world causing an enormous health and economic burden. From 1.5 to 2.7 million people die each year, most of them being children (http://www.who.int/malaria). In Morocco, until 2004, malaria was endemic being transmitted mainly by *Anopheles labranchiae* Falleroni 1926, species belonging to the *Anopheles maculipennis* (Meigen 1818) complex (WHO & Ministry of health, 2007). After this time, malaria disappeared due to several successful interventions by the ministry of health, consisting of sanitation activities, DDT use, active case detection and the treatments (WHO & Ministry of health, 2001). Despite these efforts, the risk concern of malaria re-emergence in Morocco is still considerable dueto the presence and abundance of potential vector species, *A. labranchiae* and the possible presence of gametocyte carriers across the country (Faraj *et al.*, 2008).

Different species of the *A. maculipennis* complex, morphologically indistinguishable, were the main responsible vectors of malaria transmission in Europe, from the Scandinavian Peninsula to the south of Italy, as far as in the Maghreb countries (Guy *et al.*, 1976). In Morocco, *A. maculipennis* complex was described for the first time in Salé by D'Anfreville in 1916 (D'Anfreville, 1916). Afterwards, several authors reported its presence

in different geographic regions across the country (Seguy, 1930; Bates, 1940; Gaud, 1949; Guy *et al.*, 1976; White, 1978; Himmi, 1991; Faraj *et al.*, 2008, 2010). Early reports of *maculipennis* group in Morocco reported the presence of *Anopheles sicaulti* Roubaud 1935 (Roubaud, 1935) and *A. labranchiae* (Guy, 1976). *A. sicaulti* was described by Sicault who declared that it was closely related to *A. labranchiae* in its biology and behaviours based on egg morphology (Roubaud, 1935). Four years later, Bates (1940) synonymised *A. sicaulti* with *A. labranchiae*. In subsequent studies on the *Anopheles* of Morocco (Bates, 1940), *A. sicaulti* was considered both a variety (Romeo, 1950) and a subspecies of *A. labranchiae* (Senevet & Andarelli, 1956). However, later genetic studies of *A. labranchiae* and *A. sicaulti* populations recollected in the province of Tetouan argued convincingly that *A. sicaulti* was a geographical variety of *A. labranchiae* (Zulueta *et al.*, 1983). Whereas, in Morocco, despite many studies were undertaken on *A. maculipennis* complex*,* there is still little lacking knowledge among members of the mosquito's group species. Precise and correct identification of *A. maculipennis* complex species certainly improves control strategies in targeting the potential vectors. The present study aimed to assess the composition of *A. maculipennis* complex in different regions in northern Morocco using multiplex PCR.

2. MATERIALS AND METHODS

Study areas

The study was carried out in 8 provinces in the North of Morocco where high incidence of malaria was reported in the past, namely, Tanger (35°46′00″N, 5°48′00″W), Tetouan (35°34′00″N; 5°22′00″W), Chefchaouen (35°10′17″N; 5°16′11″W), Larache (35°11′00″N; 6°09′00″W), Al Houceima (35°14′57″N; 3°55′58″W), Taza (34°13′00″N; 4°01′00″W), Salé (34°01′46″N; 6°50′09″W) and Khémisset (33°49′00″N; 6°04′00″W) (**Figure 1**).

Mosquito's collection

A. maculipennis s.l. larvae and adults were collected during the dry season in 2007-2009. Collections of adult mosquitoes were carried out in animal shelters and inside houses, using mechanical aspirators and CDC light traps. The *Anopheles* larvae were collected by standard dipping technique (WHO, 1975). The data and the sites of the collection are reported in table 1. Morphological identification of mosquito specimens was carried out according to the key of Brunhes (Brunhes *et al.*, 2000). Until processing adult specimens of *A. maculipennis* s.l. were stored in silica-gel and larvae in 70% ethanol.

Laboratory processing

The DNA extraction of single larvae was carried out using the Genomic DNA MINI kit (INVITROGEN®) following the recommended protocols from the manufacturer instructions. *A. maculipennis* s.l. specimens were identified at the species level by multiplex PCR according to Proft *et al*. (1999). PCR reactions were performed in a total volume of 25µl using 5µl of DNA for larvae and for the adult legs or wings directly set inside the PCR tubes. Concerning the *Anopheles* adult, the reaction mixture contained 1µl of each specific primer of *A. maculipennis* complex, 0.5µl of Taq polymerase (Eurogentec), 0.5µl of dNTPs (0.2 mM), 5µl of 5X reaction buffer, 1µl MgCl2 (1mM). The amplification program was : denaturation at 95°C for 2 min, followed by 35 cycles of annealing at 95°C for 20 seconds and 53°C for 30 seconds and extension at 72°C for 1 min with 6 min extra extension time in the last cycle. For *Anopheles* larvae, the reaction mixture contained 0.25µl of each specific primer of *A. maculipennis* complex, 0.125µl of Taq polymerase (Promega), 0.5µl of dNTPs (0.2 mM), 5µl of 5X reaction buffer with MgCl (25mM). The amplification program was : denaturation at 94°C for 5 min, followed by 35 cycles of annealing at 94°C for 30s and 55°C for 30s and extension at 72°C for 1min with 7 min extra extension time in the last cycle. The target amplified DNA was run on 1.6% agarose gel. Gels were stained with 0.5µl ethidium bromide and bands were visualized by UV transillumination.

3. RESULTS AND DISCUSSION

On the whole 407 *A. maculipennis* s.l. specimens, in particular 222 larvae and 185 adult females, were collected from 2007 to 2009, during the dry season. The multiplex PCR analysis of all specimens showed that *A. labranchiae* represented the only species of the complex present in the eight studied provinces during the period of mosquito collection (**Table 1**,

Figure 2). These finding confirmed the results of previous studies carried out in the same areas. In particular, Faraj *et al.* (2004), in their study of ITS2 sequences analysis of Moroccan specimens of *A. maculipennis* s.l., reported *A. labranchiae* as the only species of *A. maculipennis* group. Recently, Laboudi *et al.* (2011) showed that *A. labranchiae* was the only species of *A. maculipennis* group recorded in Larache, Chefchaouen and Khémisset provinces based on mtDNA sequences (COI barcode region) of *A. maculipennis* s.l.

A. labranchiae was recognized as an important malaria vector around the Mediterranean basin. It was still present in high population density in same coastal areas in central and southern Italy using particularly breeding sites, such as rice-fields, rivers and streams (Romi *et al.,* 1997; Di luca *et al.,* 2009). This species was also reported in Corsica in breeding sites such as small pools with fresh water and marshes (Senevet & Andrelli, 1956; Toty *et al.*, 2010). It is also highly abundant and widespread in the Maghreb countries: Morocco (Gaud, 1953; Faraj *et al.*, 2010), Algeria (Senevet & Andrelli, 1956) and Tunisia (Chahed *et al.*, 2001). In Morocco, *A. labranchiae* was found in different environmental sites including the clear water lodgings of river, grassy ponds with *Typha* and filamentous algae (El Ouali *et al.,* 2010). In the country, high abundance of *A. labranchiae* corresponded to the regions where the relative humidity was higher due to the water evaporation from rice crop that constitutes a focal point for larva hatching (Gaud *et al.*, 1953).

The geographical range of this species covers, in Morocco, almost the whole country, being *A. labranchiae* found along the Atlantic coastal plains right down to south of Agadir (Gaud, 1953) and it is the predominant species in the North West of the country (Faraj *et al.*, 2010). While, the inland range of this malaria vector include the Rif and middle Atlas Mountains, being the southern boundary represented by the northern slopes of the high Atlas Mountains.

A. labranchiae was recognized as endophilic and very anthrophilic mosquito in the Mediterranean basin (Hanon, 1958). Despite its importance in malaria transmission, little is known about vectorial capacity, vector competence and blood feeding preference of this species in Morocco. Recently, in Larache province, previously endemic for malaria, Faraj *et al.* (2008), reported that *A. labranchiae* was present from February to November; nevertheless its vector capacity was considerably high during the summer. In this period corresponding to rice cultivation period, also the abundance of the species was very high.

Regarding vector competence, *A. labranchiae* was known to be very efficient to transmit *Plasmodium vivax* Grassi & Feletti 1890 strains. Conversely, it was known to have low vector competence for the tropical strains of *Plasmodium falciparum* Welch 1897. A recent study showed that in artificial infection experiments the development of oocysts in the midget of Moroccan *A. labranchiae* specimens was at very low number (Faraj *et al.*, 2010).

In the light of the previous studies and from our survey results, we can reasonably conclude that appropriate vector control measures and constant surveillance activities, are essential particularly in northern country where *A. labranchiae* is considered the main malaria vector and its abundance remains considerably high during the hot season.

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(27 réf.)

Table 1: Collection sites in 8 provinces of North of Morocco and numbers of *Anopheles maculipennis* s.l. specimens collected in 2007-2009 and identified to be *Anopheles labranchiae* using multiplex PCR.

Provinces	District	Locality	Year	Sample	Number of collected specimens
El houceima	*Ikaouen*	*Azib jro*	2009	Larvae	25
		Azibouflou	2009	Larvae	17
Tanger	*Gzeinaya*	*Gzeinaya*	2009	Larvae	16
	Chrada	*Badrouine*	2009	Larvae	10
		Sefah	2009	Larvae	15
	Gzeinaya	*Gzeinaya*	2007	Larvae	12
Tétouan	*Oued law*	*Chroda*	2009	Larvae	13
		Tizgharite	2009	Larvae	15
Chefchaouen	*Bab berrad*	*Imizegane*	2007	Adult	8
Larache	*Kasba*	*Beggara*	2007	Adult	25
	Laouamra	*Boucharen*	2007	Adult	130
			2009	Larvae	80
Taza	*Oued Amlil*	*Oued Amlil*	2007	Larvae	10
Salé	*Bouknadel*	*Shoul*	2009	Larvae	9
Khémisset	*Roumani*	*Ait Hamousghir*	2007	Adult	22

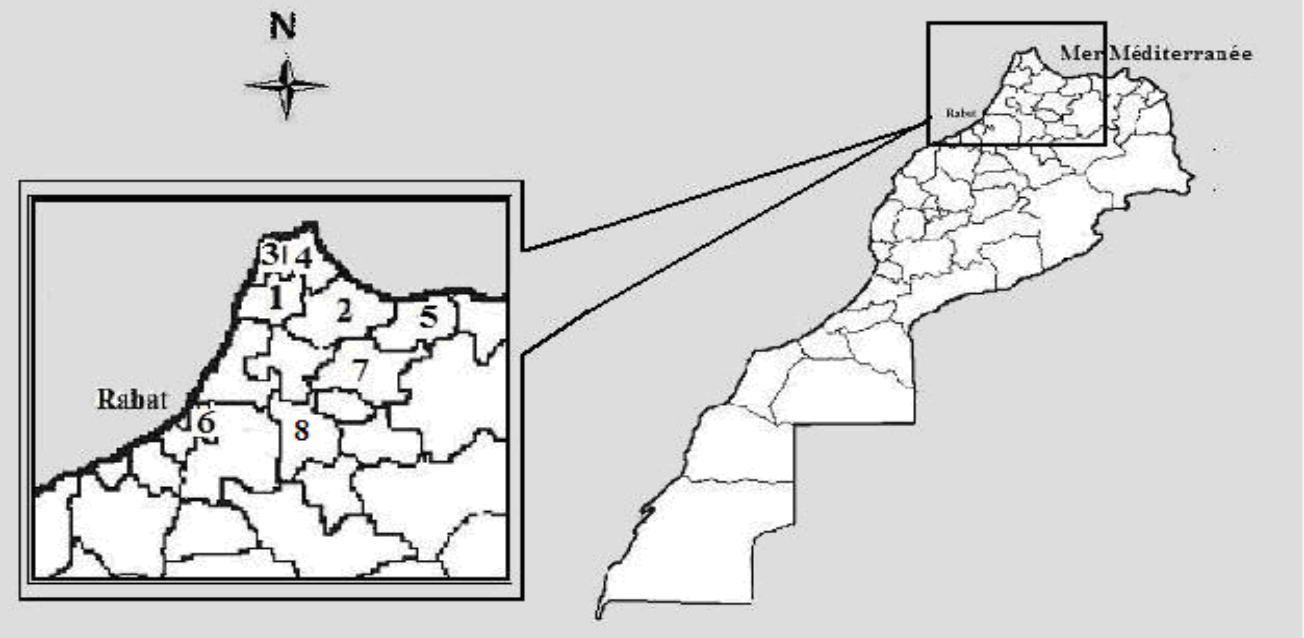

Figure 1: Map of the collection sites of *Anophles maculipennis* complex in Northern Morocco; (1), Larache (2), Chefchaouen (3), Tanger (4), Tetouan (5) Elhoceima (6), Salé (7) Taza and (8) Khemisset provinces.

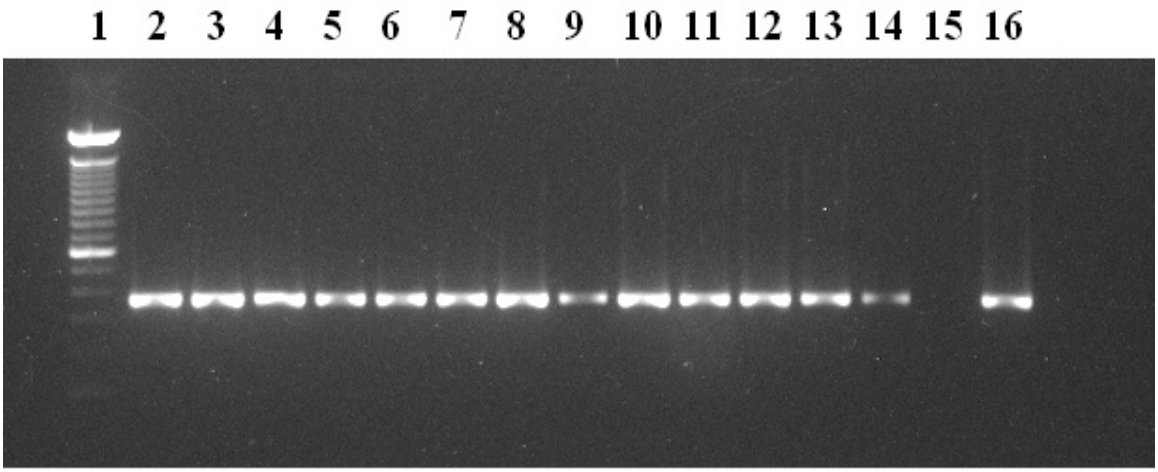

Figure 2: A representative PCR identification of *Anopheles labranchiae* among *Anopheles maculipennis* group among provinces: Lane 1: weight seize markers, lanes 1-14: *A. labranchiae* (345bp), lane 15: negative control, lane 16: positive control.