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Pianificazione di strategie di controllo della zanzara *Ochlerotatus caspius* in Nord Italia attraverso un approccio genetico-molecolare

s.s.d.: BIO/07

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INDICE

PREFAZIONE

Il lavoro presentato in questa tesi è stato svolto presso il laboratorio di Ecologia evoluzionistica del Dipartimento di Genetica e Biologia Molecolare, Università di Roma "La Sapienza", sotto la supervisione della Prof.ssa Sandra Urbanelli. Esso nasce da una collaborazione tra il suddetto laboratorio e la sezione di Entomologia medico-veterinaria del Centro Agricoltura Ambiente "G. Nicoli" di Bologna diretta dal Dott. Romeo Bellini.

Il capitolo 1 contestualizza il lavoro svolto e la finalità della ricerca. Il capitolo 2 della presente tesi è nella forma di manoscritto attualmente *in press* presso il *Journal of Applied Ecology*, mentre il lavoro presentato nel capitolo 3 è in fase di revisione presso il *Journal of Medical Entomology*. Nel capitolo 4 è presentata una discussione generale. La letteratura citata è stata posta in un unico capitolo finale (Cap. 5).

1. Inquadramento della ricerca

Gli insetti, con oltre 900.000 specie, sono i più abbondanti e diffusi fra tutti gli animali terrestri e la storia dell'uomo da sempre è legata a quella di tali organismi (Storer *et al.* 1990; Black IV e Kondratieff 2004). Se molti di loro, entomofagi o impollinatori, sono fonte di importanti risorse, molti altri danneggiano le colture o sono vettori di gravi malattie. Oltre il 30-40% della produzione agricola viene persa annualmente a causa di fitofagi e parassiti, percentuale che sale al 60-70% se si considerano anche le perdite successive alla raccolta durante le fasi di stoccaggio (Thomas 1999). Altri insetti, soprattutto ditteri, sono vettori di patogeni che causano gravi malattie per l'uomo e gli animali. Malaria, dengue, febbre gialla, leismaniosi, provocano ogni anno milioni di morti nei Paesi in via di sviluppo dove tali malattie sono ancora endemiche ed oggi, sempre più crescente, è la consapevolezza di una potenziale diffusione di tali malattie anche nelle aree temperate in seguito alla introduzione e diffusione dei vettori e delle condizioni ambientali sempre più favorevoli (Lounibos 2002).

L'uomo contribuisce alla diffusione di pest direttamente, attraverso la loro introduzione in nuove aree geografiche, o indirettamente in seguito ai cambiamenti apportati al territorio. L'urbanizzazione di vaste aree e l'utilizzo agricolo del territorio hanno determinato l'instaurarsi di condizioni ambientali, quali elevata disponibilità di nutrienti ed assenza di predatori, estremamente favorevoli alla diffusione di fitofagi e vettori. (Matson *et al.* 1997; Western 2001; Juliano e Luonibos 2005). Di difficile stima sono i costi in termini economici ed ecologici sia dei danni provocati da tali specie, sia dei tentativi stessi da parte dell'uomo di controllarne la diffusione. L'applicazione di insetticidi di sintesi è stata la principale forma di controllo attuata durante gli ultimi 50 anni. I problemi di inquinamento e il conseguente rischio per la salute umana, l'accumulo nelle reti trofiche, la rarefazione degli insetti utili, nonchè l'insorgenza di fenomeni di resistenza rappresentano i costi e i limiti di tale approccio (Casida e Gary 1998). E' quindi emersa sempre più la necessità di individuare strategie alternative o di supporto alla lotta chimica in un piano di gestione integrato (Integrated Pest Management) (Kogan 1998; Thomas 1999). I progressi fatti negli ultimi decenni nei campi della genetica e biologia molecolare hanno fornito agli entomologi una serie di strumenti attraverso i quali studiare aspetti fondamentali della biologia ed ecologia

delle specie di maggiore importanza economica e medico-sanitaria. Le conoscenze acquisite sono state quindi utilizzate e finalizzate alla pianificazione di strategie di controllo portando ad una vera e propria rivoluzione in tale ambito (Collins *et al.* 2000). Da un lato, si è mirato a razionalizzare ed ottimizzare l'uso di composti chimici di sintesi, dall'altro sono state rivalutate, alla luce delle nuove metodologie a disposizione, strategie abbandonate da tempo (p.es. lotta autocida), o ne sono state sviluppate di nuove (utilizzo di organismi transgenici).

La presente ricerca ha per oggetto *Ochlerotatus caspius* (Figura 1.1), zanzara di grande interesse dal punto di vista medico-sanitario ed economico ed obiettivo generale è stato quello di fornire indicazioni per la pianificazione di strategie di controllo nell'area della pianura Padana, dove tale specie è ampiamente diffusa ed assume carattere infestante.

La pianura Padana, situata tra l'arco alpino e l'Appennino si estende su una superficie totale di 30.000 Km^2 dal confine italiano con la Francia al mar Adriatico. Il paesaggio attuale è il risultato di un'intensa, continua e capillare azione dell'uomo, che è intervenuto sin dalla preistoria sulla vegetazione originaria, apportandovi modifiche sempre più radicali. A partire dagli inizi del 1900, industrializzazione, urbanizzazione e sviluppo agricolo sono i fenomeni che hanno contribuito al volto attuale di tale area. Tra le attività agricole la risicoltura ricopre certamente un ruolo di primo piano con una superficie attuale di circa 220.000 ettari che comprende principalmente le regioni Piemonte e Lombardia (Ente Nazionale Risi, www.enterisi.it) (Figura 1.2). Una produzione di circa 1 milione e 400 mila tonnellate annue fa dell'Italia il maggior produttore europeo di riso. Se da un lato la risicoltura rappresenta una così importante risorsa economica, dall'altro essa presenta non pochi disagi per gli abitanti, tra cui la proliferazione di zanzare. Attualmente, le aree risicole italiane sono focolai per specie quali *Culex pipiens* e *C. modestus,*, le specie del complesso *Anopheles maculipennis* e *Ochlerotatus caspius,* quest'ultima oggetto di studio della presente ricerca.

Oc. caspius è una zanzara caratteristica degli ambienti a sommersione temporanea e l'acqua dei siti di riproduzione può presentare un ampio range di concentrazione salina, da dolce a salmastra (Becker *et al.* 2003). In Nord Italia quindi tale specie è ampiamente diffusa sia nell'entroterra in cui le risaie costituiscono siti di riproduzione ottimali sia nell'area costiera del delta del Po (Bellini 1998; Bellini e Veronesi 2001; Marasca e Bellini 2001) (Figura 1.3). Nelle stagioni di maggiore sviluppo (maggio-luglio) la specie raggiunge densità

elevatissime dando origine a vere e proprie infestazioni. Data la sua marcata aggressività nei confronti dell'uomo e dei grandi mammiferi di allevamento (equini e bovini), *Oc. caspius* costituisce un grave problema di ordine medico-sanitario e zootecnico. Sono stati stimati valori medi di 22 punture in 10 minuti su uomo (con un picco di 73) e 130 punture su cavallo, con un picco di 430 (Tabalashi, personal comunication). Inoltre, le infestazioni da parte di *Oc. caspius,* riducendo notevolmente la vivibilità, costituiscono un freno allo sviluppo economico e turistico di tali aree.

Nonostante gli sforzi economici intrapresi per il controllo (10 milioni di euro nel 2004, Bellini personal comunication), essi risultano modesti rispetto all'entità del problema in quanto condotti su una piccola porzione delle aree interessate alle infestazioni e senza una adeguata coordinazione tra gli Enti preposti (Regioni, Province e Comuni). Infine, la paucità di studi e ricerche di carattere biologico, ecologico e genetico sulla specie non consentono una pianificazione delle azioni di controllo basata su profonde conoscenze della specie stessa, condizione questa indispensabile per ogni piano di gestione che voglia essere efficace (Kogan 1998). Obiettivo generale della presente ricerca è stato dunque quello di colmare, almeno in parte, tale lacuna e fornire indicazioni sulla pianificazione di strategie di controllo di *Oc. caspius* in pianura Padana. In particolare si è voluto:

1. studiare il grado di connettività delle popolazioni al fine di fornire indicazioni sulla pianificazione dei programmi di controllo in termini di:
   - definizione dell'appropriata scala geografica per la loro attuazione
   - valutazione del rischio di diffusione di eventuali alleli conferenti la resistenza;

2. valutare quali fattori possono aver influito sulla storia demografica delle popolazioni in relazione:
   - all'efficacia dei programmi di controllo effettuati fino ad oggi;
   - a cambiamenti nell'uso del territorio da parte dell'uomo;

3. approfondire lo studio dei sistemi di difesa cellulare della specie nei confronti di insetticidi di sintesi.

E' stato utilizzato un approccio genetico-molecolare che ha visto, da un lato lo studio dei pattern diversità genetica delle popolazioni attraverso l'uso di marcatori nucleari e citoplasmatici (obiettivi 1 e 2), dall'altro lo studio e l'isolamento di particolari regioni genomiche putativamente codificanti per geni coinvolti nella difesa cellulare agli insetticidi (obiettivo 3). Nei paragrafi che seguono vengono presentati in dettaglio i singoli obiettivi.

1.1 Obiettivi specifici della ricerca

1.1.1 Connettività delle popolazioni e pianificazione dei programmi di controllo

I problemi ambientali e di natura medico-sanitaria legati all'uso degli insetticidi e l'insorgenza di fenomeni di resistenza hanno evidenziato la necessità di razionalizzare l'uso dei composti di sintesi. Inoltre, l'attuale fase di crisi economica del Paese incide sensibilmente sulle risorse messe a disposizione per il controllo di pest che risultano largamente insufficienti ad affrontare il problema in maniera adeguata. E' necessario, dunque, ottimizzare le risorse disponibili concentrando gli sforzi in aree limitate rispetto all'intera superficie territoriale mediante attività mirate ed efficaci. La conoscenza delle capacità di dispersione degli individui e dei pattern di connettività tra le popolazioni sono di fondamentale importanza per definire l'appropriata scala geografica su cui effettuare i controlli, così come il pattern di diffusione di un eventuale allele conferente la resistenza. Tali informazioni sono spesso difficili da ottenere tramite metodi diretti quali esperimenti di marcaggio-ricattura per specie come le zanzare. Essi infatti permettono di descrivere i pattern di migrazione solo su brevi distanze e la misura del movimento degli individui non necessariamente riflette il movimento di geni tra le popolazioni dal momento che gli individui migranti devono poi effettivamente riprodursi nella nuova località perchè vi sia flusso genico (Roderick 1996; Hagler e Jackson 2001). Un approccio indiretto viene dagli studi di genetica di popolazioni condotti mediante l'uso di marcatori molecolari (Collins *et al.* 2000; Rollins *et al.* 2006). L'analisi dei pattern di diversità genetica all'interno e tra le popolazioni consente infatti di valutare il ruolo delle diverse forze evolutive (deriva genetica, flusso genico, selezione) e dei fattori ambientali nel determinare la struttura e dinamica delle popolazioni.

Attualmente, i programmi di controllo di *Oc. caspius* in pianura Padana sono legati ad una logica basata sui confini amministrativi, senza tener conto della reale struttura delle popolazioni. La specie è descritta come una buona volatrice (Becker *et al.* 2003), tuttavia ad oggi non esistono dati sui pattern di migrazione tra le popolazioni e sui fattori che possono influenzarne la connettività. Nel presente studio si è voluto, quindi, attraverso l'uso di marcatori genetici nucleari (13 loci allozimici) e citoplasmatici (parte della sequenza del gene mitocondriale codificante per la citocromo ossidasi II) studiare la diversità genetica e la struttura delle popolazioni di *Oc. caspius* in Nord Italia al fine di fornire indicazioni per la pianificazione di programmi di controllo attraverso: 1) la valutazione del possibile ruolo della distanza geografica e dell'eterogeneità dei siti di riproduzione (concentrazione salina dell'acqua del sito) sul pattern di differenziamento delle popolazioni; 2) valutando il pattern di dispersione e il grado di connettività delle popolazioni.

1.1.2. Storia demografica delle popolazioni

Un altro importante contributo degli studi di genetica di popolazioni al controllo di pest riguarda la possibilità di ricostruirne la storia demografica. Drastiche riduzioni nella taglia delle popolazioni ("colli di bottiglia") o espansioni lasciano infatti dei segni nella costituzione genetica delle popolazioni stesse, che possono essere rilevati ed interpretati (Cornuet e Luikart 1996; Templeton 1998 e riferimenti all'interno). Nella presente tesi si è voluto, attraverso lo studio dei pattern di diversità genetica, valutare se e quali fattori abbiano influito sulla taglia delle popolazioni in relazione sia ai programmi di controllo effettuati fino ad oggi, sia in relazione all'uso del territorio da parte dell'uomo.

Fino al 1991 l'unico metodo adottato per il controllo delle popolazioni di *Oc. caspius* nell'area del delta del Po era basato su irrorazioni notturne di insetticidi a largo spettro d'azione ripetute a calendario contro gli adulti. Data l'importanza ecologica delle aree umide da circa 15 anni i programmi di controllo sono stati indirizzati essenzialmente sui siti di riproduzione e mediante l'utilizzo di formulati a base di *Bacillus thuringensi* var. *israelensis* (*Bti*) contenendo le irrorazioni notturne di insetticidi solo in seguito al superamento di una "soglia di intervento adulticida". Nelle aree dell'entroterra le risaie costituiscono il principale ambiente di sviluppo della specie. Nonostante i notevoli disagi causati dalle infestazioni di *Oc. caspius*, solo recentemente (dal 2000) sono stati improntati programmi di

controllo nelle aree risicole di Piemonte e Lombardia. Tali azioni riguardano principalmente l'uso di formulati di *Bti* e, in congiunzione con questo, è stata proposta e in diversi casi attuata, l'introduzione di pesci larvivori quali quelli dei generi *Gambusia* e *Carassius* (Bellini e Veronesi 2001). Ad oggi nessuna valutazione è attualmente disponibile del beneficio che tali controlli abbiano dato in termini di riduzione a medio-lungo termine della densità di popolazioni di *Oc. caspius*, nonostante al contrario, siano noti o comunque stimabili i costi di tali azioni sia in termini economici (nel 2004 attorno ai 10 milioni di Euro per i programmi nel Delta del Po, nella pianura Padana Veneta ed Emiliana, nelle aree risicole Lombarde e Piemontesi), sia in termini ambientali. Sebbene, infatti, il *Bti* sia stato considerato altamente specifico, in condizioni sperimentali la tossina determina effetti citopatologici su animali di laboratorio, *in vivo* e *in vitro*, simili a quelli prodotti sugli organismi bersaglio (Mayes *et al.* 1989; Cahan *et al.* 1994, 1995). Inoltre, numerose evidenze si sono accumulate sugli effetti in natura di singoli e ripetuti trattamenti con *Bti* sulle specie non target (soprattutto invertebrati) con gravi conseguenze sull'intera rete trofica (Jackson *et al.* 2002). Anche per quel che riguarda l'introduzione di pesci larvivori, perlopiù specie alloctone, numerose sono le evidenze degli effetti negativi della loro introduzione sulla fauna locale (Goodsell e Kats 1999; Dore *et al.* 2000). Tra gli obiettivi della presente tesi si è voluto, quindi, attraverso lo studio dei pattern di diversità genetica, valutare se i programmi di controllo effettuati fino ad oggi abbiano determinato significativi cambiamenti nella taglia delle popolazioni.

Come detto nel paragrafo precedente, le risaie costituiscono i principali siti di riproduzione di *Oc. caspius* in pianura Padana. Le tecniche di coltivazione del riso attualmente utilizzate e messe in atto a partire dagli anni '60 comportano il ricorso più volte nella stagione alla pratica delle asciutte, le quali rispondono a precise esigenze agronomiche (diserbo mediante trattamenti con fitofarmaci, concimazione) (Ardizzone *et al.* 1993; Latino *et al.* 2004). L'eliminazione temporanea dell'acqua nelle vasche porta alla scomparsa, spesso irreversibile, delle popolazioni di molti organismi acquatici o anfibi predatori delle larve delle zanzare. Fra una fase di asciutta e la successiva non intercorre un tempo sufficiente che consenta agli esemplari adulti delle forme anfibie di tornare a deporre le uova. I cicli riproduttivi di tali organismi hanno la durata di diverse settimane o mesi. Le zanzare, invece, riescono a completare diversi cicli riproduttivi in questi cicli di asciutte/allagamenti. Le

pratiche colturali adottate in risaia potrebbero dunque aver inciso sulla presenza quali-quantitativa delle zanzare. Mancano tuttavia dati oggettivi a sostegno di questo presunto incremento, in quanto gli studi quantitativi sulle popolazioni sono carenti e di scarsa utilità nei casi in cui le popolazioni sono molto grandi. In questo studio si è voluto quindi valutare e far luce su questo importante aspetto della storia recente di *Oc. caspius* nell'area padana.

1.1.3. Sistemi di difesa cellulare e insetticidi di sintesi

L'insorgenza di fenomeni di resistenza costituisce uno dei principali limiti della lotta chimica. Essa ha importanti conseguenze sulla gestione delle specie nocive in quanto la minore suscettibilità degli individui può portare ad un incremento nel numero e nella frequenza di applicazione degli insetticidi con conseguente aumento del carico ambientale. Inoltre, i composti appartenenti a diverse classi agiscono in alcuni casi sullo stesso sito target, per cui l'insorgenza di resistenza multipla rende inutilizzabili simultaneamente più composti (Hemingway e Ranson 2000). Da tali considerazioni emerge quindi la necessità, da un lato di ridurre il rischio di insorgenza della resistenza utilizzando in modo ottimale i composti già conosciuti, dall'altro di comprendere maggiormente i meccanismi di interazione tra cellule e sostanze tossiche al fine di individuare nuovi potenziali siti target. In questo contesto si inserisce, dunque, tale parte della presente ricerca, volta allo studio, in *Oc. caspius*, di un specifico meccanismo di detossificazione cellulare a diverse classi di insetticidi.

Le P-glicoproteine (P-gp), o "trasportatori multidrug", sono proteine di membrana in grado di trasportare fuori dalla cellula eventuali sostanze tossiche una volta entrate diminuendone così la concentrazione intracellulare (Figura 1.4). Esse costituiscono un sistema apparentemente capace di proteggere le cellule sensibili, i tessuti e quindi gli organismi da un ampio spettro di composti chimici citotossici diversi chimicamente e strutturalmente e sono considerate la *prima linea di difesa* della cellula (Blackmore *et al.* 2001; Borges-Walmsley *et al.* 2003; Chang 2003). Nonostante non sia ancora conosciuto il loro normale ruolo fisiologico, le P-gp sono ampiamente diffuse nel mondo vivente, dai batteri all'uomo e un'amplificazione dell'attività di trasporto delle P-gp ha come conseguenza l'insorgenza della resistenza multidrug (resistenza MDR). Tale fenomeno è stato osservato per la prima volta in linee cellulari tumorali nell'uomo divenute resistenti

non solo al farmaco somministrato inizialmente, ma anche ad altri che non erano mai stati somministrati in precedenza (Lage 2003). L'insorgenza della resistenza MDR, legata a proteine di trasporto transmembrana omologhe alle P-gp, è stata evidenziata anche in batteri (p.es nei generi *Streptomyces* e *Lactococcus*), funghi (*Schizosaccharomyces pombe, Saccharomyces cerevisiae*, *Candida albicans*) e parassiti protozoi (generi *Plasmodium, Entoameba, Leishmania*).

Fra i substrati dei trasportatori multidrug vi sono anche composti chimici come gli insetticidi. Inoltre, gli studi condotti su *Chironomus riparius* e *Manduca sexta* per la prima volta hanno evidenziato un possibile coinvolgimento dei trasportatori multidrug nella difesa cellulare e nella resistenza a composti insetticidi quali l'ivermectina suggerendo l'ipotesi che anche negli insetti possa esistere un tale meccanismo di difesa (Gaertner *et al.* 1998; Podsiadlowski *et al.* 1998). Lanning *et al.* (1996), inoltre, in un'altra specie di fitofago del tabacco, *Heliothis virescens***,** ha evidenziato l'esistenza di un meccanismo legato all'attività delle P-glicoproteine in linee resistenti al thiodicarb. Più recentemente è stato osservato che l'inibizione dell'attività di trasportatori multidrug aumenta la suscettibilità di campioni larvali della zanzara *Culex pipiens* agli insetticidi piretroidi ed organoclorati (Buss *et al.* 2002).

Le P-glicoproteine potrebbero quindi costituire sia un meccanismo di difesa che un potenziale meccanismo di resistenza agli insetticidi. L'esistenza di un meccanismo, quale è quello legato all'attività delle P-gp, che protegge le cellule da un ampio spettro di composti insetticidi in condizioni normali, potrebbe essere selezionato come sito per la resistenza, provocando come conseguenza un notevole impatto negativo sulle attuali strategie di lotta. Al contrario, tuttavia, esso rappresenterebbe un sito su cui potenzialmente poter intervenire per aumentare l'efficacia dei trattamenti. L'inibizione di tale meccanismo di difesa, aumenterebbe infatti la suscettibilità degli individui agli insetticidi utilizzati per il controllo, consentendo così un loro minor impiego con conseguente diminuzione dell'impatto sull'ambiente. Obiettivo della presente parte della ricerca svolta è stato quello di *i*) isolare e caratterizzare in questa specie sequenze genomiche codificanti per traspotatori multidrug del tipo P-gp e *ii*) valutare in *Oc. caspius* il possibile ruolo di tale meccanismo di difesa cellulare contro insetticidi quali temephos, ivermectina e diflubenzuron. Il primo di tali composti è attualmente utilizzato, in stretta misura in Italia, ma largamente nelle risaie della Grecia e

Francia per il controllo di tale specie. L'ivermectina e il diflubenzuron sono composti attualmente in uso in via sperimentale in progetti pilota nelle risaie italiane e verso i quali c'è particolare interesse per un ben più ampio uso.

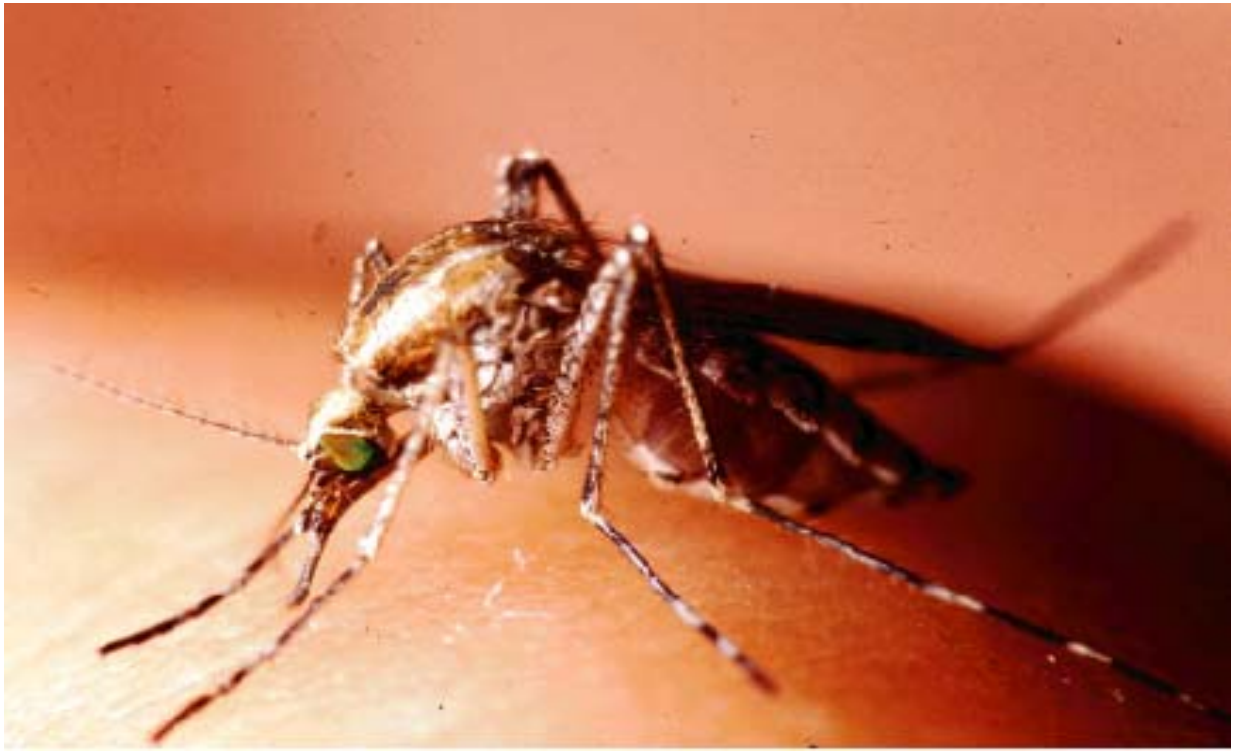

Figura 1.1- Femmina di *Ochlerotatus caspius* (Pallas, 1771) fotografata durante il pasto di sangue. Tale specie presenta una distribuzione Paleartica (Europa, Asia a nord dell'Himalaya e del fiume Huang Ho, Africa settentrionale a nord del Sahara). Il limite nord dell'areale corrisponde a circa 60° di latitudine nord, mentre a sud corrisponde a circa 30° di latitudine nord. L'Oceano Atlantico rappresenta il confine a ovest mentre a est questo è ancora impreciso (Becker *et al.* 2003).

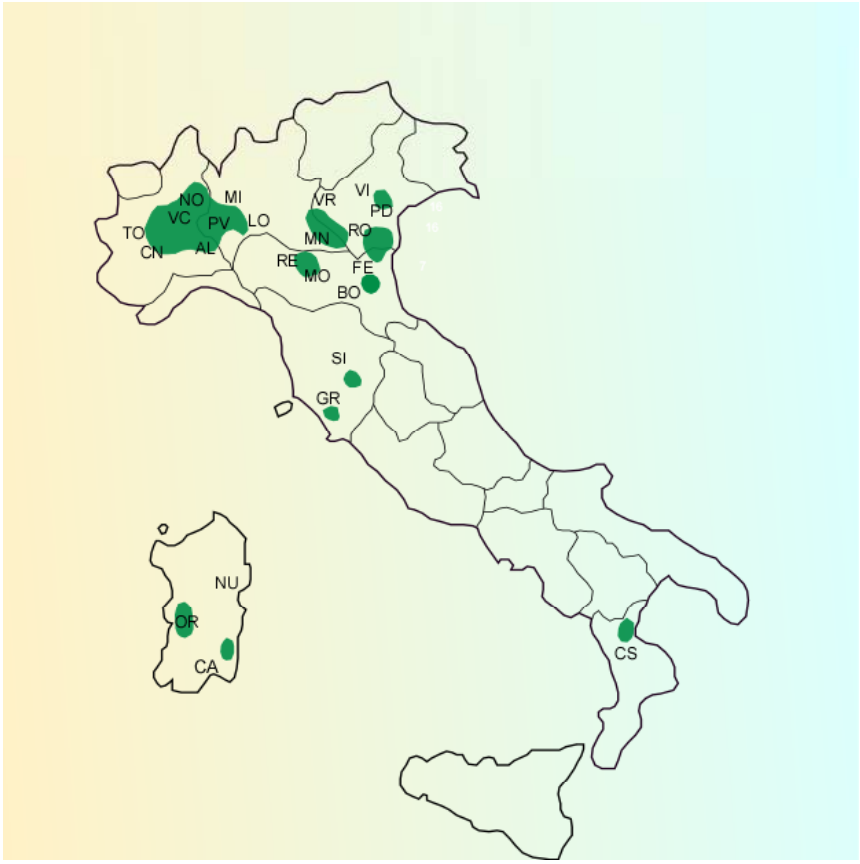

Figura 1.2. Distribuzione della superficie risicola in Italia. Con circa 220 mila ettari di risaie e una produzione di 1 milione e 400 mila tonnellate/anno quella italiana rappresenta lo 0,25% della produzione mondiale di riso (da Ente Nazionale Risi www.enterisi.

Figura 1.3. Siti di sviluppo e di riproduzione di *Ochlerotatus caspius:* ambiente costiero e risaia

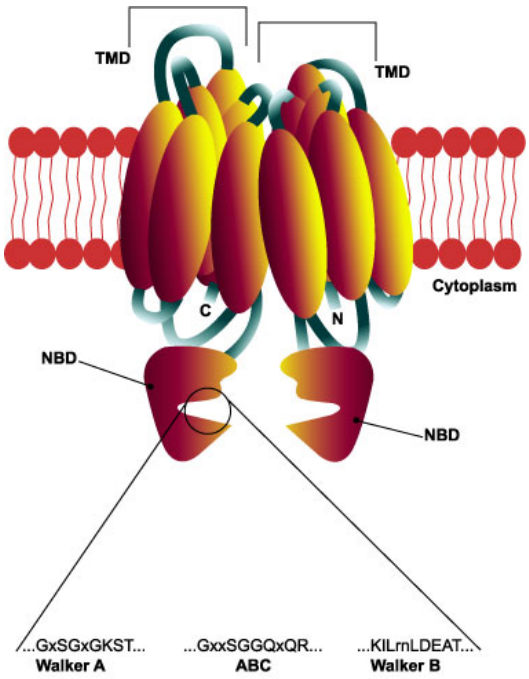

Figura 1.4. Modello di struttura di un trasportatore del tipo P-gp. La proteina attraversa la membrana citoplasmatica ed è costituita da due subunità, ognuna con un dominio transmembrana (TMD) e un dominio citoplasmatico per il legame del nucleotide (NBD). Sono mostrate le sequenze consenso Walker A, ABC segnature e Walker B (modificata da Borges-Walmsley *et al.* 2003).

2. IMPROVING INSECT PEST MANAGEMENT THROUGH POPULATION GENETIC DATA: THE CASE STUDY OF THE MOSQUITO *OCHLEROTATUS CASPIUS*

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Summary

1. Rationalising the use of chemicals in pest control programmes is a fundamental goal that is aided by the knowledge of patterns of population connectivity and historical demography.
2. In this study, we used both mitochondrial and nuclear markers to investigate the genetic structure and diversity of the Northern Italian populations of *Ochlerotatus caspius*, a mosquito of great public health and economic impact in Mediterranean countries.
3. A substantial genetic homogeneity was found among populations, with no association of pairwise population differentiation with the geographical distribution or the environmental heterogeneity of the breeding sites.
4. On a regional scale, we hypothesize a historical demographic expansion, probably associated with late Pleistocene paleoclimatic events. Furthermore, our results suggest that ever since the expansion event, an extensive gene flow is still the major evolutionary force shaping the overall genetic pattern observed.
5. At a local geographical scale, evidence of recent growth in the size of the population was found in several sites. The increased availability of large breeding sites recently offered by rice paddies seem to have played a major role in giving rise to the observed demographic expansions. Indeed, these sites have become more numerous and undergone changes in the management technique (increased number of dry/flood cycles), which enhanced their suitability as breeding habitats.

6. *Synthesis and applications***.** The migration pattern among populations of *Oc. caspius* in Northern Italy appears to be high enough to maintain an extensive genetic homogeneity. Control programmes on a small geographic scale are thus unlikely to be effective. The modern agronomic techniques and the growth of rice paddies seem to have played a role in the recent demographic history of the species in the Po plain. Therefore, satisfactory control programmes should: *i)* be mainly focused on rice paddies as breeding habitats, *ii)* favour agronomical techniques involving a reduced number of dry/flood cycles, *iii)* be carried out on a wide (regional) geographical scale, *iv)* periodically monitor the susceptibility of populations to the compounds used in control campaigns and adopt strategies aimed to avoid the possible spread of resistance alleles.

Key Words: allozymes; demographic changes; genetic diversity; landscape use; mosquito populations; mtDNA; *Ochlerotatus caspius;* pest control; rice paddies

Introduction

The wide range of insecticides used to control insect pest populations has given rise to problems associated with the disruption of pre-existing biological equilibriums, the rarefaction of useful insects, pollinators and entomophages (Stark & Banks 2003), the contamination of the environment with toxic residues that are hazardous to human health (Alavanja, Hoppin & Kame 2004) and the appearance and spread of resistance to major pesticides (reviews in Hemingway & Ranson 2000). However, chemical measures remain central to pest control plans, especially when insect density, health risks and the consequent economic damage are very high (Casida & Gary 1998). Several authors highlight the necessity to adopt control programmes with enhanced specificity for the target species and at the appropriate geographical scale, so as to optimise their effectiveness (reviewed in Kogan 1998). The risk of the appearance and spread of resistance alleles should also be evaluated, as well as the short- to medium-/long- term effect of control programmes (PAN Germany 2004). The planning of such actions must necessarily be based on the thorough knowledge of the biology and natural history of the target species (Kogan 1998). To this aim, knowledge of the dispersal abilities and migration patterns of the target populations is of utmost importance to determine the appropriate geographical scale for carrying out effective treatments and to predict the geographical spread pattern of a resistance allele. This

information is often difficult to obtain for species like mosquitoes by using classical direct methods, based on mark-recapture trials (Roderick 1996; Hagler & Jackson 2001). These methods allow migration patterns to be described only over small distances and the direct measurements of individual dispersal do not necessarily reflect gene movement, as migrating individuals must effectively reproduce in the new location in order for there to be a gene flow.

To acquire these and other data for the planning of control strategies, interest is growing in the use of genetic markers and the study of the levels and patterns of distribution of genetic diversity, as is indicated by the burgeoning number of studies published on this subject about malaria vectors and other vector insects (Neigel 1997; Simard *et al.* 2000; Collins *et al.* 2000; Pinto *et al.* 2002; Foley, Russell & Bryan 2004). Quantifying genetic differentiation among populations is important for determining the taxonomic status of the target organisms (Walton *et al.* 1999). The use of genetic markers has led to the recognition of a number of cryptic species and divergent evolutionary lineages (Schaffner, Raymond & Pasteur 2000; Ravel *et al.* 2002; Chen, Harbach & Butlin 2004), whose vector competence and/or susceptibility to insecticides are significantly different in several cases (Mousson *et al.* 2002; Yébakima *et al.* 2004). Moreover, it enables us to evaluate the levels of gene flow among populations in different geographical areas, which is an indicator of gene exchange and diffusion across the populations themselves (Chevillon *et al.* 1999; Weill *et al.* 2000; Urbanelli *et al.* 2000). Evaluating genetic diversity on both a large geographical scale and locally is crucial for gaining insight into the role of different evolutionary forces (genetic drift, gene flow, selection) and environmental factors in determining population structure and dynamics (David *et al.* 2003). The past history of a given population may also be inferred from genetic data, i.e. population size changes, range expansions or contractions as well as the relative influence of distinct causal factors (climatic, anthropogenic, etc.) (Cornuet & Luikart 1996; Templeton 1998 and references therein).

Ochlerotatus caspius (Pallas) is a wide spread mosquito (Diptera, Culicidae) of great public health and economic impact in Mediterranean countries such as Spain, France, Italy and Greece. Given its marked anthropophily, it significantly impairs the habitability of large areas and has thus become a major target of mosquito control programmes implemented by local authorities. The species breeds in several kinds of temporary and shallow waters bodies including salt, brackish and freshwaters (Becker *et al.* 2003). In Northern Italy *Oc. caspius* is widespread both in the coastal plain and inland, where rice fields constitute optimal breeding sites (Bellini 1998;

Bellini & Veronesi 2001; Marasca & Bellini 2001). The total yearly cost of control programmes in the Po Delta area, Po plain in the Veneto and Emilia regions and rice-growing areas in Lombardy and Piedmont was estimated at about 10 million euros in 2004.

In this paper, we used both nuclear (13 allozymes) and cytoplasmatic markers (partial sequences of the Cytochrome-Oxidase II mitochondrial gene) to investigate the genetic diversity and population structure of the mosquito populations *Oc. caspius* in Northern Italy in order to assist the planning of control programmes by: 1) assessing the possible role of the geographical distance and ecological heterogeneity of breeding sites in the differentiation patterns of the populations; 2) evaluating the patterns of dispersal and the degree of connectivity between populations of this species in the Po plain; 3) assessing the relative contribution of historical demography to the observed patterns at both the regional and the local geographic scale.

Material and methods

Sampling

Samples of *Oc. caspius* were collected during the summers of 2000-2002 from eleven locations in the Po plain in Northern Italy. Samples were collected both on the coastal plain and inland, in breeding sites with different salt concentrations (i.e. freshwater in rice fields and brackish water in coastal lagoons), and subject to control programmes of varying length, if any (Table 1). A comparatively larger number of samples were drawn from rice paddies as these are presently the most important class of breeding sites in the study area. The taxonomical identification of collected specimens was carried out following the morphological key by Romi, Pontuale & Sabatinelli (1997) and Schaffner *et al.* (2001). Samples were collected as larvae, brought in the laboratory and reared to adults. Adults were kept in cages, fed a sugar solution for five days, then frozen (-80° C) and stored for subsequent genetic analysis. A total of 457 specimens (221 males, 236 females) were analysed.

Allozyme

On the whole 11 enzyme systems were analyzed, representing 13 putative loci: glycerol-3-phosphate dehydrogenase (*G-3-pdh,* EC 1.1.1.8), malate dehydrogenase (*Mdh-1* and *Mdh-2,* EC 1.1.1.37), malate dehydrogenase-$NADP^+$ (*Mdhp-1,* EC 1.1.1.40), isocitrate dehydrogenase (*Idh-*

1, EC 1.1.1.42), 6-phosphogluconate dehydrogenase (6-*Pgdh*, EC 1.1.1.44)*,* superoxide dismutase (*Sod-1,* EC 1.15.1.1), aspartate aminotransferase (*Aat-1* and *Aat-2,* EC 2.6.1.1), hexokinase (*Hk-1,* EC 2.7.1.1), aconitase (*Aco*-1, EC 4.2.1.3), glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9), phosphoglucomutase (*Pgm*, EC 5.4.2.2). Alleles were numbered in order of decreasing mobility from the most anodal one, whilst alleles at each *locus* were named numerically according to their mobility with respect to the most common allele (*100*) in a reference population (Ticineto). The electrophoretic procedures are reported in detail in Urbanelli *et al.* (1996 and 2000).

Allele frequencies and the parameters of genetic variability (namely, the average number of alleles per locus, percentage of polymorphic loci, observed heterozygosity and Nei's (1978) unbiased estimate of expected heterozygosity) were estimated for each sampled population. Deviations from the expected Hardy-Weinberg equilibrium were evaluated by calculating the exact significance probabilities through a test analogous to Fisher's exact test as implemented in BIOSYS-2 (Swofford, Selander & Black 1997). Linkage disequilibria between pairs of loci were also tested for each locality, using GENEPOP 1.2 (Raymond & Rousset 1995). Weir & Cockerham's (1984) estimate of Wright's F_{ST} was calculated over all populations and for each population pair using FSTAT 2.9.3 (Goudet 2001). The significance of the estimates was assessed by means of 1000 randomisations; their standard errors, by jackknifing over loci. For multiple tests, the significance threshold (5%) was corrected by applying the Bonferroni correction (Rice 1989).

In order to verify the null hypothesis of the existence of equilibrium conditions between gene flow and drift at the regional scale, we used the approach proposed by Hutchison & Templeton (1999). The relative importance of these forces can be evaluated by predictable and contrasted patterns of relationship between genetic and geographical distances, as well as by the degree of scatter of genetic distance over geographic distance (Hutchison & Templeton, 1999). The relationship between geographical (as ln [geographic distance in km]) and genetic distances (as F_{ST}/1- F_{ST}; Rousset 1997) was evaluated with a Mantel test (with 10000 permutations), as implemented in the web version of IBD software (Jensen, Bohonak & Kelley 2005). The reduced major axis regression was used to evaluate the strength of the relationship and to calculate regression statistics.

Evidence of recent population size changes (expansions or bottlenecks) were found by testing for significant departures of genetic diversity from the expectation under mutation-drift equilibrium, as suggested by Cornuet & Luikart (1996). We used the Wilcoxon's signed-ranks test as implemented in the BOTTLENECK software (Piry, Luikart & Cornuet 1999), assuming an infinite-allele model of mutation which has been shown to be the most appropriate model for allozyme data (Chakraborty, Fuerst & Nei 1980).

mtDNA

Mitochondrial DNA was used, in conjunction with allozymes, to make inferences at a large geographic scale. Therefore, a subset of the sample studied with allozymes representative of all the geographic areas and breeding site types was screened. The specimens homogenized for allozyme analysis were frozen and subsequently used for mtDNA analyses.

DNA was extracted following the protocol by Collins *et al.* (1987). Partial sequences of the mtDNA gene encoding for cytocrome oxidase II (CO II) were obtained through PCR-amplification. Initially we used the primers pair TW-J-1305 and TK-N-3782 (Mitchell *et al.* 2002) for the amplification and sequencing of a product of ~2000 bp in length, including the regions CO-I, tRNA-Leu and CO-II. The following specific primers were designed in the CO II gene region and used for further analyses: *Ochc*COII-f 5'-GGCAACATGAGCAAATTTAGG-3' and *Ochc*COII-r 5'-CAAATTTCTGAACATTGACCAAA-3'. The PCR cycling procedure was: 95°C for 5 min followed by 33 cycles of 93°C for 1 min, 52°C for 45 s, 72°C for 1 min 30s, and a single final step at 72°C for 10 min. Sequences were obtained using ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Weiterstadt, Germany) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. All specimens analysed were double sequenced. The sequencing chromatograms were analysed with the program CHROMAS (Technelysium Pty Ltd, Australia). Alignments were done using the CLUSTALX 1.81 software (Thompson *et al.* 1997). All haplotype sequences were deposited in Genbank (Accession Number: DQ300479 - DQ300499). The network representing genealogical relationships among haplotypes was constructed using the statistical parsimony algorithm described by Templeton, Crandall & Sing (1992) and implemented in TCS software (Clement, Posada & Crandall 2000).

Mitochondrial DNA variability within populations was estimated as haplotype diversity (h) and nucleotide diversity (π) as defined by Nei (1987). Genetic differentiation among

populations was evaluated by calculating pairwise values of F_{ST} from haplotype frequencies, as estimated by parameter θ in Weir & Cockerham (1984). The significance of the F_{ST} estimates was assessed by 10000 random permutations. These, as well as the analyses that followed, were performed with ARLEQUIN 2.0 software (Schneider, Roessli & Excoffier 2000). The relationships between geographical and genetic distances were investigated using the same methodological approach as for allozyme data.

To find evidence of past demographic expansions we carried out a mismatch distribution analysis (Rogers & Harpending 1992; Rogers 1995). This analysis was also used to estimate the expansion parameters θ_0 and θ_1 (which equal $2\,N_0\mu$ and $2\,N_1\mu$, respectively, where N_0 and N_1 are the initial and final population sizes and μ is the mutation rate per sequence per generation), and τ (Tau), the time of the expansion measured in units of mutational time ($Tau=2\mu t$; t is the time in generations). To statistically assess the significance of the inferred expansion, we calculated the Raggedness index *r*, a measure of the smoothness of the distribution (Harpending, 1994) the significance of which was tested through 1000 replicates. To further corroborate indication of a past population expansion, we also calculated Fu's parameter F_S (Fu 1997). Large negative values of F_S indicate an excess of recent mutations, which is expected both under departures from selective neutrality and in populations which have experienced a recent demographic expansion.

Results

Allozymes

Ten loci (*Mdh-1, Mdph-1, Idh-1, 6-Pgd, Aat-1, Aat-2, Hk-1, Aco-1, Gpi and Pgm*) out of the 13 studied were found to be polymorphic, whereas loci *Mdh-2, Sod-1* and *G-3-pdh* were monomorphic for the same allele in all samples. (Data concerning frequencies and F_{IS} estimates of the polymorphic loci are available upon request). Significant departures (5%) from the expected genotype frequencies under Hardy-Weinberg equilibrium were found in 4 out of 110 tests performed. However they did not concern specific populations or loci, and were not significant after Bonferroni correction. No significant linkage disequilibria were observed across loci.

Estimates of genetic variability for each sampled population are given in Table 2. No significant differences were found in the distribution of genetic variability levels (χ^2 tests; all $P >$

0.05) with respect to the geographic location of samples (coastland or inland), nor to the environmental characteristics of the breeding sites (freshwater or brackish water) or to control programmes, if any.

The overall value of Weir & Cockerham's (1984) F_{ST} was 0.024 (± 0.051 *SE*). Pairwise values of F_{ST} (see Table 3) varied from 0.001 (between Spina-Salicornieto and Lidi di Comacchio and Comacchio) to 0.099 (between Sacca di Bellocchio and Ticineto). The correlation analysis revealed the lack of a significant association between genetic and geographic distances at the level of the entire dataset (Mantel test: $P > 0.05$). Moreover, the shape of the scatterplot (Fig. 1) conforms well to Hutchison & Templeton's (1999) case II - i.e. with a non significant association coupled with low differentiation between populations and a low degree of scatter -, in which the lack of regional equilibrium between gene flow and drift can be attributed to the prevalence of the former.

The possible occurrence of recent demographic size changes was tested for each population by evaluating departures from mutation/drift equilibrium under neutral expectations by Wilcoxon's test (Cornuet & Luikart 1996). Population size changes were inferred for all the samples studied except for the ones from Crevalcore and Bologna. Size change appeared in the direction of a population expansion, in the significant heterozigosity deficiency indicated by one-tail Wilcoxon tests (all $P < 0.05$).

mtDNA data

Consensus sequences of 526 bp of the mitochondrial cytochrome oxidase II gene were obtained for 173 specimens from seven population samples (Table 2). The *Oc. caspius* sequences were very A-T rich (on average 76% of the sequence) as reported for several other mosquito species (Chen, Harbach & Butlin 2004). On the whole 21 unique haplotypes were identified from 22 variable positions (4.2% of the sequence); all transitions, of which 16 occur at the third codon position, 5 at the second and 1 at the first. Three substitutions resulted in amino acid changes: one from Trp to Arg, one from Ala to Val, and one from Val to Ile.

The statistical parsimony network representing genealogical relationships among haplotypes is shown in Fig. 2. The haplotypes at the highest frequency were 15 and 21, the latter occupying a central position in the network. The majority of low-frequency haplotypes differed

from high-frequency haplotypes by one or two mutational steps, giving rise to a star-like haplotype network.

Estimates of population haplotypic (h) and nucleotid (π) diversity are given for each sampled population in Table 2. Similar to what was described for allozymes, no significant differences were observed in the distribution of genetic variability levels (χ^2 tests; all $P > 0.05$) with respect to the geographic location of samples (i.e. coastland/inland), the environmental characteristics of the breeding sites (freshwater/brackish water), or among samples subject to control programmes of varying length, if any. Table 3 shows that F_{ST} values between population pairs ranged from 0.001 to 0.029 (average value was F_{ST}= 0.007± 0.01), with no statistically significant differentiation among samples. Similar to the allozyme data, pairwise comparisons between geographic and genetic distances among populations revealed no significant correlation between these measures (Fig. 1; Mantel test: $P > 0.05$).

The distribution of pairwise nucleotide differences among the CO-II haplotypes found showed a bell-shaped distribution (Fig. 3), due to an excess of small pairwise differences, as predicted for populations that have undergone a rapid demographic expansion (Rogers & Harpending 1992). A fitting of the observed to the expected distribution under the sudden expansion model cannot be rejected on the basis of Harpending's raggedness index, which was low (0.041) and non significant ($P = 0.39$). The estimates of the expansion parameters were θ_o = 0.000 and θ_1 = 15.677 and τ = 2.457 (low bound 0.480, up bound 4.524). A large, negative and highly significant value of Fu's F_s statistic (F_s = – 8.373; $P < 0.01$) again suggests the past occurrence of a demographic expansion.

The time since the expansion began can be estimated from the value of τ (the time in generations since the expansion started, in units of mutational time), by dividing by 2μ and multiplying by the generation time (Rogers & Harpending 1992). Assuming $\mu = 1 \cdot 10^{-8}$ (Powell *et al.* 1986; Walton *et al.* 1999; Chen, Harbach & Butlin 2004) and 3 to 6 generations/year (R. Bellini, personal observations), the expansion event would have taken place between 85000 and 10000 years ago.

Discussion

Patterns of genetic diversity and appropriate geographical scale for control planning

Both allozymes and mitochondrial markers concordantly indicated the lack of significant genetic structure in *Oc. caspius* in the Po plain area (Tables 3 and 4, Fig. 1). Moreover, neither allozyme nor mitochondrial data showed any significant differences in the distribution of genetic variability levels (χ^2 tests; all $P > 0.05$) in relation to the environmental characteristics of the breeding sites (freshwater vs. brackish water) or the geographic location of samples (coastland vs. inland areas).

On a regional scale, both of the two types of markers used indicated that there was no equilibrium between migration and genetic drift in the area (see Fig. 1). Genetic distance and its variance were, on the whole, small and did not increase with increasing geographical distances between populations, as would be expected under equilibrium conditions (Hutchison & Templeton 1999). This pattern conforms well to Hutchison & Templeton's (1999) case II, in which the lack of regional equilibrium between gene flow and drift can be attributed to a prevalence of the latter, and suggests a situation resulting from a recent colonization event and the demographic expansion of a relatively homogeneous source population (Hutchison & Templeton 1999). Further evidence in favour of a recent demographic expansion comes from the mitochondrial dataset. Such a scenario is consistent with the star-like shape of the haplotype network shown in Fig. 2. Moreover, the mismatch distribution was smooth and unimodal, fitting the distribution expected under a sudden expansion model (Rogers & Harpending 1992). The significant excess of recent mutations (as evidenced by the significantly large and negative value of Fu's F_s statistic; Fu 1997) is also consistent with this hypothesis. This expansion event, estimated from the observed mismatch distribution using the parameter τ, would have taken place between 85000 and 10000 years ago. In this time span two paleoclimatic events occurred that led to significant changes in the geography of the Po plain, which could have driven demographic expansion: A) during the last glacial period the Adriatic sea retreated and the Po plain extended southward; B) by the end of the last glacial period, glaciers started to withdraw, across Europe the climate became warmer and more humid, and the Adriatic sea shoreline started its northward migration (Van Andel & Tzedakis 1996). On the basis of our data, we cannot distinguish between these two possible scenarios. However, considerations about

temperature, moisture and rainfall regimes, together with paleoenvironmental reconstructions (Amorosi, Cotalongo & Fusco 1999, Cremaschi 2003 a, b) for the said periods, have led us to suggest the second historical event as the most plausible causal factor for the inferred demographic expansion.

For the purpose of the present study it is important to understand whether the observed pattern of genetic diversity at the regional scale can be attributed to historical population size changes or to contemporary gene flow. According to Hutchinson & Templeton (1999), after a range expansion a pattern reflecting panmixia (i.e. with a low degree of pairwise population differentiation and a small variance of the differentiation estimates) as the one we have observed, is expected if gene flow remains the main evolutionary force. In view of these considerations we argue that since the expansion event, gene flow is still the major evolutionary force shaping the genetic patterns observed and our data does not suggest the existence of any significant barrier to gene flow among *Oc. caspius* populations in the Po plain region.

The first implication of these results is that, since gene flow appeared high enough to maintain an extensive genetic homogeneity among populations in the study area, control programmes based on isolated treatments (i.e. at a small geographic scale) are unlikely to be effective. For about 15 years *Oc. caspius* populations have been the target of permanent larval and adult control operations in the Po Delta Emilia-Romagna region. Only recently (since 2000) have the Piedmont rice fields been included within a patchwork of control strategies (Bellini 1998; Bellini & Veronesi 2001). The control programmes targeting some local populations do not seem to have left any long-term marks on their genetic structure and do not seem to have reduced the genetic diversity of the populations themselves. Thus coordinated treatments of breeding habitats, conducted on a wide geographical scale, seem to be the only option for a satisfactory control of *Oc. caspius* populations.

A further implication of our findings is an overall high risk of spread of resistance alleles, due to the abundant gene flow among populations. The main active compounds used for control are pyrethroids, organophosphates and *Bacillus thuringiensis* ssp *israelensis* and the appearance of resistance alleles have been reported for several mosquito species (Hemingway & Ranson 2000). Cases of resistance to such compounds have not yet been reported in *Oc. caspius*. However, the above results call for monitoring programmes aimed at periodically assessing the level of susceptibility of individual populations to these compounds, as well as implementing

insecticide rotation, integrated control and/or other strategies (e.g. Lenormand & Raymond 1998) to maintain the efficacy of the compounds themselves.

Local demographic changes and landscape use

The analysis of possible recent population size changes indicated significant departures from the expectation under mutation-drift equilibrium, in the direction of significant heterozygote deficiency ($P < 0.05$), in all samples studied except Bologna and Crevalcore. Such deficiencies are expected as a result of the recent size growth for some populations, whether accompanying a range expansion or not (Cornuet & Luikart, 1996).

Two main reciprocally non-exclusive causes may have contributed to the observed population size changes: *i)* the change in rice cultivation techniques toward an increasing number of dry/flood cycles; *ii)* the size growth of areas devoted to rice paddies. Rice paddies have long been recognized as an important source of breeding sites for the species. At present, rice fields in the Po plain are mainly managed through a succession of flooding and drying periods, a technique that was introduced in the 1950s. Since then, the number of dry/flood cycles has progressively increased in most part of the study region (Ardizzone *et al.* 1993; Latino *et al.* 2004). This technique enhances the suitability of rice paddies as breeding sites for *Oc. caspius*, because the species oviposit on moist ground and egg hatching occurs when the sites are flooded, therefore the number of generations per year is directly associated with the number of dry/flood cycles. The increasing amount of land devoted to rice fields may have played an important role in the observed demographic expansions. As summarized in Figure 4, populations showing evidence of a recent demographic growth are all located in areas where larger portions of land are being used as rice paddies (since the beginning of the 1980s). Vercelli has the highest number of rice fields and there has been no change in land use but the number of dry/flood cycles has increased. The finding that this population has also expanded is further evidence of the role of agronomic changes in the recent demographic history of *Oc. caspius*. In Bologna there has also been an increase in the number of dry cycles, but the drastic reduction of rice areas might have contrasted demographic growth, resulting in the observed lack of size change.

An interesting approach to the control of *Oc. caspius* in a rice-growing environment might thus be the reduction or elimination of multiple dry phases, in favour of the traditional farming technique that entails a single annual flooding. In this context, agronomic

experimentation takes on a fundamental role. There are recent agronomic experiences in which planting is done on a dry field (with brief flooding, if necessary, such as to help germination without enabling mosquito development), followed by a final submersion with water treated with herbicides (Riso secondo natura® Vercelli-Italy, organic farming). Such a practice would significantly curb growth in the populations of *Oc. caspius* by reducing egg-laying opportunities.

Conclusions

At present, control programmes in the Po plain follow local administration boundaries, without any reliable knowledge of the structure of populations. Our study shows there is substantial lack of genetic structure for *Oc. caspius* populations in the study area. Therefore, control programmes based on isolated treatments are unlikely to be effective and there is a high risk of a fast spread of resistance alleles. Furthermore, there is evidence indicating that in the study area rice paddies have played a major role in the recent demographic history of the species. Therefore, satisfactory programmes to control *Oc. caspius* populations in Northern Italy should: *i)* be mainly focused on rice paddies as breeding habitats, *ii)* favour agronomical techniques involving a reduced number of dry/flood cycles, *iii)* be carried out on a wide (regional) geographical scale, *iv)* involve the periodical assessment of the susceptibility of individual populations to the compounds used in the control campaigns.

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Table 1. Collection site features of *Ochlerotatus caspius* in the Po plain and number of specimens analysed for allozymes and mitochondrial cytochrome oxidase II gene

Code	Locality	Sampling date	Geographic location	Breeding site (°/°° NaCl)	Starting date of control Measures*	Sample size	
						Allozymes	mtDNA
1	Ticineto	May 2002	44°45'N-8° 33' E	Rice paddies	2000	58	29
2	Vercelli	May 2002	45°20'N-8° 23' E	Rice paddies	2000	84	20
3	Milano	June 2000	45°29'N-9° 4' E	Rice paddies	Never	61	28
4	Crevalcore	July 2000	44°44'N-11° 7' E	Freshwater	Never	33	-
5	Bologna	June 2001	44°32'N-11° 15' E	Rice paddies	Never	24	24
6	Comacchio	July 2002	44°43'N-12° 8' E	Rice paddies	Never	40	26
7	Lidi di Comacchio	August 2000	44°42'N-12° 15' E	Saltemarsh (26)	1990	32	-
8	Spina-Salicornieto	August 2000	44°41'N-12° 6' E	Freshwater	1990	23	-
9	Lidi di Spina	May 2001	44°36'N-12° 17' E	Saltemarsh (28)	1990	27	20
10	Sacca di Bellocchio	July 2002	44°33'N-12° 12' E	Saltemarsh (48)	1990	35	-
11	Orsi Mangelli	July 2002	44°28'N-12° 9' E	Freshwater	1990	40	23

*Bellini 1998; Bellini and Veronesi 2001

Table 2. Estimates of genetic diversity at allozymes and mitochondrial cytochrome oxidase II gene for *Ochlerotatus caspius* samples studied.

	Allozymes				mtDNA		
Locality	**Mean number of alleles per locus**	**Percentage of polymorphic loci**	**Mean observed heterozygosity (± SE)**	**Mean heterozygosity expected under Hardy-Weinberg equilibrium**	**Haplotypes**	**Haplotipic diversity (± DS)**	**Nucleotidic diversity (± DS)**
1 Ticineto	2.2 ± 0.4	55.6	0.143 ± 0.051	0.140 ± 0.048	1(2), 7, 10(2), 12, 14, 15(8), 16(2), 21(12)	0.761 ± 0.059	0.003 ± 0.002
2 Vercelli	2.0 ± 0.3	55.6	0.173 ± 0.058	0.167 ± 0.060	6, 7, 10(2), 11(2), 12(2), 13(2), 14, 15(2), 16(2), 17(2), 21(6)	0.917 ± 0.036	0.004 ± 0.002
3 Milano	2.6 ± 0.4	66.7	0.176 ± 0.046	0.190 ± 0.055	9(3), 12(2), 14, 15(8), 16(4), 19(2), 21(10)	0.807 ± 0.043	0.004 ± 0.003
4 Crevalcore	2.3 ± 0.4	66.7	0.196 ± 0.051	0.246 ± 0.073			
5 Bologna	2.4 ± 0.3	66.7	0.213 ± 0.071	0.203 ± 0.062	7, 9, 10(2), 12(2), 15(2), 16(2), 17(2), 19(2), 20(2), 21(6)	0.917 ± 0.036	0.004 ± 0.003
6 Comacchio	2.8 ± 0.5	77.8	0.203 ± 0.061	0.200 ± 0.060	2(2), 4(2), 6, 10(2), 14, 15(7), 18, 21(10)	0.788 ± 0.058	0.004 ± 0.002
7 Lidi di Comacchio	2.4 ± 0.3	66.7	0.125 ± 0.030	0.158 ± 0.045			
8 Spina-Salicornieto	2.2 ± 0.3	66.7	0.150 ± 0.045	0.170 ± 0.052			
9 Lidi di Spina	2.3 ± 0.4	44.4	0.169 ± 0.049	0.157 ± 0.057	4, 8(2), 10(4), 12(3), 15(4), 21(6)	0.837 ± 0.044	0.004 ± 0.002
10 Sacca di Bellocchio	2.2 ± 0.3	66.7	0.163 ± 0.048	0.186 ± 0.066			
11 Orsi Mangelli	2.5 ± 0.4	77.8	0.185 ± 0.046	0.167 ± 0.044	3, 4(2), 5, 6(2), 7(2),11(3), 13, 14, 15(3), 16(2), 21(5)	0.921 ± 0.032	0.005 ± 0.003

SE, standard error; DS standard deviation

Table 3. Weir and Cockerham's (1984) F_{ST} between population pairs for allozyme variation.

Samples	1	2	3	4	5	6	7	8	9	10	11
1 Ticineto	*****										
2 Vercelli	0.045	*****									
3 Milano	0.014	-0.005	*****								
4 Crevalcore	0.049	0.016	-0.007	*****							
5 Bologna	0.035	0.034	0.028	0.041	*****						
6 Comacchio	0.021	0.011	0.004	0.021	0.008	*****					
7 Lidi di Comacchio	0.042	0.035	0.021	0.054	0.014	0.009	*****				
8 Spina- Salicornieto	0.029	-0.005	-0.007	0.022	0.018	0.001	-0.001	*****			
9 Lidi di Spina	0.061	0.023	0.012	0.036	0.037	0.026	-0.005	0.002	*****		
10 Sacca di Bellocchio	0.099	0.006	0.020	0.033	0.044	0.031	0.029	-0.002	0.010	*****	
11 Orsi Mangelli	0.041	0.044	0.021	0.047	0.024	0.024	-0.009	0.009	-0.007	0.040	*****

Table 4. Weir and Cockerham's (1984) F_{ST} estimates between pairs of *Ochlerotatus caspius* samples studied for mitochondrial cytochrome oxidase II sequence

Samples	1	2	3	5	6	7	11
1 Ticineto	****						
2 Vercelli	0.019	****					
3 Milano	-0.011	0.016	****				
5 Bologna	0.022	-0.017	0.008	****			
6 Comacchio	-0.018	0.021	0.002	0.023	****		
7 Lidi of Comacchio	0.011	0.002	0.029	0.016	-0.001	****	
11 Orsi Mangelli	0.027	-0.018	0.018	0.005	0.018	0.020	****

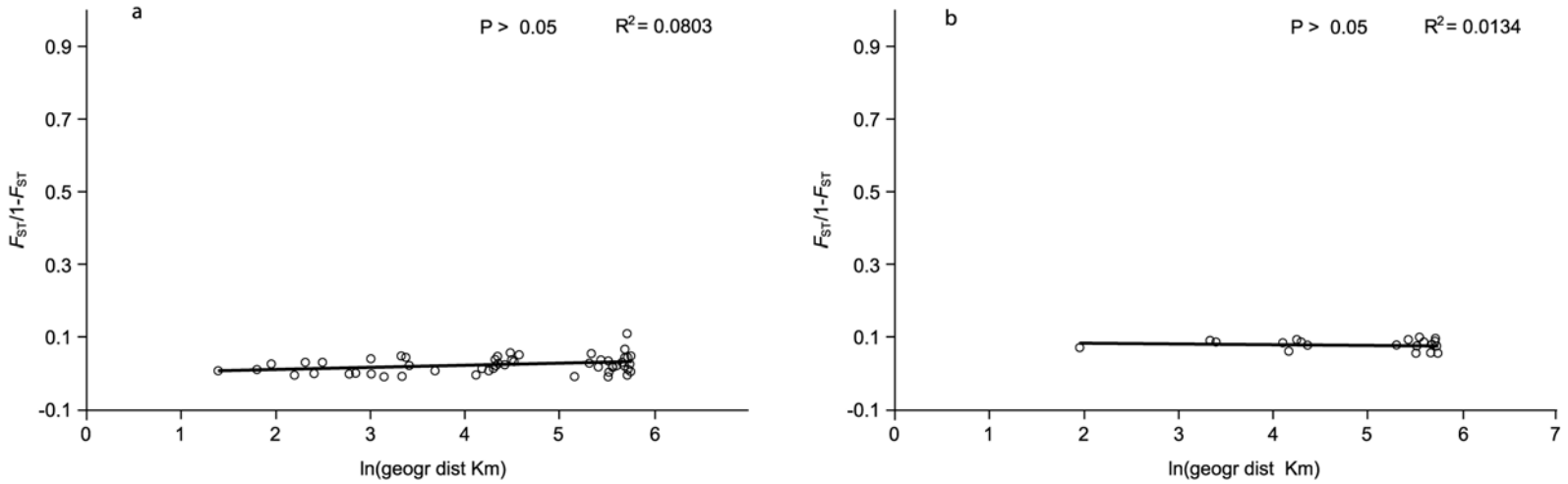

Fig.1. Pairwise $F_{ST}/1\text{-}F_{ST}$ genetic distances for allozyme loci (a) and mtDNA cytochrome oxidase II sequences (b) regressed on corresponding ln (geographic distance in Km). *P*-values are the significances of the observed correlations as estimated by a Mantel test (10000 permutations).

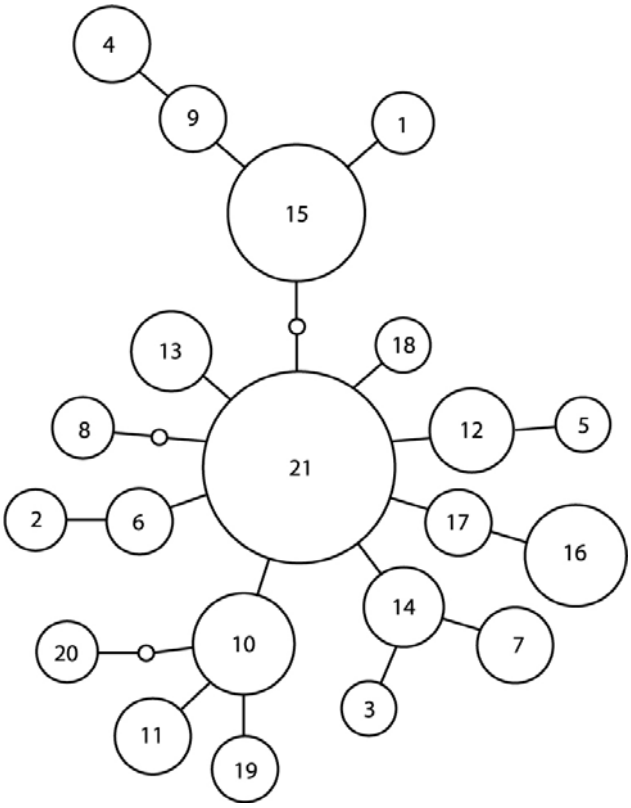

Fig. 2. Statistical parsimony network representing genealogical relationships among haplotypes observed in all samples of Po plain for the cytochrome oxidase II encoding gene. The relative abundance of each haplotype is indicated by the circle size.

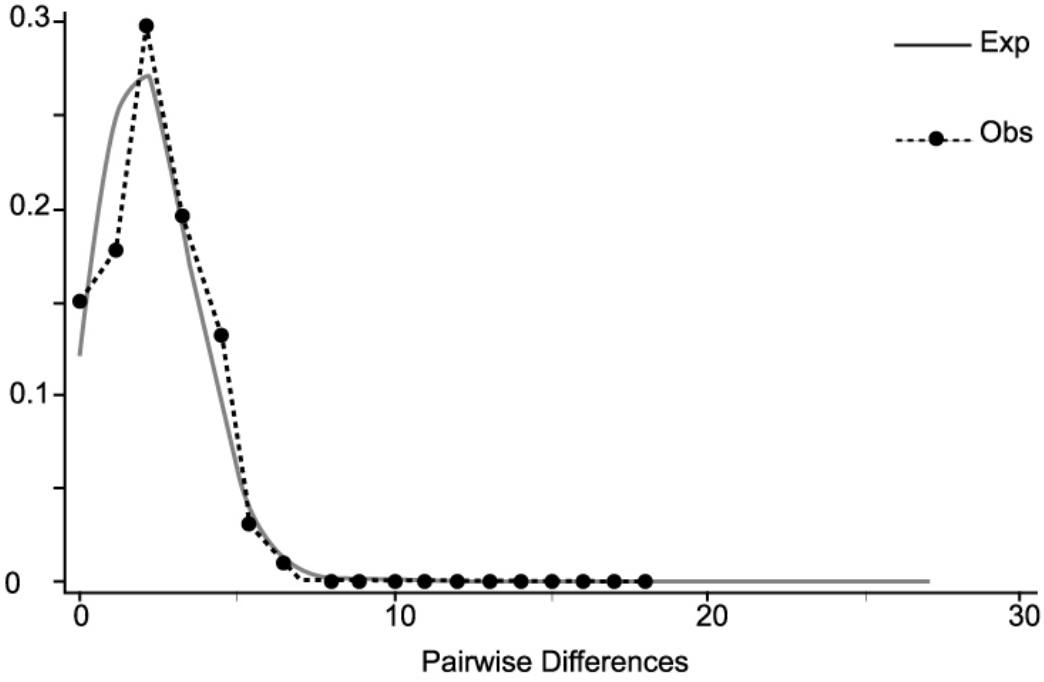

Fig. 3. Mismatch distributions for all sampling localities in Po plain: grey line, expected; dashed line with circles, observed.

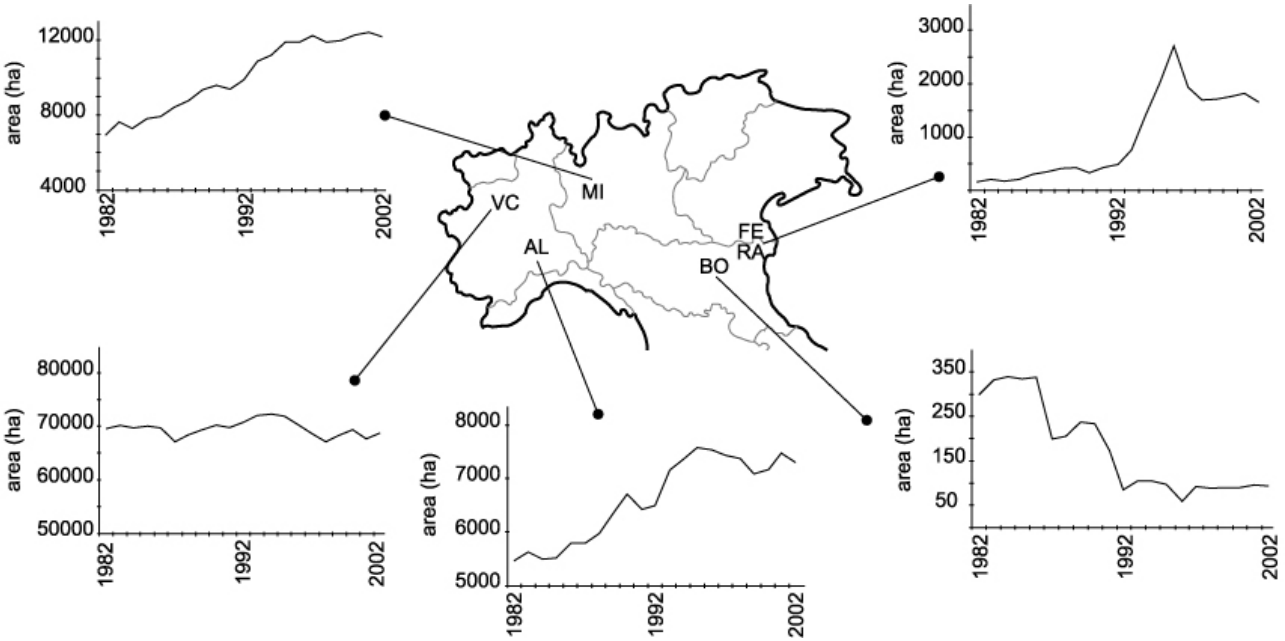

Fig. 4. Areas devoted to rice-growing from 1982 to 2002. VC, Vercelli; AL, Alessandria; BO, Bologna; RA, Ravenna; FE, Ferrara; MI, Milano; ha, hectares (modified by Ente Nazionale Risi, http//www.enterisi.it/index.jsp).

3. P-GLYCOPROTEIN EFFLUX PUMPS AS A DEFENCE MECHANISM AGAINST INSECTICIDES IN THE MOSQUITO *OCHLEROTATUS CASPIUS* (DIPTERA: CULICIDAE)

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Abstract

P-glycoproteins (P-gps) are efflux transporters found in all living cells that protect them from multiple structurally dissimilar toxic compounds. Their action consist in transporting toxic substances outside the cell, reducing the concentration inside cells as much as possible. The purpose of this study was to examine in the mosquito *Ochlerotatus caspius* the possible role of P-gps in the defence against insecticides temephos, ivermectin and diflubenzuron and to isolate genomic DNA sequences encoding for P-gp transporters. Mosquito larvae were treated with insecticides alone and in conjunction with a sublethal dose of P-gp inhibitor verapamil. The inhibition of P-gps reduced the LD50 values of temephos and diflubenzuron by a factor of 3.5, and 16.4 respectively, suggesting the potential involvement of P-gps in insecticide defence. Using a polymerase chain reaction (PCR)-based approach, a 476-base-pair sequence was isolated, whose inferred nucleotide sequence shows high homology with the C-terminal sequence of known P-gps. The isolation and characterization of a putative P-gp sequence from *Oc. caspius* is the first step to a better molecular understanding of the role played by multidrug transporters in the defence against insecticides in this species. This knowledge could open the way to a novel control strategy based on the inhibition of pest defences. The beneficial consequences of the inhibition of efflux pumps in improving insecticide performance are discussed.

Key words: P-glycoprotein; insecticide toxicity; mosquito control; *Ochlerotatus caspius,* verapamil; temephos; diflubenzuron.

Introduction

The development of insecticide resistance in arthropods is one of the main limits of the chemical control of agricultural pest and vector species of public health concern (WHO 1992; Denholm *et al.* 1998; Hemingway and Ranson 2000). This phenomenon has important implications in pest management, as chemical measures remain central to pest control plans, especially when insect density, health risks and the consequent economic damage are very high (Casida and Gary 1998). Increased number and rates of application due to reduced level of control in resistant populations may lead to a higher environmental load. There is a limited number of classes of available insecticides, even fewer insecticidal modes of action and cross-resistance enables simultaneously the use of different insecticide compounds. The discovery and development of new insecticides is slow as new products have to meet rising standards of environmental as well as toxicological safety (Ware 2000). Therefore, the evolution of pesticide resistance must be slowed down by making optimal use of existing compounds and improving our knowledge about the interaction between cells and toxic substances in order to find novel target sites for compounds with a low, if any, environmental impact (Kogan 1998).

Metabolic detoxification is known in arthropods as the main defence mechanism against on a broad array of substrates, including both naturally occurring plant allelochemicals and artificial pesticides. Three enzymatic systems are mainly involved in detoxification: esterase, glutathione-S-transferase and cytochrome P450-dependent monooxygenase. An increase in the activity of one of these systems is correlated with the development of insecticide resistance (reviews in Hemingway and Ranson 2000). More recently, several studies have pointed to another possible cellular defence mechanism which is not associated with the metabolic conversion of toxic compounds. Implicated in this mechanism are ATP-dependent membrane proteins, denominated P-glycoproteins (P-gps), whose action consists in transporting toxic substances outside the cell and reducing their concentration inside the cells as much as possible (Blackmore *et al.* 2001; Borges-Walmsley *et al.* 2003; Chang 2003). They belong to ABC (ATP-Binding- Cassette) transporters and have been suggested to act as the cells' "first line of defence", relying on a mechanism other than detoxification, which may come into play later, working in synergy with the transporters. Membrane transporters are capable of acting upon a broad range of structurally and chemically different compounds (*multidrug transporters*). In bacteria and in man they have been well characterised and widely studied in connection with anti-tumour chemotherapy treatments, since the amplification of

these transporters is correlated with the resistance to a broad range of drugs that tumour cells have acquired (*multidrug resistance*) (Lage 2003).

In the past decade evidence emerged that insecticides act as substrates for P-gps, and proteins similar to P-gps have been found in insects such as *Chironomus riparius* and *Manduca sexta* (Gaertner *et al.* 1998; Podsiadlowski *et al.* 1998). Lanning *et al*. (1996) found evidence supporting the role of P-gps in the development of pesticide resistance, i.e., in the tobacco budworm (*Heliothis virescens*) against thiodicarb. Only recently P-gps have also been found in the mosquito *Culex pipiens*, and they are thought to be involved in defence mechanisms against insecticides (Buss *et al*. 2002). The existence of such a mechanism protecting insects from a broad spectrum of chemical products and thus the possibility that other sites are involved in endowing them with resistance poses an additional challenge to pest control planners. However, identifying P-gp sites with the ultimate aim of inhibiting them could represent a new control strategy. By increasing the susceptibility of organisms to insecticides, it would be possible to reduce their doses and frequency of application. The implementation of such a strategy, which could support the use of insecticides, requires the availability of species-specific inhibitors in order to avoid the serious consequences that would derive from a generic inhibition of P-gp in non-target organisms. All the compounds currently capable of inhibiting P-gps act upon protein activity as competitive inhibitors being substrates to P-gp themselves (e.g. verapamil) or non-competitive inhibitors inducing changes in P-gps that inhibit linkage with ATP and, consequently, the transportation of the cytotoxic compound (reviewed in Thomas and Coley 2003). Both types of inhibitors are generic, hence the need for the molecular characterization of the multidrug P-glycoprotein genes by which it will be possible to find out species-specific inhibitors of the genes themselves.

We investigated in the mosquito *Ochlerotatus caspius* (Pallas) the existence of P-gp multidrug transporters and their potential involvement in cell defence against insecticides: temephos, ivermectin and diflubenzuron. At this purpose we conducted bioassays with insecticides and chemical inhibitors of P-gp activity. In addition we aimed to identify and isolate genomic DNA sequences belonging to genes encoding P-gp transporters through a polymerase chain reaction (PCR) based approach. *Oc. caspius* is a mosquito species of great public health and economic impact in Mediterranean countries such as Spain, France, Italy and Greece, where it is widely spread. Given its marked anthropophily, this species has significantly impaired the liveability of large areas. In Northern Italy this species is the major

target of the pest control programs implemented by local authorities (Bellini 1998; Bellini and Veronesi 2001; Marasca and Bellini 2001).

Materials and Methods

Mosquito samples

The *Ochlerotatus caspius* samples used in this study were collected as 3rd stage larvae in Comacchio area (Ferrara province, Northern Italy). For the taxonomical identification of collected specimens we used the morphological key by Schaffner *et al.* (2001). Larvae were brought in the laboratory and directly used in the experiments.

Chemicals

Three insecticides with different chemistry and modes of action were used in the experiments: temephos (technical grade: 90,60 %), obtained from Industria chimica Leica S.p.A. Forlì, Italy, ivermectin (technical grade: minimum 90 % ivermectin B1A) obtained from Sigma-Aldrich S.r.l. Milan, Italy, diflubenzuron (technical grade: 90,1 %) obtained from Chemtura Italy, Latina, Italy.

Temephos is an organophosphate (OP) pesticide. It acts by inhibiting the acetylcholinesterase, resulting in acetylcholine accumulation in neuromuscular synapses. The acute toxic effects of organophosphate pesticides are due to the hyperstimulation of muscarinic and nicotinic receptors, resulting in symptoms that range from increased secretions to death by respiratory depression (Ware 2000)

Ivermectin is a semi-synthetic drug derived from 22,23-dihydro avermectin B1 that is produced by *Streptomyces avermectilis*. The observed effects of ivermectin on the mosquito larvae are probably correlated with chloride channel activation on cell membranes. The bind of ivermectin to GABA results in the activation of chloride channels of postsynaptic membranes. This promotes an influx of chloride ions and irreversible hyperpolarization, with the consequent inhibition of transmission signals (Turner and Schaeffer 1989). Mahmood *et al.* (1991) observed that in *Aedes aegypti* ivermectin directly or indirectly affected at least three major organ systems (nervous, digestive, and reproductive). Interference in the Malpighian tubules and water balance as well as alterations in fat body were also observed (Strong 1993; Alves *et al.* 2004).

Diflubenzuron belongs in a class of insecticides that act as insect growth regulators (IGRs). They interfere with chitin synthesis, and are taken up more by ingestion than by

contact. When the larvae ingest the active ingredient, it disrupts the development of the exoskeleton, resulting in death of the larvae (Ware 2000). With increasing environmental regulations worldwide, the IGR family is a good answer to the demand for safer insect control products.

Verapamil (>99 purity, Sigma-Aldrich S.r.l.), a known modulator of P-glicoproteins, was used in conjuction with insecticides to inhibit P-gp activity. It is itself substrate of P-gps and thus works by competing with cytotoxic compounds for efflux by the membrane pumps (Thomas and Coley 2003).

Bioassays

Assays were conducted on fourth-instar mosquito larvae using standard methods (WHO 1981). Insecticides were weighted and dissolved in acetone to make the mother solutions, which then were diluted with water into test solutions. All tests were conducted using six insecticide doses planned to have mortality in the range 1 – 99 %. Each experiment was replicated four times. Groups of 25 larvae were put in plastic glasses of 300 ml with 100 ml of water and treated with insecticide and insecticide + verapamil. Additional groups of larvae were treated with only water and acetone as controls. Larvae were held at constant temperatures of 25 ± 2 ° C. Mortality was assessed at 24 h post-treatment. The larvae were considered dead if immobile and unable to reach the water surface. The subletal dose of verapamil used in bioassays with insecticides was evaluated treating larvae with six concentrations of verapamil ranging from 10 to 320 μM, following the same protocol described above .

The data were subjected to probit regression analysis (Finney 1971) using POLO-PC (LeOra Software POLO-PC, Berkeley, California) to determine the LD_{50} values as well as their 95% confidence intervals (CI). Failure of 95% CI overlap was used as the criteria for identifying significant differences among LD50 values of insecticide alone and insecticide + verapamil.

Molecular analysis

Degenerate oligonucleotides design. To identify and isolate genomic DNA sequences homologous to genes encoding P-gp multidrug transporters a polymerase chain reaction (PCR) approach was applied (Mäser and Kaminsky 1998; Mendes do Nascimento *et al.* 2002). All eukaryotic ABC transporters present two highly conserved segments, the so-called

Walker A and Walker B motifs in the ATP-binding domains (Walker *et al.* 1982). Thus we aligned Walker motifs of P-gp type transporters of different organisms available in GenBank to design oligonucleotide primers. For alignments CLUSTALX software was used (Thompson *et al.* 1997) and two pairs of degenerate oligonucleotide primers were synthesized (MWG biotech, Milan Italy): ABC-cas_1 (5'GTYGGTTCHTCHGGHTGYGGWAA-3'), and
ABC-cas_3 (5'-AAGGKSARACGSTBGCCCTGGTTGGA-3'), forward;
ABC-cas_2 (5'-RTCYAAAGCDGADGTDGCYTCATC-3') and
ABC-cas_4 (5' GAGGTBGCYTCGTCCAGCAGVAGGA -3'), reverse.

PCR amplification and cloning. DNA was extracted from single mosquito adults following the protocol by Collins *et al.* (1987). PCR analysis was performed in a reaction mixture consisting of 2.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 μM (each) dATP, dCTP, dGTP, and dTTP, 1 μg of each primer, 0.5 U of Taq DNA polymerase (Promega, Milan Italy), and 50 ng of template DNA. Manipulations were carried out with dedicated DNA-free pipettes in a sterile field to minimize the risk of contamination. Amplification was performed in a PTC-150 Minicycler™ MJ Research. PCR cycling procedure was: 95°C for 5 min followed by 33 cycles of 93°C for 1 min, 52°C for 45 s (ABC-cas_1 x ABC-cas_2 and ABC-cas_3 x ABC-cas_4), 72°C for 1 min 30s, and a final step at 72°C for 10 min. The amplified products were resolved by electrophoresis on a 1% agarose gel TBE buffer. Then, the PCR products of the expected size corresponding to Walker A-Walker B motifs (~500 base pairs) were selected and purified with the QIAquick gel extraction kit (QIAgen, Milan Italy). The purified PCR fragments were cloned using a pGEM-T easy kit (Promega, Milan Italy). Ligation into plasmid vector, transformation of competent cell, plamid preparations, and sequencing of the inserts followed standard protocols (Sambrook *et al.* 1989). Each insert was sequenced on both strands with ABI PRISM 377 DNA sequencer (PE Applied Biosystems) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. The sequencing chromatograms were analysed with the program CHROMAS (Technelysium Pty Ltd, Australia). The data base for EMBL (European Molecular Biology Laboratory) and NCBI (Nucleic Center for Biotecnology Information) DNA and proteins was thus screened using the TBLASTX option in BLAST searches (Basic Local Alignment Search Tool, Altschul *et al.* 1990) in order to characterize by homology the sequences isolated in *Oc. caspius*. This program translates the nucleotide sequence in all six frames and compares the output against all the translated sequences in the database. It therefore maximises the potential for retrieving sequences similar to the sequence of interest.

Southern blotting analysis. The genomic organization of the P-gp sequences isolated was investigated by Southern blots. DNA was extracted from single adult mosquitoes according to methods previously described (Collins *et al.* 1987) and digested with *Eco*RI restriction endonuclease. Fifteen micrograms of digested DNA were separated on a 0.8% agarose gel and transferred to a Hybond N^+ membrane (Amersham Pharmacia, Piscataway, NJ, USA). Hybridization was carried out under high stringency conditions (65 °C) with the probe labelled with the non-radioactive DIG DNA labelling and detection kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. After hybridization, the blot was blocked and incubated with antidigoxigenin antibodies (Boehringer Mannheim, Indianapolis, IN). Immunoreactive bands were visualized using the CSPD detection system (Boehringer Mannheim, Indianapolis, IN).

UPGMA analysis. The DAMBE software (Xia and Xie 2001) was used to align the ABC cassette sequences identified in *Oc. caspius* and the C-terminal ATP-binding cassette homologous obtained from GenBank. The PHYLIP 1.0 package (Felsenstein 1993) was used to construct a genetic distance matrix from protein sequence data (PROTDIST programme). Cluster analysis was thus performed by the unweighted pair group method with arithmetic averaging (UPGMA, Sokal and Michener 1958). The robustness of the phylogenetic trees was evaluated by comparing the dendrograms by bootstrap analysis (Felsenstein 1985) with 1000 replicates.

Results

Bioassays

The LD_{50} values resulting from mortality data subject to probit regression analysis against log [insecticide concentration] are reported in Tab.1.

No died larvae were found in all control samples treated with water and acetone.

The addition of verapamil significantly increased the toxicity of temephos (3.5 fold) and diflubenzuron (16,4 fold) (Table 1). The treatment ivermectin + verapamil gave non linear dose/mortality results (4 replicates) which prevent from LD_{50} calculation.

Isolation of P-gp type genomic sequences

Identification of ATP-binding cassettes by PCR. Degenerate oligonucleotide primers corresponding to the sequences of the Walker A and B motifs in the ATP-binding domains (Walker *et al.* 1982) were used for PCR amplifications. Agarose gel electrophoresis of PCR

products revealed bands at the expected size of ~500 bp for only the ABC-cas_3 x ABC-cas_4 primer combination. This band was excised from the gel and DNA fragments were cloned. By screening the sequences from about 34 transformant colonies we identified one fragment, designated as *Ochc*F10, which contained typical ATP-binding boxes and ABC signature sequences (accession number DQ869035, Fig. 1).

Sequence comparison. The ABC cassette newly identified in *Oc. caspius* was compared against databases using TBLASTX (Altschul *et al.* 1990) algorithm. Alignment revealed a pronounced homology of 91% identical residues to the C-terminal ATP-binding cassette of the protein EAT37643 of *Aedes aegypti* (Fig. 2). Homologies were found also with the C-terminal ATP-binding cassette of the the protein ENSANGP00000021663 of *Anopheles gambiae* (90% of identity), proteins Mdr49, Mdr50 and Mdr65 of *Drosophila melanogaster* (72, 67 and 68%, respectively of identical residues, Wu *et al.* 1991; Gerrard *et al.* 1993), proteins GA10136-PA and GA21135-PA of *Drosophila pseudoobscura* (66 and 67% of identical residues, respectively Richards *et al.* 2005) and with the P-glicoprotein MDR1 of *Homo sapiens* (65% of identical residues, Chen *et al.* 1990). Accession numbers for all gene products described in this report are listed in Figure 2.

UPGMA analysis. Cluster analysis was performed using the ATP binding cassette identified in *Oc. caspius* and the C-terminal ATP-binding cassette of human, mosquitoes and *Drosophila* P-gp proteins from GenBank which presented the highest homology by TBLASTX analysis. Three clusters were observed: one that comprises the *Ochc*F10 sequence isolated in *Oc. caspius*, the protein EAT37643 of *Aedes aegypti,* ENSANGP00000021663 of *Anopheles gambiae* and the Mdr49 of *Drosophila melanogaster*; a cluster grouping the protein GA21135 of *D. pseudoobscura* and Mdr50 of *D. melanogaster*; a third cluster constituted by the proteins GA10136 of *D. pseudoobscura*, Mdr65 of *D. melanogaster* and human MDR1 (Fig. 3).

Southern blotting. The PCR product of the *Ochc*F10 clone was used as probe for the hybridisation. Figure 4 shows the autoradiographs of Southern blots containing DNA from of *Oc. caspius* samples digested with *Eco*RI restriction endonuclease. Two fragments were observed of about 4.2 and 5.2 kb.

Discussion

P-glycoproteins are membrane efflux pumps whose presence has been detected in all eukaryotic and prokaryotic cells. In this paper we investigated in the mosquito *Ochlerotatus*

caspius the involvement of P-gp transporters in cell defence against insecticides: temephos, ivermectin and diflubenzuron. To determine whether P-gps influenced insecticide toxicity, a classical approach was used: bioassays with insecticides alone and with chemical inhibitors of P-gps activity (Podsiadlowski *et al.* 1998; Buss *et al*. 2002; Challagan and Denny 2002).

Of the insecticides tested diflubenzuron showed the highest toxicity (LD_{50} 0,001 mg/L) followed by temephos and ivermectin (LD_{50} 0,0028 and 0,01 mg/L, respectively). A significantly increase in the toxicity of all insecticides was observed in presence of verapamil at sublethal concentration. Such results indicate the involvement of P-gp activity in defence against these compounds. However some differences were observed: for temephos, mortality rate with the addition of verapamil resulted in a significantly reduction of LD_{50}; a stronger effect of verapamil was observed for diflubenzuron (Table 1); in the case of ivermectin+verapamil in order to fit in the 1-99 % mortality range it has been necessary to reduce the ivermectin dosage of about 300 times, and despite the 4 replicates conducted we were not able to obtain linear dose/mortality results permitting probit analysis. Verapamil synergic effect on ivermectin was the most pronounced we observed in the three insecticides analysed.

Three main issues could account for the different behaviour of temephos, ivermectin and diflubenzuron in our experiments and in the literature.

1) The molecular mechanism by which multidrug transporters can extrude multiple structurally unrelated substrates is currently topic of debate as their behaviour cannot be explained by the establishing of a precise network of hydrogen bonds or other specific interactions characteristic of traditionally studied enzymes and receptors. Recent advances in the structural analysis of a number of soluble multidrug-recognizing proteins show that these proteins have large hydrophobic binding sites. These results suggest that they could bind their substrates through a combination of a hydrophobic effect and electrostatic attraction (reviewed in Neyfakh 2002). Thus the differences could be ascribed to the characteristic transport mechanism of P-gps and/or the different biochemical features of these compounds.

2) Another issue is the target site of these insecticides. Organophosphates like temephos act on insect nervous system, whereas ivermectin seems to affect directly or indirectly the nervous, digestive, and reproductive systems (Mahmood *et al.*1991). Interference with the Malpighian tubules and water balance was also observed (Strong 1993; Alves 2004). Analogously, diflubenzuron presents a wider spectrum of effects, interfering with chitin synthesis and being absorbed rather by ingestion than by contact (Ware 2000).The presence of

P-gp-like transporters was observed in the Malpighian tubules and intestinal tube of insects, and their involvement in cellular defence against toxic compounds was shown (Gaertner *et al.* 1998; Leader and O'Donnell 2005). Therefore, P-gp's could be a better defence than temphos against invermectina/diflubenzuron because these compounds act either on several target organs or on target organs with abundant P-gp's, or both.

3) Finally, substrate specificities in different P-gp genes could explain the differences observed in toxicity alteration of verapamil against organophosphates in *C. pipiens* and *Oc. caspius* (Buss *et al*. 2002).

A second topic of interest emerging from toxicological data, concerns pest control planning. In *Oc. caspius*, as well as for *Chironomus riparius* and *Culex pipiens,* evidence has been found of P-glycoprotein involvement in cellular defence response against insecticides (Podsiadlowski *et al.* 1998, Buss *et al.* 2002). This class of membrane transporters thus represents a potential resistance-inducing mechanism and hence a potential target of inhibitory agents to be used in synergy with insecticides so as to enhance the efficiency of the latter. However, in order for such a strategy to be practicable, specific inhibitors must be identified for the target species. The use of non-specific inhibitors could have a severe impact on non-target organisms, depriving them of an important defence mechanism against toxic compounds of varying nature. Identifying specific inhibitors requires that multidrug transporters be molecularly characterized in terms of both protein and gene components, which would in turn lead to a better understanding of their role and specificity, mechanisms of action and evolution in different taxa. To our knowledge, no member of the class of multidrug transporters has been described at a molecular level in mosquito species (Diptera: Culicidae), with the exception of *Anopheles gambiae* and *Aedes aegypti* (genome sequencing project). Therefore, the second part of this paper aimed to isolate P-gp genomic sequences in *Oc. caspius*. The oligonucleotide primers ABC3xABC4 allowed us to isolate in *Oc. caspius* a nucleotide sequence (denominated *Ochc*F10) containing the Walker motifs characteristic of P-gp's. High homology has been found among the amino acidic sequence inferred by nucleotide sequence of the clone *Ochc*F10 and the C-terminal sequence of P-gp homologous to MDR1 of humans (Chen *et al.* 1990). By UPGMA analysis *Ochc*F10 clustered together P-gps of *Ae. aegypti*, *An. gambiae*, and Mdr49, of *D. melanogaster* (Fig. 3). Thus, the characterization by homology of the sequence *Ochc*F10 isolated in *Oc. caspius*. suggests that it may corrispond to the portion of P-gp related to the gene homologous to Mdr49 of *D. melanogaster*. The restriction pattern observed by Southern hybridization using the sequence *Ochc*F10 as probe

suggests a single copy organization in *Oc. caspius* genome. The double bands observed could be result by a polymorphism in or around the P-gp gene sequence. Alternatively, as P-gp genes present two domains containing Walker motifs, the double bands may result from hybridization of the probe with both such regions. This hypothesis is no well supported by the high stringency condition (65° C) of hybridization or by the high degree of differentiation observed between the amino acid sequence of the C- and N-terminal domains of the same P-gp (i.e. Mdr49, Mdr50, Mdr65 of *D. melanogaster*).

In summary, the isolation and preliminary characterization of a P-gp putative sequence from *Oc. caspius* is the first approach to a better molecular understanding of the role played by multidrug transporters in the defence of this species against insecticides,which could open the way to novel control strategies based on the inhibition of pest defences. In the last few years, medical research has pointed out the numerous potentially beneficial consequences of the inhibition of efflux pumps in improving the clinical performance of various antibiotics (Wright 2000; Lewis 2001; Lomovskaya and Watkins 2001). Nature has already done it: plant amphipathic cations, the berberine alkaloids, are good MDR substrates. Berberis plants produce 5'-methoxyhydnocarpin-D, an MDR inhibitor that enhances the action of berberine (Stermitz *et al.* 2000). Emulating nature's strategy by empowering insecticides with P-gp inhibitors can be an effective strategy against pest insects with a lower toxicological burden for the environment.

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Table 1. Toxicity of verapamil and insecticides against *Ochlerotatus caspius* larvae. n, larvae used in bioassays.

Insecticide	**n**	**Slope (± SE)**	**LD_{50} (95% CI)**
Verapamil	600	2.607 ± 0.283	268.001 µM (230.0-326.3)
Temephos	600	5.108 ± 0.453	0.0028 mg/L (0.0026-0.003)
+ verapamil (30µM)	600	0.616 ± 0.090	0.0008 mg/L (0.0004-0.0013)
Ivermectin	600	3.335 ± 0.271	0.010 mg/L (0.009-0.010)
+ verapamil (30µM)	600	-	-
Diflubenzuron	600	1.487 ± 0.099	0.001 mg/L (0.001-0.001)
+ verapamil (30µM)	600	0.649 ± 0.090	0.000061 mg/L (0.000032-0.000096)

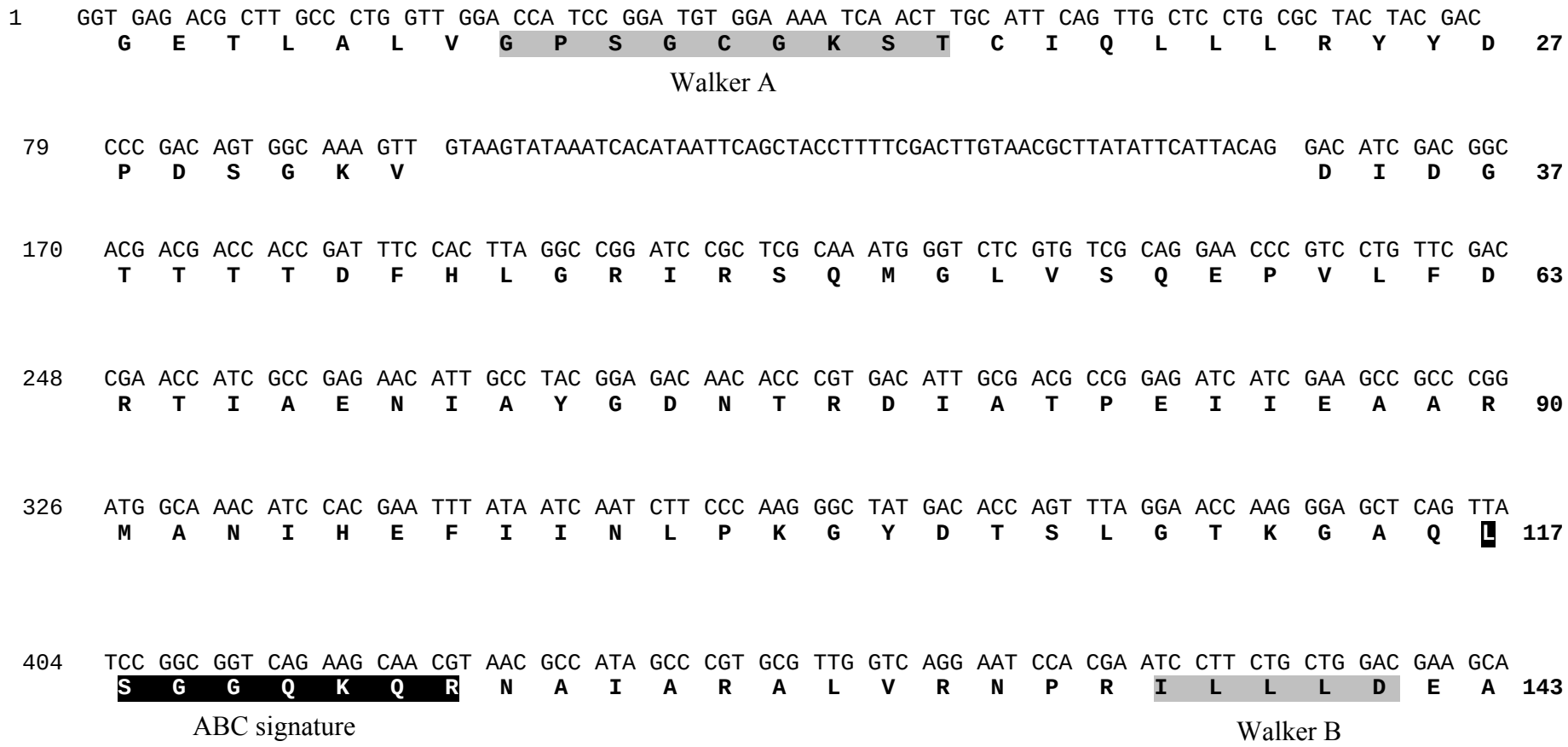

Figure 1. Nucleotide and deduced amino acid sequence of *Ochc*F10 clone. Numbers on the left refer to nucleotide sequence; numbers on the right indicate amino acid positions. Walker ATP-binding motifs A and B, and ABC signature (Walker *et al.* 1982) are shaded in grey and black, respectively.

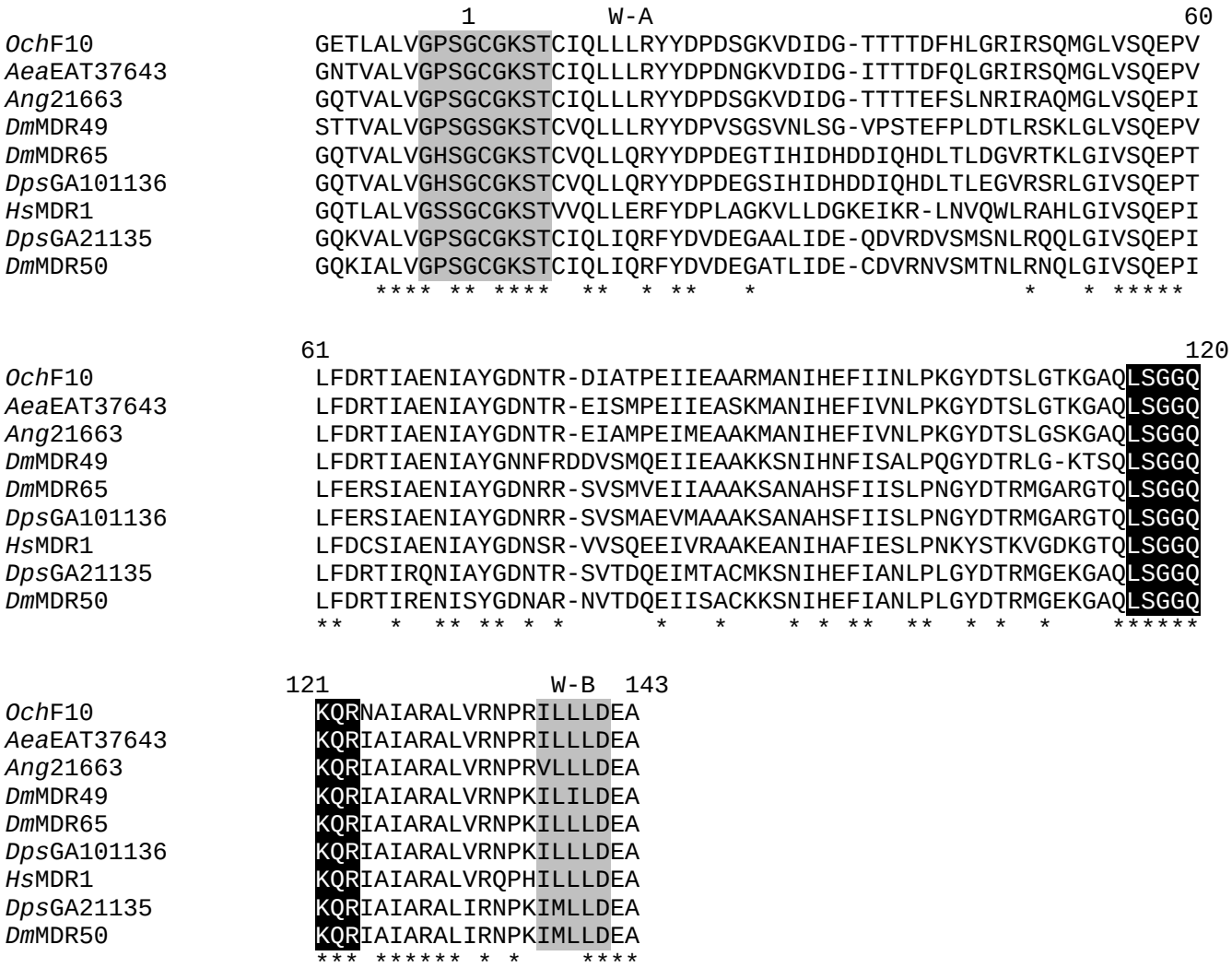

Figure 2. Alignment of the deduced amino acid sequence of the *Ochc*F10 clone and C-terminal ATP-binding cassette of *Aedes aegypti* protein EAT37643 (accession number, EAT37643), *Anopheles gambiae* protein ENSANGP000000211663 (XM315658), *Drosophila melanogaster* proteins MDR49 MDR65 MDR50 (M59076, M59077 and NM_079016, respectively), *Drosophila pseudoobscura* proteins GA101136 (EAL31274) and GA21135 (EAL26456); *Homo sapiens* protein MDR1 (M14758). Walker ATP-binding motifs A and B are shaded in grey (W-A W-B); ABC signature is shaded in black. Asterisks indicate the identical amino acid residues.

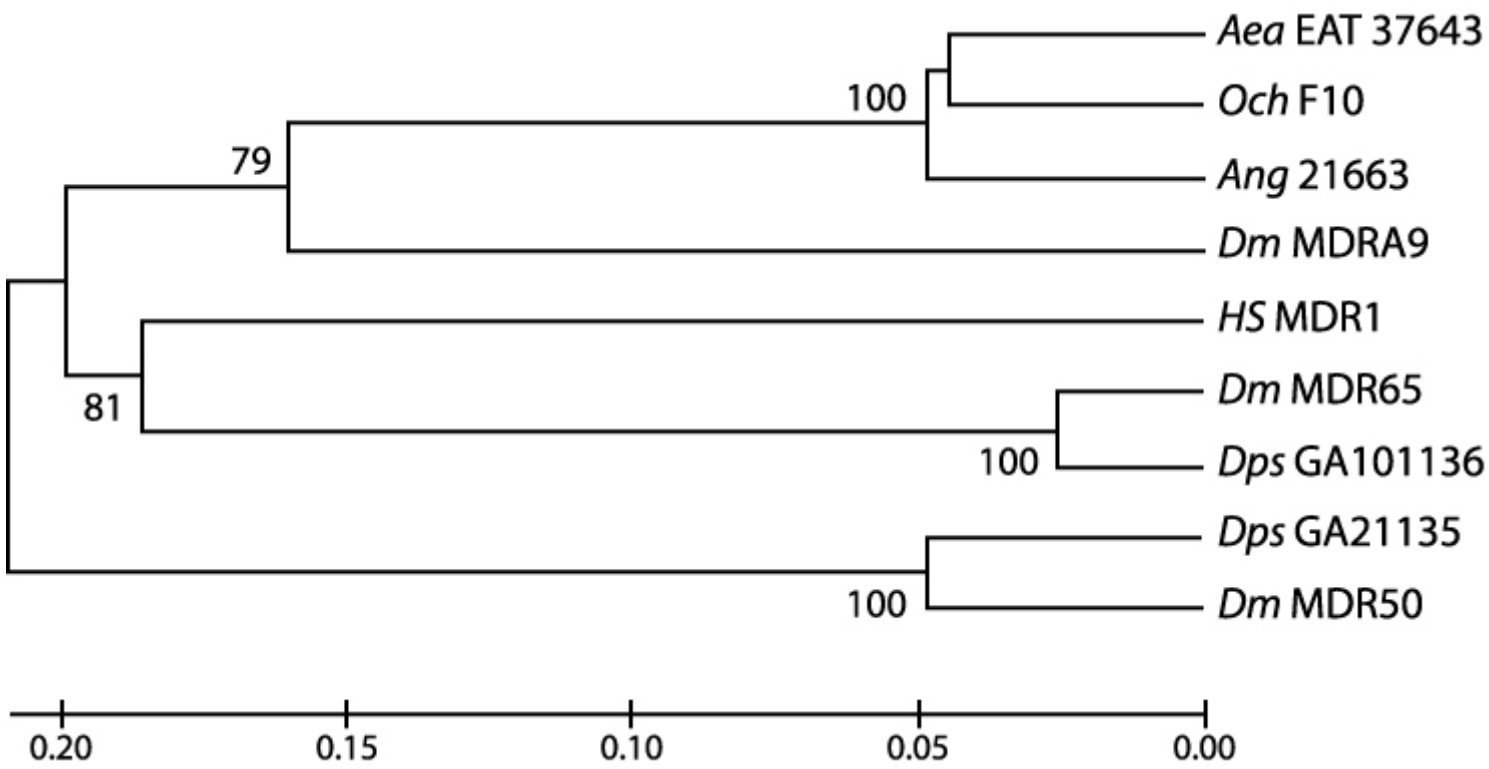

Figure 3. UPGMA analysis of the deduced amino acid sequence of the *Ochc*F10 clone and C-terminal ATP-binding cassette of *Aedes aegypti* protein EAT37643 (*Aea*), *Anopheles gambiae* protein ENSANGP000000211663 (*Ang*), *Drosophila melanogaster* proteins MDR49 MDR65 MDR50 (*Dm*), *Drosophila pseudoobscura* proteins GA101136 and GA21135 (*Dps*); *Homo sapiens* protein MDR1 (*Hs*). Bootstrap values based on 1000 replications are shown at the nodes.

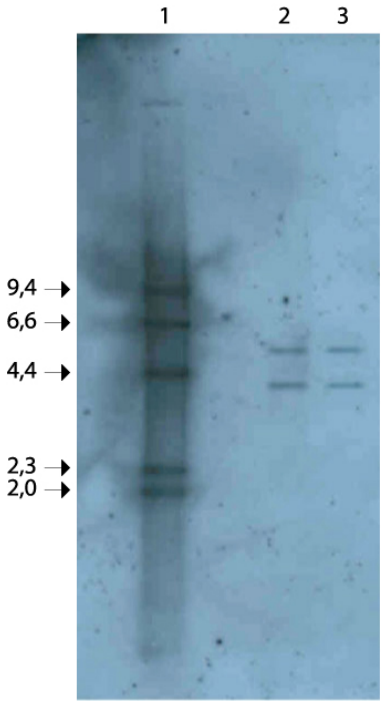

Figure 4. Southern blot of *Ochc*F10 sequence with genomic DNA from 2 individuals of *Ochlerotatus caspius* digested with *EcoRI* (lane 3-4). Size markers (lane 1 in kilobase pairs Kbp) are λ DNA digested with *Hind*III.

4. DISCUSSIONE GENERALE

La presente ricerca ha riguardato una specie, *Ocherotus caspius*, di grande interesse economico e sanitario per i Paesi del Mediterraneo e l'Italia in particolare, per la sua spiccata aggressività e per il carattere infestante che essa assume nelle aree in cui è presente ad alte densità. Da un lato si è voluto studiarne la costituzione genetica delle popolazioni nell'area della pianura Padana, dall'altro è stato indagato il ruolo e l'esistenza, in tale specie, di un potenziale meccanismo di difesa cellulare e di resistenza a diverse classi di insetticidi legato a proteine di membrana con funzione di trasporto. Obiettivo generale del lavoro era quello di fornire chiare indicazioni sulla pianificazione di strategie di controllo di *Oc. caspius* nell'area padana, efficaci e a basso impatto ambientale. Il controllo di pest, è un obiettivo di primo piano delle scienze applicate e grande interesse c'è oggi per le potenzialità applicative degli studi di genetica e biologia molecolare. I risultati ottenuti nella presente ricerca quindi, non solo sono di rilievo per la specie oggetto di studio, ma costituiscono anche un interessante caso di studio nell'ambito di un campo di indagine di più generale interesse, soprattutto per specie quali le zanzare.

L'analisi genetica ha evidenziato come le popolazioni di *Oc. caspius* costituiscano in pianura Padana un'unica entità panmittica. Una sostanziale mancanza di differenziamento genetico è stata infatti rilevata sia in relazione alla distanza geografica, sia in relazione all'eterogeneità dei siti di riproduzione. E' stato ipotizzato che l'attuale pattern di diversità genetica osservato sia il risultato di un'espansione delle popolazioni in tale area, legata a cambiamenti paleoclimatici pleistocenici, cui è seguito un elevato flusso genico. Quest'ultimo rappresenta ancora la principale forza evolutiva che agisce sulle popolazioni. I programmi di controllo a cui sono sottoposte alcune popolazioni da circa 15 anni non sembrano aver lasciato segni a lungo termine nella costituzione genetica delle popolazioni. Al contrario, l'aumento della superficie risicola e i cambiamenti nelle tecniche agronomiche avvenute nella seconda metà del '900 (incremento del numero di asciutte) sembrano aver determinato una più recente espansione delle popolazioni a livello locale. Quindi, interventi mirati a contenere la densità delle popolazioni non hanno avuto un significativo impatto a medio- lungo termine, mentre una pratica di altra natura (un cambiamento delle tecniche agronomiche) ha portato a cambiamenti significativi nella storia demografica recente delle popolazioni stesse. Da questo punto di vista, la storia recente di *Oc. caspius* in pianura Padana, esemplifica accuratamente come cambiamenti indotti dall'uomo nell'uso del territorio possano profondamente

influenzare la distribuzione di una specie nociva, costituendo questo un altro aspetto di interesse generale dello studio.

Attualmente, i programmi di controllo di *Oc. caspius* in pianura Padana sono legati ad una logica basata sui confini amministrativi, senza tener conto della reale struttura delle popolazioni. Dai risultati ottenuti emergono chiare indicazioni, quali la necessità di azioni di controllo su ampia scala geografica e focalizzate principalmente nei siti di riproduzione risicoli. Nonostante infatti i molteplici focolai di sviluppo larvale che si formano, anche se per brevi periodi di tempo, nelle zone rurali non risicole ed in collina legati all'irrigazione per sommersione di mais, barbabietola e pioppeti, agli scarichi inquinati, cisterne per l'irrigazione o abbeveratoi per animali, resta indubbio che la principale fonte di infestazione sia rappresentata dalle zanzare che si sviluppano in risaia. Rivolgere l'attenzione ai focolai urbani e rurali rappresenta quindi un impegno che non si può tradurre in una diminuzione del fastidio causato dalle zanzare, se prima non viene risolto il problema delle risaie. L'auspicio è quello di un approccio alla gestione delle popolazioni di *Oc. caspius* basato su diverse strategie (dai cambiamenti nell'uso del territorio, alle tecniche agronomiche, all'uso di composti insetticidi) mediante piani coordinati tra i Comuni, le Province e le Regioni interessate.

Il principale intervento per il controllo di *Oc. caspius* dovrebbe riguardare un radicale cambiamento nelle attuali pratiche agricole verso la riduzione o eliminazione delle fasi di asciutta che si succedono nella tecnica di coltivazione attuale. Oltre agli allagamenti ed asciutte vi sono, tuttavia, una serie di situazioni intermedie che consentono la schiusa di *Oc. caspius* e sulle quali si può intervenire. In particolare anche quando l'acqua viene mantenuta nella camera della risaia, le perdite per evaporazione e percolazione obbligano l'agricoltore a frequenti rabbocchi. Questo determina una lieve ma continua variazione di livello dell'acqua che scorre e sommerge aree più o meno vaste, soprattutto in corrispondenza dei bordi ma anche in zone dove il terreno non è perfettamente livellato, causando una schiusa continua e quindi la presenza di larve disetanee (Donati e Bellini 2006). Quindi, parallelamente alla riduzione delle asciutte, anche altri aggiustamenti nell'esecuzione delle pratiche colturali potrebbero ridurre il numero di schiuse, come mostrato dai risultati di alcuni progetti pilota realizzati nella provincia di Alessandria (Drago 2006). Da tali studi è stato evidenziato che un'aratura di 20 cm è sufficiente ad interrare le uova e ad impedirne la schiusa risultando un efficiente strumento di contenimento della prima generazione di *Oc. caspius*. Analogamente il rinnovamento annuale degli argini tramite ribaltamento del terreno è risultato efficace nell'impedire la schiusa delle uova svernanti presenti in tale parte delle camere risicole.

All'approccio preventivo per il contenimento di *Oc. caspius* attraverso gli interventi agronomici va associato il controllo diretto attraverso l'uso di composti larvicidi. Abbassamento della densità larvale e riduzione del rischio ambientale sono le due esigenze cui necessariamente bisogna far fronte. Il *Bti*, attraverso un uso moderato, sembra essere quello che meglio incontra entrambe le necessità. Tuttavia, poichè esso agisce efficacemente solo nei confronti delle larve di primo e secondo stadio, in un contesto così complesso i risultati possono non essere soddisfacenti. Se a ciò aggiungiamo la presenza di alghe che spesso creano uno strato superficiale, diminuendone la diffusione, si comprende come le prestazioni di tale larvicida possano essere anche fortemente ridimensionate rispetto alle condizioni ideali di applicazione. Ciò ha portato alla proposta di usare composti di sintesi quali temephos e diflubenzuron. Nonostante entrambi sembrino aver portato a significativi contenimenti delle popolazioni con un impiego di dosi molto basse e con effetti sulla fauna non target confrontabili con quelli del *Bti* (Drago 2006), il loro ipotetico impiego deve essere considerato con estrema cautela. In tale prospettiva, lo studio del ruolo delle P-glicoproteine quale meccanismo di difesa agli insetticidi, oltre ad avere una valenza generale nella comprensione delle interazioni cellula-sostanze tossiche, acquista un particolare interesse per la realtà *Oc. caspius*. L'aumento di suscettibilità delle larve di 3 e 16 volte rispettivamente per il temephos e il diflubenzuron, a causa dell'inibizione delle P-glicoproteine, indica l'importanza di tale meccanismo di difesa e le potenzialità della sua inibizione. L'isolamento e la preliminare caratterizzazione di una sequenza genomica codificante per una P-glicoproteina in *Oc. caspius* è il primo passo per una maggiore comprensione del ruolo giocato dai trasportatori multidrug nella difesa in questa specie contro gli insetticidi ed apre la porta per la realizzazione di una nuova strategia di controllo basata sull'inibizione delle difese della specie target.

Poichè il problema delle zanzare è strettamente connesso con la realtà economica sociale e politica del territorio padano, sembra opportuno fare le seguenti ulteriori considerazioni. Nel periodo 1970-2000 si è assistito ad una progressiva diminuzione del numero di aziende risicole (nel solo Piemonte scese da 8.818 a 2.360, -73%) in seguito ad un processo di concentrazione aziendale con conseguente aumento della loro dimensione. A questo si è aggiunta una marcata specializzazione produttiva ed un' elevata meccanizzazione delle pratiche colturali che ha visto una riduzione degli addetti impiegati in tale settore (attualmente 50.000). La ricchezza proveniente dal riso è quindi legata all'imprenditore a fronte di una diminuzione dei posti di lavoro e di ridotta vivibilità delle aree con conseguenti danni economici in un'area pari ad 1/3 dell'intero territorio nazionale. A tali considerazioni è interessante aggiungere alcuni dati sull'attuale situazione del mercato risicolo. Il nostro Paese

è il principale produttore di riso in Europa con circa 1 milione e 400 mila tonnellate annue di cui il 90% prodotto nelle risaie piemontesi e lombarde. Il fatturato complessivo del riso italiano si aggira intorno ai 700 milioni di euro, in gran parte generato da una commercializzazione in piccola confezione e dalla esportazione verso i Paesi europei. Il settore tuttavia attraversa un momento di difficoltà da collegare ai problemi che tutta l'agricoltura sta affrontando a seguito dell'internazionalizzazione dell'economia. Il riso inoltre sconta le conseguenze di una serie di misure di politica agraria comunitaria: a) il dimezzamento del prezzo d'intervento (vale a dire il livello dei prezzi al di sotto del quale scatta il ritiro pubblico della produzione dal mercato); b) la concessione di numerose facilitazioni ai paesi che esportano riso sul mercato europeo; c) l'entrata in vigore degli accordi WTO (World Trade Organization) con la progressiva apertura del mercato ad una dimensione globale. Nel 2009, in base ad un accordo tra l'Unione Europea ed i Paesi Meno Avanzati, 48 di questi, potranno esportare verso l'Europa anche il loro riso a dazio zero.

A fronte di tale situazione critica, da parte del mondo della produzione e delle Istituzioni si rende indispensabile una riconsiderazione della politica gestionale ed economica del settore risicolo. In tale bilancio, aggiungiamo, non si può non tener conto del costo in termini di gestione delle zanzare in ambiente risicolo e della riduzione di vivibilità del territorio in relazione alla presenza delle risaie stesse. Ci si chiede quindi, quanto costi mantenere questo tipo di risicoltura. Se non si debba puntare su un ridimensionamento della superficie risicola riconvertendo il territorio ad altri usi e puntando su una produzione minore, ma di alta qualità. Se gli aiuti statali economici che oggi vanno al settore risicolo (destinati nel prossimo futuro ad aumentare in risposta alla crisi imminente), non debbano andare solo ai produttori che puntino sulla qualità e su una risicoltura che comporti il minor numero di costi aggiuntivi in termini di controllo di pest o inquinamento ambientale o ridotta vivibilità delle aree. Se non si debba adottare lo stesso principio adottato per l'inquinamento industriale secondo cui "chi inquina paga", (D.lgs. 152 11 maggio1999). Domande forse provocatorie, ma necessarie soprattutto in un periodo così delicato per il mondo della risicoltura italiana ed europea cui si chiedono importanti cambiamenti politici e strutturali. E' forse proprio questa un'occasione per apportare cambiamenti radicali e tesi ad ottenere benefici a lungo termine. Questo richiederà un chiaro e duro confronto tra tutte le parti interessate (classe politica, i produttori, la popolazione locale) per il raggiungimento di un punto d'incontro tra i diversi interessi economici, politici e medico-sanitari. Un tavolo di discussione in cui si spera non manchi la voce della ricerca scientifica.

5. BIBLIOGRAFIA

Alavanja M.C.R. and Hoppin J.A., Kame F. 2004. Health effects of chronic pesticide exposure: cancer and neurotoxicity. *Ann Rev of Pub Health,* 25: 155-197.

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. 1990. Basic local alignment search tool. *J Mol Biol.* 215 (3): 403-10.

Alves S.N., Serrão J.E., Mocelin G. and De Melo A.L. 2004. Effect of ivermectin on the life cycle and larval fat body of *Culex quinquefasciatus*. *Braz Arch Biol Technol*. 47 (3): 433-439.

Amorosi A., Cotalongo M.L. and Fusco F. 1999. Gladio-eustatic control of continental-shallow marine cyclicity from late quaternari deposits of the southeastern Po plain, northern Italy. *Quaternary research*, 52: 1-13.

Ardizzone F., Baldi G., Berruto R., Bottonelli F., Finessi A., Ghiotti G., Giudici M.L, Guttardi P., Mazzali E., Moletti M., Piccarolo P., Politi A., Setti G. 1993. IL RISO. Edagricole S.p.a., Bologna, Italy.

Becker N., Petric D., Zgomba M., Boase C., Dahl C., Lane J. and Kaiser A. 2003. Mosquitoes and their control. Kluwer Academic/Plenum Publisher, 498 pp.

Bellini R. 1998. Linee di sviluppo per la lotta alle zanzare in Piemonte. In Atti del Convegno "Esperienze di lotta biologica alle zanzare in Piemonte", Casale Monferrato, 24-25 Nov, pp. 5-9.

Bellini R. and Veronesi R. 2001. Gestione delle zanzare nel Parco del Delta del Po emiliano-romagnolo con riferimento agli aspetti fitoecologici. Atti Conv. "Gestione delle risorse agro-forestali in aree protette" *Informatore Botanico Ialiano*, 33, (1):190-191.

Black IV W.C. and Kondratieff B.C. 2004. Biology of disease vectors, second edition, W. Marquardt editor, pag. 4-9.

Blackmore C.G., Mcnaughton P.A. and. Van Veen H.W. 2001. Multidrug transporters in prokaryotic and eukaryotic cells: physiological functions and transport mechanisms. *Mol Membr Biol.* 18: 97-103.

Borges-Walmsley M.I., Mckeegan K.S. and Walmsley A.R. 2003. Structure and function of efflux pumps that confer resistance to drugs. *Biochem J.* 376: 313-338.

Buss D.S., McCaffery A.R. and Callaghan A. 2002. Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. *Med Vet Entomol.* 16: 218-222.

Cahan R., Shainberg A., Malik Z. and Nitzan Y. 1994. Biochemical and morphological changes in rat muscle cultures caused by 28,000 mol. wt toxin of *Bacillus thuringiensis israelensis. Toxicon* 32:1125-1136.

Cahan R., Shainberg A., Pechatnikov I. and Nitzan Y. 1995. A 28,000 mol. wt toxin from *Bacillus thuringiensis israelensis* induces cation transport in rat muscle cultures. *Toxicon* 33: 943-951.

Callaghan, A. and Denny N. 2002. Evidence for an interaction between p-Glycoprotein and cadmium toxicity in cadmium-resistant and -susceptible strains of *Drosophila melanogaster*. *Ecotox Enviro Safe*. 52: 211-213.

Casida, J.E. and Gary B.Q. 1998. Golden age of insecticide research: Past, Present or Future? *Annu Rev of Entomol.* 43: 1-16.

Chakraborty R., Fuerst P.A. and Nei M. 1980. Statistical studies on protein polymorphism in natural populations. III. Distribution of allele frequencies and the number of alleles per locus. *Genetics* 94: 1039-1063.

Chang G. 2003. Multidrug resistance ABC transporters. *FEBS Letters* 555: 102-105.

Chen C.J., Clark D., Ueda K., Pastan I., Gottesman M.M. and Roninson I.B. 1990. Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins. *J Biol Chem.* 1 (5): 506-514.

Chen B., Harbach R.E., Butlin, R.K. 2004. Genetic variation and population structure of the mosquito *Anopheles jeyporiensis* in southern China. *Mol Ecol* 13, (10): 3051-3056.

Chevillon C., Raymond M., Guillemaud T., Lenormand T. and Pasteur N. 1999. Population genetics of insecticide resistance in the mosquito *Culex pipiens*. *Biol J of the Linn Soc* 68, (1-2): 147-157.

Clement M., Posada D. and Crandall K.A. 2000. TCS: a computer program to estimate gene genealogies. *Mol Ecol* 9: 1657–1659.

Collins F.H., Mendez M.A., Rasmussen M.O., Mehaffey P.C., Besansky N.J. and Finnerty V. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *Am J Trop Med Hyg* 37: 37– 41.

Collins F.H., Kamau L., Ranson H.A., Vulule J.M. 2000. Molecular entomology and prospects for malaria control. *Bullettin of the World Health Organization*, 78: 1412-1423.

Cornuet J.M. and Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144: 2001-2014.

Cremaschi M. 2003a. Mutamenti del clima nel Quaternario: le linee generali. In: Italia preistorica (Guidi, A. and Piperno, M. eds.), pp 3-15. Edizioni La Terza, Italy.

Cremaschi M. 2003b. La penisola italiana nel Quaternario: aspetti geologici e geoarcheologici. In: Italia preistorica (Guidi, A. and Piperno, M. eds.), pp 15-39. Edizioni La Terza, Italy.

David J.P., Huber K., Failloux A.B., Rey D. and Meyran J.C. 2003. The role of environment in shaping the genetic diversity of the subalpine mosquito, *Aedes rusticus* (Diptera, Culicidae). *Mol Ecol* 12, (7):1951-1961.

Denholm I., Pickett J.A. and Devonshire A.L. 1998. Insecticide resistance: from mechanisms to management. *Philosophical Transactions of the Royal Society,* Series B: *Biol Sciences* 353: 1673–1795.

Donati L. e Bellini R. 2006. Metodi agronomici per ridurre le zanzare in risaia. L'informatore agrario 29: 58-62.

Dore B., Bonelli S. e Pattono P. 2000. L'impatto della lotta biologica alle zanzare sulle popolazioni di anfibi. Atti del I Congresso Nazionale della *Societas Herpetologica Italica*. 727-734.

Drago A. 2006. Relazione Triennale Progetto per l'ottimizzazione dei metodi di controllo delle zanzare in Monferrato. Provincia di Alessandria Assessorato Agricoltura Caccia e Pesca.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

Felsenstein J. 1993. Documentation for PHYLIP (Phylogeny Inference Package) version 3.5C. University of Washington, Seattle, Wash.

Finney D.J. 1971. Probit analysis. Cambidge University Press, Cambridge, UK.

Foley D.H., Russell R.C. and Bryan J.H. 2004. Population structure of the peridomestic mosquito *Ochlerotatus notoscriptus* in Australia. *Med and Vet Entomol* 18, (2): 180-190.

Fu Y.-X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics,* 147: 915-925.

Gaertner L.S., Murray C.L. and Morris C.E. 1998. Transepithelial transport of nicotine and vinblastine in isolated malpighian tubules of the tobacco hornworm (*Manduca sexta*) suggests a P-glycoprotein-like mechanism. *J Exp Bio*. 201 (18): 2637-2645.

Gerrard B., Stewart C. and Dean M. 1993. Analysis of Mdr50: a *Drosophila* P-glycoprotein/Multidrug resistance gene homolog. *Genomics* 17: 83-88.

Goodsell J.A. and Kats L.B. 1999. Effect of Introduced Mosquitofish on Pacific Treefrogs and the Role of Alternative Prey. *Conser Biol*, 13 (4): 921-924.

Goudet J. 2001. *FSTAT, a Program to Estimate and Test Gene Diversities and Fixation Indices Version 2.9.3*. Available from http://www.unil.ch/izea/softwares/fstat.html.

Hagler J. R., and Jackson C.G. 2001. Methods for marking insects: current techniques and future prospects. *Ann Rev Entomol,* 46:511–43.

Harpending H. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology* 66: 591-600.

Hemingway J. and Ranson H. 2000. Insecticide resistance in insect vectors of human disease. *Ann Rev Entomol*, 45: 371-391.

Hutchison D.W. and Templeton A.R. 1999. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution* 53, (6):1898-1914.

Jackson J.K., Horwitz R.J. and Sweeney B.W. 2002. Effects of *Bacillus thuringiensis israelensis* on Black flies and nontarget macroinvertebrates and fish in a large river. *Transactions of the American Fisheries Society* 131: 910-930.

Jensen J.L., Bohonak A.J. and Kelley S.T. 2005. Isolation by distance, web service *BMC Genetics*, 6: 13.

Juliano S.A. and Luonibos L.P. 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. *Ecology Letters* 8: 558-574.

Kogan M. 1998. Integrated pest management: Historical Perspectives and Contemporary Developments. *Ann Rev Entomol* 43: 243-270.

Lage H. 2003. ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int J Antimicrob Ag.* 22: 188-199.

Lanning C.L., Ayad H.M. and Abou-Donia M.B. 1996. P-Glycoprotein involvement in cuticular penetration of ^{14}C thiodicarb in resistant tobacco budworms. *Toxicol Lett.* 85: 127–133.

Latino G., Peira G., Percivale F., Perosino M., Saglia A., Soster M., Spanna F., Varese E., Cressano G. and Lovisetto M. 2004. Un Piemonte a tutto riso. Regione Piemonte. Direzione sviluppo dell'Agricoltura.

Leader J.P. and O'Donnell M.J. 2005. Transepithelial transport of fluorescent p-glycoprotein and MRP2 substrates by insect Malpighian tubules: confocal microscopic analysis of secreted fluid droplets. *J Exp Biol.* 208: 4363-4376.

Lenormand T., & Raymond M. 1998. Resistance management: the stable zone strategy. *Proc R Soc London (B)* 265:1985-1990.

Lewis K. 2001. In search of natural substrates and inhibitors of MDR pumps. *J Mol Microb Biotech.* 3 (2): 247-254.

Lomovskaya O. and Watkins W. 2001. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. *J Mol Microb Biotech.* 3 (2): 225-236.

Lounibos L.P. 2002. Invasion by insect vectors of human disease. *Ann Rev Entomol* 47: 233-266.

Mahmood F., Walters L.L, Guzman H. and Tesh R.B. 1991. Effect of ivermectin on the ovarian development of Aedes aegypti (Diptera: Culicidae*). J Med Entomol.* 28 (5): 701-7.

Marasca S. e Bellini R. 2001. Le zanzare in aree umide di nuova formazione. *Terra e Vita*, 27: 75-77.

Mäser P. and Kaminsky R. 1998. Identification of three ABC transporter genes in *Trypanosoma brucei* spp. *Parasotiol Res.* 84: 106-111.

Matson P.A., Parton W.J., Power A.G., Swift M.J. 1997. Agricultural intensification and ecosystem properties. *Science* 277: 504-509.

Mayes M.E., Held G.A., Lau C., Seely J.C., Roe R.M., Dauterman W. C. and Kawanishi C. Y. 1989. Characterization of the mammalian toxicity of the crystal polypeptides of *Bacillus thuringiensis* subsp. *israelensis*. *Fundam Appl Toxicol.* 13: 310-322.

Mendes do Nascimento A., Goldman M.H.S. and Goldman G.H. 2002. Molecular characterization of ABC transporter-encoding genes in *Aspergillus nidulans*. *Gen Mol Res.* 1 (4): 337-349.

Mitchell A., Felix A.H., Sperling H. and Hickey D.A. 2002. Higher-level phylogeny of mosquitoes (Diptera:Culicidae): mtDNA data support a derived placement for Toxorhynchites. *Ins Sys and Evol* 33: 163-174.

Mousson L., Vazeille M., Chawprom S., Prajakwong S., Rodhain F., Failloux A.-B. 2002. Genetic structure of *Aedes aegypti* populations in Chiang Mai (Thailand) and relation with dengue transmission. *Trop Med Int Health* 7, (10): 865-872.

Nei M. 1978. Estimation of average eterozygosity and genetics distance from a small number of individuals. *Genetics,* 89: 583-590.

Nei M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, NY, USA.

Neigel J.E. 1997. A comparison of alternative strategies for estimating gene flow from genetic markers. *Ann Rev in Ecol and Syst* 28:105-128.

Neyfakh A.A. 2002. Mystery of multidrug transporters: the answer can be simple. *Mol Microbiol*. 44 (5): 1123-1130.

PAN Germany 2004. Moving towards Pesticide Reduction. http://www.pangermany.org/download/bap.pdf

Pinto J., Donnelly M.J., Sousa C.A, Gil V., Ferreira C., Elissa N., Do Rosário, V.E., Charlwood J.D. 2002. Genetic structure of *Anopheles gambiae* (Diptera: Culicidae) in São Tomé and Príncipe (West Africa): implications for malaria control. *Mol Ecol* 11: 2183-2187.

Piry S., Luikart G. and Cornuet J-M 1999. bottleneck: a computer program for detecting recent reductions in the effective population size using allele frequency data. *J of Heredity*, 90: 502–503.

Podsiadlowski L., Matha V. and Vilcinskas A. 1998. Detection of a P-glycoprotein related pump in Chironomus larvae and its inhibition by verapamil and cyclosporin A. *Comp Biochem and Physiol.* Part B. 121: 443-450.

Powel J.R., Caccone, A., Amato G.D. and Yoon C. 1986. Rates of nucleotide substitution in Drosophila mitochondrial DNA and nuclear DNA are similar. *PNAS USA*, 83: 9090-9093.

Ravel S., Hervé J.-P., Diarrassouba S., Kone A. and Cuny G. 2002. Microsatellite markers for population genetic studies in *Aedes aegypti* (Diptera: Culicidae) from Côte d'Ivoire: evidence for a microgeographic genetic differentiation of mosquitoes from Bouaké. *Acta Tropica,* 82, (1): 39 – 49.

Raymond M. and Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Heredity*, 86:248-249

Rice W.R. 1989. Analysing tables of statistical tests. *Evolution*, 43: 223-225.

Richards S., *et al.* 2005. Comparative genome sequencing of *Drosophila pseudoobscura*: Chromosomal, gene, and cis-element evolution. *Genome Res.* 15: 1-18.

Roderick G.K. 1996. Geographic structure of insect populations: gene flow, phylogeography, and their uses. *Ann Rev Entomol* 41: 325-352.

Rogers A.R. and Harpeting H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Mol Biol and Evol* 9: 552-569.

Rogers A.R. 1995. Genetic evidence for a Pleistocene population explosion. *Evolution* 49: 608-615.

Rollins L.A., Woolnough A.P. and Shervin W.B. 2006. Population genetic tools for pest management: a review. *Wildlife research* 33: 251-261.

Romi R., Pontuale G. and Sabatinelli G. 1997. Le zanzare italiane: generalità e identificazione degli stadi preimaginali (Diptera: Culicidae). *Fragmenta Entomologia,* 29: 1-141.

Rousset F., 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* 154 (4): 1219-1228.

Sambrook J., Fritsch E.F and Maniatis T. 1989. Molecular cloning: A laboratory manual, Second Edition. Cold Spring Harbour Laboratory, NY.

Schaffner F., Raymond M. and Pasteur N. 2000. Genetic differentiation of *Anopheles claviger s.s*. in France and neighbouring countries. *Med and Vet Entomol* 14, (3): 264-271.

Schaffner F., Angel G., Geoffroy B., Hervy J.-P., Rhaiem A. and Brunhes J. 2001. Les moustiques d'Europe. CD-ROM, IRD Edition.

Schneider S., Roessli D. and Excoffier L. 2000. Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Simard F., Lehmann T, Lemasson J.-Js, Diatta M., and Fontenille, D. 2000. Persistence of *Anopheles arabiensis* during the severe dry season conditions in Senegal: an indirect approach using microsatellite loci. *Insect Mol Biol* 9, (5): 467-479.

Sokal R.R., and Michener C.D. 1958. A statistical method evaluating systematic relationships. Sci. Bull., University of Kansas. 38: 1409-1438.

Stark J.D. and Banks J.E. 2003. Population-level effects of pesticides and other toxicants on arthropods. *Ann Rev Entomol,* 48: 505-519.

Stermitz F.R., Lorenz P., N.J. Tawara, Zenewicz L.A. and Lewis K. 2000. Synergy in a medical plant: antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. *Proc. Natl. Acad. Sci. USA* 97 (4): 1433-1437.

Storer T.I., Usinger R.L., Stebbins R.C., Nybakken J.W. 1990. Zoologia, ed. Zanichelli, Bologna, pag. 653-657.

Strong L. 1993. Overview: the impact of ivermectin on pastureland ecology. *Vet Parasit*. 48: 3-17.

Swofford D.L., Selander R.B. and Black W.C. 1997. *BIOSYS-2: A computer program for the analysis of allelic variation in population genetics and biochemical systematics.* Champaign, IL: Illinois Natural History Survey.

Templeton A.R., Crandall K.A. and Sing C.F. 1992. A cladistic analysisof phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132: 619–633.

Templeton A.R. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Mol Ecol* 7: 381-397.

Thomas M.B. 1999. Ecological approaches and the development of "truly integrated" pest management. *Proc. Natl. Acad. Sci. USA* 96: 5944-5951.

Thomas H. and Coley H.M. 2003. Overcoming Multidrug Resistance in Cancer : an update on the clinal strategy of inhibiting P-glycoprotein. *Cancer Control*. 10 (2): 159-165.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25: 4876-4882.

Turner M.J. and Schaeffer J.M. 1989. Mode of action of ivermectin. In: Campbell, W. C. (ed.). *Ivermectin and abamectin*. New York : Springer-Verlag. pp. 73-88.

Urbanelli S., Sallicandro P., De Vito E., Colonnelli E. and Bullini, L. 1996. Molecular reexamination of the taxonomy of *Ochthebius* (Calobius) (Coleoptera: Hydraenidae) from the mediterranean and macaronesian regions. *Ann. Entomol. Soc. Am.* 89 (5): 623-636.

Urbanelli S., Bellini R., Carrieri M., Sallicandro P. and Celli G. 2000. Population structure of *Aedes albopictus* (Skuse): the mosquito which is colonizing Mediterranean countries. *Heredity,* 84: 331-337.

Van Andel T.H. and Tzedakis P.C. 1996. Palaeolithic landscapes of Europe and environs, 150,000-25,000 years ago: an overview. *Quaternary Science reviews*, 15: 481-500.

Yébakima A., Charles C., Mousson L., Vazeille M., Yp-Tcha M.M. and Failloux A.-B. 2004. Genetic heterogeneity of the dengue vector *Aedes aegypti* in Martinique. *Trop Med Int Health* 9*,* (5): 582-587.

Walker J.E., Saraste M., Runswick M.J., and Gay N.J. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP syntase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J.* 1: 945-951.

Walton C., Sharpe R.G., Pritchard S.J., Thelwell N.J., Butlin R.K. 1999. Molecular identification of mosquito species. *Biol J of the Linn Society* 68, (1-2): 241-256.

Ware G.W. 2000. The Pesticide Book, 5th edn. Thomson Publications, Fresno, CA, USA.

Weill M., Chandre F., Brengues C., Manguin S., Akogbeto M., Pasteur N., Guillet P. and Raymond M. 2000.The *kdr* mutation occurs in the Mopti form of *Anopheles gambiae s.s.* through introgression. *Insect Mol Biol* 9, (5): 451-455.

Weir B.S. and Cockerham C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution,* 38: 1358-1370.

Western D. 2001. Human-modified ecosystems and future evolution. *Proc. Natl. Acad. Sci. USA* 98: 5458-5465.

WHO. 1981. Instructions for Determining the Susceptibility or Resistance of Mosquito Larvae to Insecticide. Document WHO/VBC/81.807. World Health Organization, Geneva.

WHO. 1992. Vector Resistance to Pesticides. Fifteenth Report of the WHO Expert Committee on Vector Biology and Control. Technical Report Series, 818. World Health Organisation, Geneva.

Wright G.D. 2000. Resisitng resistance: new chemical strategies for battling superbugs. *Chem Biol.* 7 (6): 127-132.

Wu C-T., Budding M., Griffin M.S., and Croop J.M. 1991. Isolation and characterization of *Drosophila* Multidrug Ressistance gene homologs. *Mol Cell Biol.* 3940-3948.

Xia X., and Xie Z. 2001. DAMBE: Data analysis in molecular biology and evolution. *J Hered.* 92: 371-373.

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