LANDSCAPE, POPULATION STRUCTURE AND GENETIC DIVERSITY OF *STOMOXYS CALCITRANS*

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Summary:

To investigate whether different landscapes could affect genetic diversity and structure of the cosmopolitan diptera *Stomoxys calcitrans*, populations from Gabon and southern France were studied using dominant amplified fragment length polymorphism (AFLP) markers. Gabon is characterized by a forested closed landscape, and southern France by an open Mediterranean landscape. The genetic diversity between Gabon and France populations did not differ significantly ($P > 0.05$). Contrary to our expectation, this study shows a moderate level of genetic differentiation between these two distant countries ($Fst = 0.0979$) and a low genetic structure among Gabonese and French populations ($Fst = 0.0291$ and 0.0275 respectively). This result could indicate the capacities of *S. calcitrans* populations to sustain a high level of gene flow, despite geographic distance and isolation.

KEY WORDS : *Stomoxys calcitrans*, population genetics, landscape ecology, AFLP.

Résumé : IMPACT DU PAYSAGE SUR LA STRUCTURE DES POPULATIONS ET LA DIVERSITÉ GÉNÉTIQUE DE *STOMOXYS CALCITRANS*

Pour étudier l'impact du paysage sur la diversité génétique et la structure des populations de Stomoxys calcitrans, *diptère cosmopolite, des populations du Gabon et du sud de la France ont été étudiées en utilisant des marqueurs AFLP (amplified fragment length polymorphism). Le Gabon est caractérisé par une forêt équatoriale, correspondant à un paysage fermé, alors que la zone d'étude dans le sud de la France correspond à un paysage largement ouvert. La diversité génétique entre les populations du Gabon et de France n'a pas montré de différence significative ($P > 0,05$). Contrairement à notre attente, cette étude a montré une différenciation génétique modérée entre les deux pays ($Fst = 0.0979$) et une structuration génétique faible au sein des populations du Gabon et de France ($Fst = 0.0291$ et 0.0275 respectivement). Ces résultats semblent indiquer la capacité des populations de* S. calcitrans *à maintenir un flux de gènes élevé, en dépit de la distance géographique et de l'isolement.*

MOTS CLÉS : Stomoxys calcitrans, *génétique des populations, paysage, écologie, AFLP.*

The relationship between landscape pattern and population structure has become a major interest of many population genetics studies. Manel *et al.* (2003) coined the term of landscape genetics to describe combination of landscape ecology and population genetics. Thus, the landscape genetics approach provides information about the interaction between landscape features and microevolutionary processes such as gene flow, genetic drift and selection (Manel *et al.*, 2003; Storfer *et al.*, 2007). Although the common thought is that environmental and ecological changes exacerbate the risk of decline or extinction of species, it is also important to consider organisms that may be favoured by current environmental changes. Particularly, insects that have an immense ecological capability of adaptation and a great biological success in diverse environments (Behura, 2006; Mock *et al.*, 2007). A number of recent studies highlighted the effects of landscape characteristics on patterns of genetic population structuring, including in insects (Keyghobadi *et al.*, 1999; Hunter, 2002).

Our team is presently working on stable fly populations. Stable fly, *Stomoxys calcitrans* (L) (Diptera, Muscidae), is a cosmopolitan insect that usually lives in close association with large domestic mammals and human beings (Zumpt, 1973). Both sexes are hematophagous. Their economic impact has been well established (Campbell *et al.*, 1987). These flies cause important economic losses in the cattle breeding, by their direct pathogenic effects (blood predation, irritation of the animals, reduction in immunizing defences, reduction in the production of milk...) and by their role in the transmission of certain pathogenic agents (Campbell *et al.*, 2001; D'Amico *et al.*, 1996). *S. calcitrans* historically has been a pest of livestock in confined operations but seldom of animals on pastures (Broce *et al.*, 2005). These flies are found essentially in rural areas near stables, slaughter houses, cattle markets and the rubbish dumps, locations related mainly to the presence of fermenting organic material (Batista *et al.*, 2005; Eesa & El-Sibae, 1993).

Describing insect diversity and ecology is important to understand their present distribution and can provide

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important information for effective management strategies. The purpose of our work is to evaluate the impact of landscape on population's structure and genetic diversity of this cosmopolitan species. We have had the opportunity to study populations from Gabon and from France. Gabon, in Africa, is characterised by an equatorial forested landscape. Contrary to Gabon, the Larzac area, located in southern France is characterized by a mediterranean karstic plateau and deforested landscape (Plagnes & Bakalowicz, 2001). Populations of *S. calcitrans* are present in both environments. Our hypothesis was that the equatorial forest in Gabon makes physical barriers between populations of flies living around isolated and distant villages.

The most commonly used molecular tools for landscape genetic studies are neutral hypervariable markers (amplified fragment length polymorphisms and microsatellites) (Storfer *et al*., 2007). To conduct this study we choose to use the amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995). AFLP was selected over other techniques because it has been demonstrated to be a powerful method for the characterization of intraspecific polymorphism among populations (Krauss, 2000). This method is relatively inexpensive, requires no initial time investment associated with primer design and has the advantage of sampling variation throughout the entire genome (Bensch & Akesson, 2005; Mendelson & Shaw, 2005).

MATERIALS AND METHODS

Study sites and sample collection

Samples of *Stomoxys calcitrans* were collected in Gabon from four sites close to the following towns: Makokou, Libreville, Mouilla and Nzadié, and in France from three sites in Larzac area (Le Caylar, Luc and other small villages grouped together). Vavoua traps (Laveissière & Grébaut, 1990) were used for captures. Trapped flies were put into freezer to kill them. Then they were preserved in 95° ethanol and identified in the laboratory according to Zumpt (1973).

DNA extraction and AFLP protocol

S. calcitrans were crushed with tungsten microballs and their DNA extracted using the DNeasy tissue Kit (QIAGEN).

AFLP analysis was performed essentially as described by Vos *et al*. (1995), using AFLP Core Reagent Kits (Invitrogen Life Technologies). The DNA of each sample was digested with the restriction enzymes *Mse*I and *Eco*RI, 1.25 U each in a volume of 12.5 µl containing reaction buffer at 37 °C for 2 h, followed by a final step of 70 °C for 15 min. 12 µl of adapter/ligation solution containing *EcoR* I/*Mse* I adapters, 0.5 µl of T_4 DNA ligase were added to the digest DNA. The solution was incubated at 20 °C for 2 h. Following ligation, a first amplification was carried out with primers containing one selective nucleotide (cytosine and adenine for *MseI* and *EcoRI* primers, respectively), 20 µl pre-amp primer mix, 2.5 µl 10 × PCR buffer plus Mg and 0.5 µl Taq polymerase were added in a total volume of 25.5 µl. PCR was performed for 20 cycles, which consisted of 30 s at 94 °C, 1 min at 56 °C and 1 min at 72 °C in a PTC-100™ MJ Research Peltier thermocycler. The PCR products were diluted 10-fold with TE buffer. The second amplification was carried out with the selective primer pair: fluorescent-labelled *EcoRI*-GAGTGCGTACCATTC-**GAC** and unlabelled *MseI*: GATGAGTCCTGAGTAA**CTC** (selective bases are in bold letters). The PCR mixture consisted of 5 µl of diluted preamplified DNA, 2 µl of 10 × PCR Buffer plus Mg, 0.8 µl of 20 mM dNTP mix, 0.2 µl of 10 µM/ml *EcoRI* primer, 0.2 µl of 10 µM/ml *MseI* primer and 0.1 µl Taq polymerase (5U/µl) in a total volume of 20 µl. The thermocycler program consisted of two segments. The first segment comprised 12 cycles with the annealing temperature decreased from 65 °C by 0.7 °C in each cycle: 30 s at 94 °C, 30 s at 65 °C to 57.3 °C and 1 min at 72 °C. The second segment consisted of 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C.

For automated fragment analysis, 1.5 µl of selective PCR products were pooled on 15 µl of deionized formamide and 0.2 µl of Genescan-500Rox, an internal size standard (Applied Biosystems) and run on an ABI 3130 sequencer. Fragments were sized using GeneMapper® Software v.3.7 (Applied Biosystems). Bands used in scoring ranged in size from 100 to 500 bp. AFLP products were scored visually as discrete character states (present/absent).

Population genetic analysis

Gene diversity was calculated after the estimation of allelic frequencies at each locus using a Bayesian method with nonuniform prior distribution of allele frequencies (Zhivotovsky, 1999) and assuming Hardy-Weinberg equilibrium implemented in the program AFLP-SURV (Vekemans *et al*., 2002) with a predefined value of Fis = 0. The frequency of the null allele at each locus is computed from two numbers, the sample size and the number of individuals in the sample that lack the AFLP fragment. This method has been shown to generate nearly unbiased estimates of null allele frequencies in dominant marker systems (Zhivotovsky, 1999). After estimating allele frequencies, statistics of gene diversity and population genetic structure were computed according to Lynch & Milligan (1994). For each population, we calculated the number (NPL) and proportion (PLP) of polymorphic loci at 5 % level and

expected heterozygosity under Hardy-Weinberg proportions (Hj) (also called Nei's gene diversity).
Additionally, AFLP-SURV uses the Lynch and Milligan (1994) method to assess the total population genetic structure by estimating total gene diversity (Ht), average gene diversity within populations (Hw) (analogous to Hs), average gene diversity between populations (Hb) (analogous to Nei's Dst), and finally Wright's Fst (Fst computed as Hb/Ht). These estimates were used, in turn, to calculate the average number of individuals that migrate to each population per generation by using the formula Nm ≅ (1-Fst)/4Fst (Wright, 1951).
Nei's genetic distance was estimated after Lynch & Milligan (1994) and Pairwise Fst between populations was also calculated to assess genetic differentiation (significance assessed by 1,000 permutations).

RESULTS

A total of 114 *S. calcitrans* were analysed, including 88 individuals from four Gabonese populations and 26 from three French populations. The two AFLP primers combinations yielded a total of 322 unambiguously scorable bands for the Gabonese populations and 216 for the French populations.
The proportion of polymorphic loci (PLP) ranged from 50 to 59.3 % for Gabonese populations and from 50.9 to 54.6 % in French populations. The expected heterozygosity (Hj) in Gabonese and French populations varies from 0.1408 to 0.1817 and from 0.1746 to 0.1988 respectively (Table I). The genetic diversity between Gabon and France did not differ significantly (Mann-Whitney U-test: PLP, P = 0.6286 and Hj, P = 0.1143) (Table I). Total gene diversity (Ht) and mean within population gene diversity (Hw) were higher for French than for Gabonese populations (Ht = 0.1922 and 0.1610 (P = 0.0238) and Hw = 0.1869 and 0.1563 (P = 0.0238) respectively) (Table II). Average gene diversity among populations (Hb) was higher for Gabonese populations (Table II).
The smallest Nei's genetic distance value was obtained between the populations from Makokou and Libreville (0.0024). The largest distance was obtained between Mouilla and Nzadié (0.0077) (Table II).
A moderate level of genetic differentiation was found when all populations were compared (Fst = 0.0727). Pairwise Fst between populations ranged from 0.0139 (between Makokou and Libreville) to 0.0409 (Mouilla and Nzadié). The Fst value among French populations did not differ significantly than that among Gabonese populations (P = 0.7143) (Table II). However, Fst values between Gabonese and French populations (Fst = 0.0979) was significantly greater from those within populations (P < 0.0001).
Gene flow (Nm) value recorded within French popu-

Populations	Sample size	Polymorphic loci (percentage)	Hj
Gabon			
Makokou (Mk)	28	180 (55.9)	0.1565
Libreville (Lib)	19	161 (50)	0.1460
Nzadie (Nz)	19	191 (59.3)	0.1817
Mouila (Mo)	22	168 (52.2)	0.1408
France			
Le Caylar (Cay)	15	113 (50.9)	0.1746
Luc (Lu)	5	114 (52.8)	0.1988
Remainder (Re)	6	118 (54.6)	0.1872

Table I. – Statistics of gene diversity within populations of *S. calcitrans*. Polymorphism expected heterozygosity (Hj) is calculated using Lynch & Milligan (1994).

	Ht	Hw	Hb	Nei's genetic distance	Fst	Nm
Between Gabonese populations			**0.0047**		**0.0291**	**8.34**
Mk/Lib	0.1534	0.1513	0.0021	0.0024	0.0139	17.73
Mk/Nz	0.1752	0.1691	0.0061	0.0070	0.0346	4.72
Mk/Mo	0.1523	0.1487	0.0036	0.0040	0.0236	10.34
Lib/Nz	0.1691	0.1639	0.0052	0.0058	0.0304	7.97
Lib/Mo	0.1474	0.1435	0.0040	0.0045	0.0270	9.00
Mo/Nz	0.1683	0.1613	0.0070	0.0077	0.0409	5.86
Between French populations			**0.0053**		**0.0275**	**8.84**
Cay/Lu	0.1926	0.1867	0.0058	0.0048	0.0302	8.02
Cay/Re	0.1845	0.1810	0.0036	0.0029	0.0193	12.7
Lu/Re	0.1994	0.1930	0.0064	0.0056	0.0322	7.51
Between Gabon-France						
	0.1456	0.1312	0.0144	0.0166	**0.0979**	**2.3**

Table II. – Differentiation between *S. calcitrans* populations and genetic structure. Calculations performed with AFLP SURV 1.0 (Vekemans *et al.*, 2002).

lations was similar to that found between Gabonese populations (Mann-Whitney U-test: P = 0.904).

DISCUSSION

We have studied the genetic diversity of *S. calcitrans* in two different landscapes: equatorial forest in Gabon and deforested open field in France. According to our AFLP markers result, genetic differentiation was not different between Gabon and France. The Fst values (< 0.05) recorded within Gabonese populations and within French populations suggest a low level of genetic differentiation between these stable fly populations. Moreover, Fst values between Gabon and France (0.0979) indicate a moderate genetic differentiation. Low Nei's genetic distances confirm the lack of differentiation between populations. Furthermore, the values of Nm indicated high levels of gene flow occurring between all populations, independently of the scale.

Ecological evidence suggests that stable flies are strongly vagile, dispersing far and wide (Eddy *et al*., 1962). These flies have great flight capacities and certain studies (Bailey *et al*., 1979; Hogsette *et al*., 1987) mention surprising displacement distances (several hundred kilometers), in search of a blood meal.

A PCR-RFLP analysis carried out by Szalanski *et al.* (1996) on *S. calcitrans* populations from Nebraska, Canada and Texas shows the same low differentiation. They recorded Nei genetic distances ranging from 0.000 to 0.0067. The number of migrants and Fst between Canadian and Kerrville (Texas) populations were respectively 3.48 individuals per generation and 0.067. Moreover, using microsatellite markers, Gilles *et al.* (2007) reveal large levels of gene flow on La Réunion Island for *S. calcitrans* (Fst = 0.02) despite the natural geographic barriers. As well, he demonstrated no genetic structuration for *S. niger* populations on this island (Fst = 0).

Recently, Marquez *et al.* (2007) studied the phylogeography of stable fly using mitochondrial COI and r16S, shows that countrywide pairwise estimates of genetic differentiation (Gst) varied from 0.08 (Kenya and Ivory Coast) to 0.71 (United Kingdom and Brazil) and that differentiation among six Nearctic populations was about 0.09. They also found that nucleotide diversities observed within and among regions were similar. Mitochondrial variation noted by Marquez *et al.* (2007) indicates greater indices of genetic differentiation than microsatellites record by Gilles *et al.* (2007) and even than our AFLP results. This corroborates Krafsur *et al.* (2005) results, which prove a greater mitochondrial differentiation than microsatellites in the house fly. So, the degree of population structuring in stable flies can be compared with that reported in other invasive, colonizing muscoid fly species. Comparing studies made by Cummings & Krafsur (2005) on house fly, Cummings *et al.* (2005) on face fly and Marquez *et al.* (2007) on stable fly, a substantial differentiation degree of population stucturation has been shown using mitochondrial markers. However, studies on gene diversity at polymorphic loci for muscoid diptera in North America show less genetic diversity in *S. calcitrans* than *Musca domestica*, *M. automnalis* and *Heamatobia irritans* (He = 0.182, 0.367, 0.305 and 0.328 respectively) (Krafsur, 1993). These speculations are explained by the fact that the mitochondrial genome has a set of characteristics (uniparentally inherited, homoplasic and non recombining) which makes it more sensitive to demographic phenomena than the nuclear genome (Avise, 2004). Moreover, as dominant marker, AFLP complicates heterozygosity analysis (Blears *et al*., 1998).

Finally, for *S. calcitrans* the different landscapes observed in Gabon and in southern France do not show any influence on the genetic differentiation of populations, using AFLP markers. Further study on this topic should combine both mitochondrial and nuclear markers.

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