

# Divergence of Scent Pheromones in Allopatric Populations of *Acanthodactylus boskianus* (Squamata: Lacertidae)

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The evolutionary phenomena associated with divergence in chemical signals between populations of the same species help to understand the process of speciation. Animals detect and react to semiochemicals and pheromones used in communication. Comparison between populations of the same species that are geographically isolated from one another allows us to determine the genetic or environmental factors responsible for chemical differentiation. *Acanthodactylus boskianus* from the east and west of Egypt were used as an example to compare the geographical diversity in chemical fingerprints of this species' femoral gland secretions and its phylogeography. Chemical analysis via GC–MS showed that the two geographically distinct populations' odor fingerprints are quantitatively different despite sharing the same components of the secretions. Phylogenetic analysis showed that the eastern and western Egyptian populations are genetically distinct and that chemical divergence of these lizards' odor profiles may be an example of signal evolution.

**Key words:** signal evolution, phylogeography, chemical ecology, reptiles, GC–MS

## INTRODUCTION

Chemical signals are widely used in vertebrate communication and are detected by the vomeronasal system (Jacobson's organ). As highlighted in a recent review by Johansson and Jones (2007), chemical signals can play significant roles in mate choice and reproductive isolation, but to date little is known about the chemical nature and the mechanisms underlying their biological functions. Chemical cues potentially allow for nest mate and kin recognition (Carlin and Hölldobler, 1983; Baker, 2008) where evolutionary shifts in signalling systems have been studied intensively (Löfstedt et al., 1986).

Scent marking is potentially a significant factor in population isolation (Pillay, 2000; Higgie and Blows, 2008). Allopatric populations have different chemical signals in African striped mouse (Pillay et al., 2006) and in *Drosophila serrata* (Higgie and Blows, 2008). In the calling songs of anurans (Smith et al., 2003), Mormon crickets (Bailey et al., 2007) and mice (Smadja and Ganem, 2008), the signals differ in allopatric populations. Population-specific communication signals have been described as the result of divergence in allopatry (Pillay, 2000).

Tongue-flicking is the quintessential squamate behaviour (Cooper, 1994). The chemical signals collected by the tongue are detected by the vomeronasal system. Tongue-flicking thus plays a role in delivery to the vomeronasal

organ, which is located in the roof of the mouth of lizards. Some lizards can use femoral (epidermal) glands to deposit semiochemicals, which may convey information about social status and competitive ability, on the substrate as they move through their home ranges (López et al., 2002), but few studies have examined the chemical nature of epidermal gland secretions in lizards. A number of studies have showed that gland secretions consist of both lipids and proteins (Weldon and Bangall, 1987; Mason and Gutzke, 1990; Weldon et al., 2008). The major compounds involved in chemical communication are believed to be lipid in nature (Cooper and Garstka, 1987; LeMaster and Mason, 2001).

The chemical composition of the femoral gland secretions has been studied in a number of lizard species. In the lacertid lizard *Acanthodactylus erythrus*, secretions include alcohols, steroids, carboxylic acids and their esters, lactones, ketones, squalene, and  $\alpha$ -tocopherol (López and Martín, 2005a). The same class of compounds were found in another lacertid lizard, *Iberolacerta monticola cyreni* (López and Martín, 2005b), but in different relative proportions. In the girdled lizard or sungazer, *Cordylus giganteus*, carboxylic acids, alcohols, ketones, esters, and steroids were detected in both males and females (Louw et al., 2007). Alcohols and steroids are the most important chemical classes in femoral gland secretions, the former were reported earlier as major components in the *Acanthodactylus* lizards; *A. erythrus* (López and Martín, 2005b) and *A. boskianus* (Khannoon et al., 2011a). Chemical and behavioural studies on the femoral gland secretions of *A. boskianus* (Khannoon, 2009; Khannoon et al., 2010, 2011a, b) showed sex-specific differences in their components and recorded cholesterol as the most abundant individual chemical.

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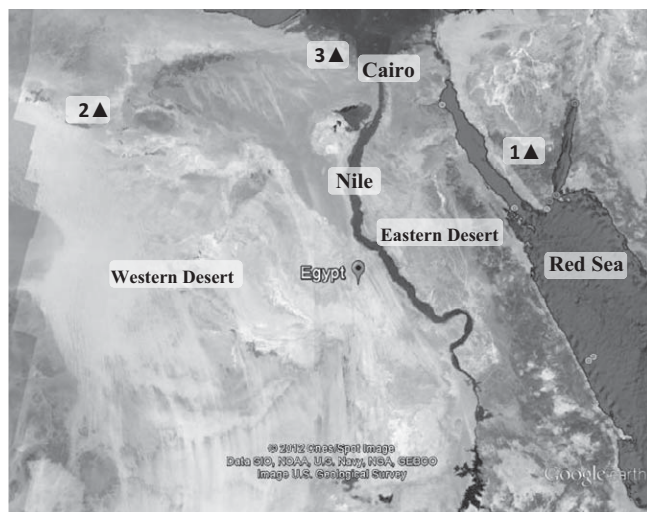
*Acanthodactylus* is a genus of ground-dwelling lizards containing 39 species that are widely distributed in arid areas of southwest Asia, North Africa, and Iberia (Fonseca et al., 2009). *A. boskianus* is one of the most widely distributed species of lizards in Egypt, recorded in the eastern desert, western desert, and northern Mediterranean coast, along the Nile River, and in Sinai (Baha El Din, 2006). Its range extends in Northern Africa, reaches the Mediterranean and Atlantic coast; south to Mali and north Nigeria, Sudan, and Abyssinia (Schleich et al., 1996).

The aim of the present study was to investigate whether there are differences in the composition of the femoral gland secretions, which we studied before (Khannoon et al., 2011a, b), between different geographically separated populations of *A. boskianus*, and to view these in a phylogenetic context. If the composition of the secretions has diverged (i.e., through the evolution of signal traits and mating preferences), we could expect population-specific odor profiles and behavioral separation upon exposure to gland secretions, and that these would reflect phylogenetic divergence.

## MATERIALS AND METHODS

### Samples

Adult *Acanthodactylus boskianus* up to 79 mm snout-vent length (SVL) from different populations were captured in Egypt during April, which coincides with the activity and mating season of these lizards. Samples were collected and identified according to Schleich et al. (1996) and Baha El Din (2006). Populations separated by the Nile River were selected as representing east and west of Egypt. The Siwa oasis (29°11'N, 25°31'E) and west of Abu Rawash (30°02'N, 30°50'E) populations represented the west of the country (Fig. 1). The Sinai population, east of the Nile River was represented by two sites: Maghara (28°45'N, 33°25'E) and Sharm El Sheikh (27°51'N, 34°16'E). The animals were transferred to the University of Hull, UK, and kept for chemical analyses and DNA experiments. Collection of secretion samples was performed just after the arrival of the animals in April and May (breeding season when the animals are sexual active). All experiments were approved by the University of Hull.



**Fig. 1.** A map of Egypt showing the locations from which the populations were collected, Sinai (1), Siwa (2) and Abu Rawash (3). Source (Google Earth).

### Chemical analysis of gland secretions

To avoid the effect of sexual variations we used only adult males from the three populations; Sinai (population 1,  $n = 17$ ), Siwa (population 2,  $n = 10$ ), and west of Abu Rawash (population 3,  $n = 13$ ). Age effects were avoided by choosing adult individuals with very narrow range of snout-vent lengths (SVL) ( $\pm 3$  mm). Secretions were collected in the chemical ecology lab, The University of Hull by gently squeezing the plugs from the femoral pores of the lizards using forceps. Secretions of each individual were collected directly into glass vials with Teflon-lined caps and then dissolved in 250  $\mu$ l of dichloromethane (DCM) (Aldrich, GC grade). The collected samples were kept at  $-18^{\circ}\text{C}$  until processing for analysis. Control samples with DCM solvent at the same conditions of collecting the secretion were used to exclude impurities.

### Derivatization

Fifty  $\mu$ l of liquid secretion was placed in a 2 ml vial, and the solvent was removed in a gentle stream of nitrogen at  $50^{\circ}\text{C}$ . The residue was taken up in 10  $\mu$ l dichloromethane and 50  $\mu$ l *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were added. The mixture was heated to  $50^{\circ}\text{C}$  for 30–60 minutes in a vial with a closed cap. The solvent and the remaining reagent were then evaporated in a gentle stream of nitrogen at  $50^{\circ}\text{C}$  and the residue was taken up in 10  $\mu$ l dichloromethane. Exactly 1.0  $\mu$ l was injected into the gas chromatography-mass spectrometry (GC-MS) system. We derivatized the extracts with MSTFA before analyses to form trimethylsilyl derivatives. Derivatization helped to identify the more polar compounds that had poor elution properties.

### GC-MS analysis

Samples were analysed using a Hewlett-Packard model 6890 gas chromatograph connected to a Hewlett-Packard model 5973 mass-selective detector equipped with a BPX-5 column: 25 m  $\times$  0.22 mm i.d., 0.25  $\mu$ m film thickness (SGE). The temperature program was as follows:  $50^{\circ}\text{C}$  for 5 minutes, then with  $5^{\circ}\text{C}/\text{min}$  to  $320^{\circ}\text{C}$ , 30 minutes hold time. Helium was used as carrier gas with 1 ml/min in constant flow mode. Accelerating voltage of MS was 70 eV. Compounds were identified by comparison of mass spectra and retention indices of derivatized and underivatized samples with those of reference compounds.

### DNA analysis (DNA extraction, amplification and sequencing)

In order to investigate the genetic relationship between these populations we sequenced three separate genes from a total of 34 individuals. Lizard tail tips were cut, stored in 100% ethanol, and kept for DNA extraction for the three populations: Sinai ( $n = 15$ ), Siwa ( $n = 14$ ), Abu Rawash ( $n = 5$ ). *A. scutellatus* from Siwa was used as an outgroup. Total genomic DNA was extracted using either DNeasy blood and tissue kit or hot sodium hydroxide-Tris solution (HotSHOT) technique (Truett et al., 2000). Fragments of 12S, ND4, and cyt b genes were amplified using the primers 12Sa and 12Sb for 12S rRNA gene (Kocher et al., 1989), ND4 and LEU for ND4 gene (Arévalo et al., 1994), and cyt b1 and CB3 for cyt b gene (Palumbi, 1996). Amplifications were performed in 25  $\mu$ l volumes containing 2.5  $\mu$ l of 10x reaction buffer, 3  $\mu$ l of 25 mM  $\text{Mg}^{2+}$ , 2.5  $\mu$ l of 8 mM dNTP, 1  $\mu$ l of 10 mM of each primer, 0.2  $\mu$ l Bio Taq DNA polymerase (500 unit), 1  $\mu$ l genomic DNA and 13.8  $\mu$ l ultrapure  $\text{H}_2\text{O}$ . Amplification started with initial cycle of denaturation at  $94^{\circ}\text{C}$  for 3 min, followed by 35 cycles of: denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 1 min, extension at  $72^{\circ}\text{C}$  for 3 min. A final extension was conducted at  $72^{\circ}\text{C}$  for 10 min. Negative controls were run for all amplifications. PCR products were sent to a commercial company (Macrogen, Korea) for purification and sequencing. Sequences for all individuals included in this study have been submitted to the international sequence databases (accession numbers: GU225704–GU225707, HM596595–HM596598, HM749619–HM749623, HM769288–HM769301, and HM778091–HM778108).

### Phylogenetic analyses

Sequence traces from 12S rDNA, cytb, and ND4 genes were aligned using CodonCode Aligner (version 3). Neighbor-joining and Maximum likelihood (ML) phylogenetic analyses were conducted using BioNJ (Gascuel, 1997) and PhyML (Guindon and Gascuel, 2003) using the general time-reversible model with a gamma distribution of rate heterogeneity and four rate categories. Confidence in nodes was assessed using both bootstraps and approximate likelihood ratio test (aLRT) support values.

### Statistical analysis

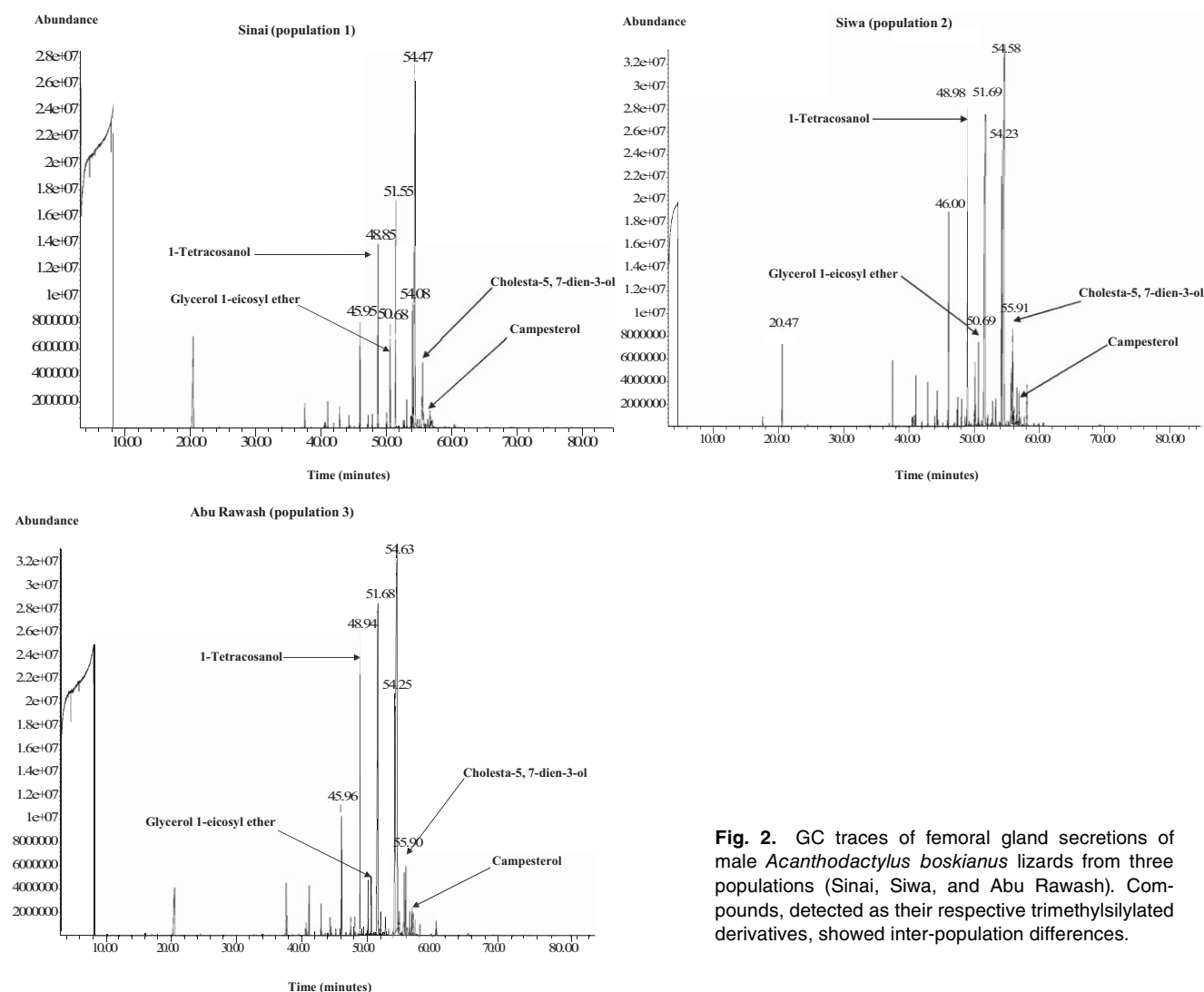
The relative amount of each component analysed chromatographically was determined as the percent of the total ion current (TIC). Chemicals included as variables (13 chemicals) in the statistical analysis were only those which were presented in all individuals and constituted > 0.1% of TIC. The relative areas of the selected peaks were transformed according to Aitchison (1986) following the formula  $Z_{ij} = \ln(Y_{ij}/g(Y_j))$ , where  $Z_{ij}$  is the standardized peak area  $i$  for individual  $j$ ,  $Y_{ij}$  is the peak area  $i$  for individual  $j$ , and  $g(Y_j)$  is the geometric mean of all peaks for individual  $j$ . For comparing the populations, these transformed areas were used as dependent variables in a series of analyses of variance (ANOVAs), with Bonferroni adjustment for the 13 multiple comparisons. These analyses and

Scheffe post-hoc test were done to determine whether and which of the populations differ in the proportions of chemicals.

## RESULTS

### Chemical composition of femoral gland secretions

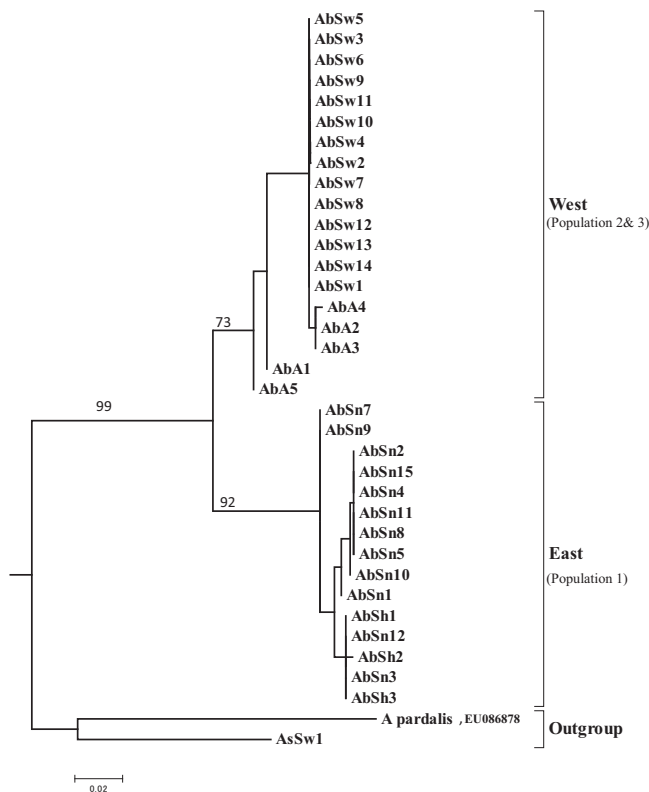
As we reported in a previous chemical study (Khannoon et al., 2011a), a total of 122 chemicals were identified in different individuals of all populations without qualitative differences (Khannoon et al., 2011a). The identified compounds, as percents of TIC, were alcohols (50.29%), steroids (35.54%), carboxylic acids (9.37%), glycerolmonoethers (3.11%), monoglycerides (1.21%), with minor fractions of alkanes, amides, aldehydes, carboxylic acid esters, and squalene. The most abundant chemical detected was cholesterol (30.78%). The GC–MS analysis of the underivatized natural extracts showed the presence of more polar compounds, which could not be identified because of their poor elution properties. Therefore the extracts were analyzed after derivatization with MSTFA to form trimethylsilyl-derivatives, thus enabling the GC–MS analysis of the more polar compounds. This method led to the identification of



**Fig. 2.** GC traces of femoral gland secretions of male *Acanthodactylus boskianus* lizards from three populations (Sinai, Siwa, and Abu Rawash). Compounds, detected as their respective trimethylsilylated derivatives, showed inter-population differences.

**Table 1.** Results of Scheffe test, showing the differences in chemical proportions between the populations: Sinai (1), Siwa (2) and Abu Rawash (3).

Chemicals	Populations ( <i>P</i> value)		
		2	3
1-Tetracosanol	1	< 0.001	< 0.001
	2		0.099
Cholesta-5,7-dien-3-ol	1	< 0.001	< 0.001
	2		0.097
Campesterol	1	0.001	< 0.001
	2		0.66
Glycerol 1-eicosyl ether	1	0.066	0.001
	2		0.47



**Fig. 3.** Maximum likelihood tree depicting phylogenetic relationships among the three populations of *Acanthodactylus boskianus*. Sequences of *A. scutellatus* and *A. pardalis* were used as outgroups. Numbers above the branches are bootstrap values. The individuals of the three populations are given short names: *A. boskianus*, AbSn and AbSh (Sinai), AbSw (Siwa), and AbA (Abu Rawash). *A. scutellatus* outgroup was collected from Siwa (AsSw).

additional components not previously reported from lizards. These components are monoglycerides and related monoethers of glycerol, 1, 3-alkanediols, and glycerol ethers of the alkanediols (Khannoon et al., 2011a).

Interpopulational differences were detected in the proportions of four chemicals (Fig. 2): 1-tetracosanol ( $F_{(2,37)} = 18.23$ ,  $P < 0.001$ ), cholesta-5,7-dien-3-ol ( $F_{(2,37)} = 23.78$ ,  $P < 0.001$ ), campesterol ( $F_{(2,37)} = 21.97$ ,  $P < 0.001$ ), and glycerol 1-eicosyl ether ( $F_{(2,37)} = 8.47$ ,  $P < 0.001$ ). Post-hoc comparisons using Scheffe test indicated that the Sinai population was significantly different from both the Siwa and Abu

Rawash populations. The latter two populations did not differ significantly (Table 1).

### Molecular analysis

The aligned sequence data set of 12S (408 bp), cytb (662) and ND4 (846 bp) totalled 1916 bp, with 34 individuals from *A. boskianus* and two outgroup sequences included. The maximum likelihood tree provides evidence of two lineages corresponding to populations from the eastern and western sides of Egypt (Fig. 3). Clade 1 (East), which corresponds to the Sinai population, represents a well-supported geographical cluster different from clade 2 (West) represented by individuals from Siwa and Abu Rawash.

### DISCUSSION

The femoral gland secretions of lizards play an important role in chemical signalling (Cooper and Vitt, 1984; Alberts, 1993; Aragón et al., 2001; Khannoon et al., 2010), and our previous work on *A. boskianus* confirms the role of these chemicals, particularly alcohols and steroids, as scent pheromones that appear to be used in territorial marking (Khannoon et al., 2011a, b). Similarly, we have shown previously that *A. boskianus* are able to discriminate conspecifics of the same or the opposite sex by differences in the chemical components of their secretions (Khannoon et al., 2010). The five main groups of compounds, alcohols, steroids, acids, glycerolmonoethers and monoglycerides detected in this study represent more than 98% of the TIC from *A. boskianus* secretions. It is very likely therefore that the chemical repertoire sampled in the present study includes the active elements responsible for these scent differences. Although the secretion components are shared between the two groups (east and west of Egypt), their quantitative composition is significantly different. Phylogenetic analysis indicates that the lizards in these two chemically divergent populations are also genetically distinct.

Signals result from multiple adaptations to the environment, and the physical properties of the environment exert important constraints on the nature and form of signals (Théry and Heeb, 2008). The differences in secretion component proportions between the eastern and western populations may thus be the result of either differences in environmental conditions and/or genetics. Evolution of signals depends on different types of constraint (phylogenetic or environmental), and can be the result of trade-off between natural and sexual selection (Théry and Heeb, 2008). Environmental conditions are different between the Sinai and Siwa habitats. While South Sinai comprises mountainous regions of up to 2641 m with a maximum temperature 29.7°C and 1–35 mm annual rainfall, the Siwa oasis lies deep in the highly arid western desert with temperatures of up to 53°C and infrequent and unpredictable rainfall (Goodman and Meininger, 1989; Kassas, 1993). The environmental and temperature variability between the two population habitats may support the environmental constraint effect on the chemical composition of secretions. Studies on birds have demonstrated the effect of environment on skin lipids (Ro and Williams 2010; Williams et al., 2012). This environmental effect may be important if these long chain lipids with lower volatility and higher chemical stability are needed in higher temperature habitats to render volatiliza-

tion and decomposition of the secretions. Only a few (four) chemicals, showed differences between eastern and western populations, and no specific long chain chemical accumulated on either side. On the other hand, Abu Rawash lies adjacent to Delta and Nile where the temperatures and aridity are much lower than in Siwa. Despite this, Abu Rawash is chemically similar to Siwa, both of which are on the western side of the Nile. This raises the possibility that the chemical composition reflects phylogenetic rather than environmental relationships.

The phylogenetic results (Fig. 3) clearly show that the samples of *A. boskianus* fall into two geographically-restricted clades. Clade 1 represents the east and clade 2 represents the west, containing individuals from both Siwa and Abu Rawash. These two clades are robust to the addition of many more samples representing populations throughout Egypt, North Africa and Arabia (Khannoon et al., unpublished data). It is likely that these groups represent a phylogeographic genetic subdivision of the species, possibly corresponding to races or subspecies. This has been reported in many organismal systems (Avice and Wollenberg, 1997; Walker and Avice, 1998).

A significant divergence in chemical characteristics of the femoral secretions and a phylogenetic split between eastern and western populations of *A. boskianus* mirrors this subdivision have been demonstrated here. It is likely therefore that the time spent in isolation by these groups, separated by the Nile River, with little or no gene flow between them has also lead to divergence in secretion characteristics. Although a robust test of an environmental basis for the secretion differences would require a carefully designed transplantation or common-garden experiment, our current data casts doubt on this as an explanation. Samples from Abu Rawash are geographically closest to those from Siwa, found in the same phylogenetic clade, and are chemically similar. Despite these similarities, the lizards inhabit very different environments with regard to temperature and rainfall.

The type of signal divergence in allopatry that we propose has been seen in other systems as well. In mammals, such as the mouse *Rhabdomys pumilio*, population-specific communication signals, and in particular olfactory cues, assortative mate choice, and pre-mating reproductive isolation, have all been described as the result of divergence in allopatry (Pillay, 2000). In anurans, call structure was diverged with allopatric populations (Smith et al., 2003) and calling songs in Mormon crickets (Bailey et al., 2007) and odorant signals of the house mouse (Smadja and Ganem, 2008) have the same patterns.

Territorial behavior and scent marking are potentially significant factors in population isolation in many species (Pillay, 2000; Higgin and Blows, 2008). Our molecular analysis shows the existence of two genetically diverged clades of these lizards in Egypt. This divergence is reflected in the equally large differences in the odour profiles of the populations. This suggests that lizards could well be capable of detecting such differences. Such odour profiles used as scent marking (Khannoon et al., 2010) may form the basis of mating preferences, kin and nest mate recognition, as well as indicators for health and immune status, as is known in house mice (Zala et al., 2004), ants (Carlin and Hölldobler,

1983) and moths (Baker, 2008). Odor-based population isolation is well known from insects, such as moths, in which pheromone bouquets vary significantly between populations (Löfstedt, 1993), and it has recently been shown that similar isolation exists in marine invertebrates (Sutton et al., 2005). The present study showed that allopatric populations are quantitatively, not qualitatively, different in their scent gland secretions. In turnip moths *Agrostis segetum*, Löfstedt (1993) described the population dependent pheromone bouquets as genetically based. It was concluded that mate choice drove selection pressures that favoured geographically differences where the compounds themselves are the same but show geographical diverse odour fingerprints.

In conclusion, we demonstrate that the inter-population chemosignal variability of *A. boskianus* most likely reflects the genetic divergence in allopatry of the populations under study. However, further studies on the behaviour of *A. boskianus* females towards male secretions of the studied populations are needed, assuming these secretions function in species isolation, which would mean their intended receiver is a female lizard. The divergence in chemical composition of femoral pore secretions will be biologically meaningful if females could differentiate between secretions from males of both population clades. Further studies on the chemical composition of the sympatric species *A. scutellatus* and other species of the same genus might give important insights into signal evolution and speciation within this group.

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