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Running title: Specific proteins in lizard chemical signals

AUTHOR CONTRIBUTION

MM, SB, RS designed research; MM, SS, RS performed research; SB, RVD, JM contributed data; MM analysed data and created figures; and MM, SB, RVD, RS wrote the paper and all authors aided in interpreting the results and contributed to editing the final paper.

Abstract. The species-specific components (SSC) of animal sexual signals can facilitate species recognition and reduce the risks of mismatching and interbreeding. Still, empirical evidence for SSCs in chemical signals is scarce and limited to insect pheromones. Based on the proteinaceous femoral glandular secretions of 36 lizard species (Lacertidae), we examine the SSC potential of proteins in lizard chemical signals. By quantitatively comparing the one-dimensional electrophoretic patterns of the protein fraction from femoral gland secretions, we first reveal that protein composition is species-specific, accounting for a large part of the observed raw variation, and allowing us to discriminate species on this basis.. Secondly, we find increased protein pattern divergence in sympatric, closely related species. Thirdly, lizard protein profiles show a low phylogenetic signal, a recent and steep increase in relative disparity, and a high rate of evolutionary change compared to non-signal traits (i.e. body size and shape). Together, these findings provide strong support for the specific-specificity of proteins in the chemical signals of a vertebrate lineage.

Key words. Chemical communication; signal evolution; species recognition; interspecific interference; proteins; lizards

INTRODUCTION

The spectacular diversity of animal signals and displays has been a great source of wonder for a long period of time (Guilford & Dawkins, 1991; Laidre & Johnstone, 2013). Species-specific components (SSCs), i.e., those features entailed in species recognition, constitute an important element of this variability (West-Eberhard, 1984; Ord & Stamps, 2009; Schaefer & Ruxton, 2015). Notable examples include bird song (Becker, 1982), the signature head-bob in *Anolis* lizards displays (Stamps & Barlow, 1973), the "whine" intro in the advertisement calls of some Leiuperinae frogs (Ryan, 1983), and the specific cuticular hydrocarbons of *Formica* ants (Martin, Helanterä, & Drijfhout, 2008), all of which exhibit striking species specificity.

Acting as a kind of species-identity badge, SSCs have been implicated in species recognition mechanisms (Wiley, 1983; Ord & Stamps, 2009), and therefore may play a role in speciation and the maintenance of reproductive isolation (Dobzhansky, 1937; Mayr, 1942, 1963; West-Eberhard, 1983; Rundle & Nosil, 2005; Sobel *et al.*, 2010; Rabosky, 2016). The "badge" may consist of a simple and distinct element of the signal, such as the stereotyped sequence of visual displays (e.g. in lizards; (Ord & Martins, 2006)), specific notes in acoustic emissions (e.g., in bird songs; (Becker, 1982)), or the presence of particular molecules (e.g., complex pheromone cocktails of wasps; (Weiss *et al.*, 2015)). In other cases, the "badge" is more complex and composed of multiple characteristics, as occur for example in the multicomponent and multimodal communication (Partan & Marler, 1999, 2005). Examples of this are the head and body combined features of Darwin's finches (Ratcliffe & Grant, 1983), the hydrocarbons profiles of crickets (Tyler *et al.*, 2015), as well as visual and chemical cues in swordtail fish (Hankison & Morris, 2003). While the evolution of a simple or complex badge may depend upon a combination of natural and sexual selection pressures (Schaefer & Ruxton, 2015), animal SSCs are expected to share some general design features and among-species variability patterns (Weber *et al.*, 2016; Tibbetts, Mullen, & Dale, 2017). Indeed, in order to ensure the accurate detection and recognition of conspecifics (Johnstone, 1997a;

Gröning & Hochkirch, 2008; Pillay & Rymer, 2012), SSCs must be highly specific, showing a narrow within-species variation, and a wide among-species variability (Becker, 1982; Ord & Stamps, 2009; Tibbetts *et al.*, 2017). Notably, SSC divergence should be strongest between sibling spatially overlapping (sympatrics and syntopics) species (West-Eberhard, 1984; Percy, Taylor, & Kennedy, 2006; Schaefer & Ruxton, 2015; Grether *et al.*, 2017), since this condition requires an enhanced accuracy in species recognition in order to avoid interbreeding (Gröning & Hochkirch, 2008; Ord & Stamps, 2009; Pfennig & Pfennig, 2009; Grether *et al.*, 2017). In this sense, it would be expected that the evolution of these traits to exhibit weaker Brownian phylogenetic signal and, possibly, higher evolutionary rates than non-signaller traits, such as morphology (especially those non-genital), or trophic ecology (Ritchie, 2007; Arnegard *et al.*, 2010; Weber *et al.*, 2016; Zozaya *et al.*, 2019; Quipildor *et al.*, 2021). Indeed, SSC is expected to diverge as speciation occurs, contributing to generally increase intra-clade variability (Symonds & Elgar, 2004; Weber *et al.*, 2016; García-Roa *et al.*, 2017b).

As one of the oldest and most widespread sensory modalities (Ache & Young, 2005), chemoreception has been shown to function for species recognition in a wide range of animal taxa (Wyatt, 2003; Smadja & Butlin, 2009). Many lizards, like other squamate reptiles, are strongly chemically-oriented and are equipped with both a nasal and a well-developed vomeronasal-lingual system that allow them to efficiently sample and process chemicals from the environment (Schwenk, 1995; Baeckens *et al.*, 2017b). Further, most lizard species carry epidermal glands (pre-cloacal or femoral glands, hereafter FG) producing chemical signals (Martín & López, 2011, 2014; Mayerl, Baeckens, & Van Damme, 2015; Zozaya *et al.*, 2019). FG secretions consist of a protein-lipid mix (Alberts, 1990; Mangiacotti *et al.*, 2019c,a) used to convey a wide range of different messages (Martín & López, 2011, 2015; Baeckens, 2019), including species identity (Gabirot *et al.*, 2010a; García-Roa *et al.*, 2016; MacGregor *et al.*, 2017; Valdecantos & Labra, 2017). The majority of our understanding of the evolution of chemical signalling in lizards and the role of FG therein originates from the analysis of the

lipophilic fraction alone. Chemical and behavioural analyses suggest that lipids primarily convey condition-related features of the signaller, such as its fighting ability, health, parasite load and body size (reviewed in (Martín & López, 2015)), but at least in some taxa, the composition of the lipid fraction varies greatly among closely related groups and therefore may also function in species-recognition (Martín & López, 2006; Zozaya *et al.*, 2019). Interestingly, phylogenetic comparative analyses revealed that lipid fraction has a weak phylogenetic signal (Baeckens *et al.*, 2018a), with specific compounds following different evolutionary patterns (García-Roa *et al.*, 2017b; Campos *et al.*, 2020). Maximizing signal efficacy is considered the main evolutionary driver of both the variability and complexity of the lipid signal (Baeckens *et al.*, 2017a, 2018a,b), as chemical signals respond to different environment constraints (Alberts, 1992). For example, xeric environments promote the increased abundance of less-volatile compounds, which guarantee a more long-lasting signal, while mesic conditions favour the use of less heavy molecules to enhance detectability (Heathcote *et al.*, 2014; Baeckens *et al.*, 2018a). Similar conclusions are drawn by the intraspecific comparison of lipid fraction variability across environmental gradients (Gabirot, López, & Martín, 2012; Martín *et al.*, 2017).

As we mentioned before about the composition of FG secretions and contrary to the lipophilic counterpart, hardly anything is known on the protein fraction.. Although a long time is recognized that FG contained proteins with a possible function in communication (Padoa, 1933; Cole, 1966; Alberts, 1990; Alberts & Werner, 1993), studies of lizard chemical communication have subsequently ignored them manifestly (Font *et al.*, 2012; Mayerl *et al.*, 2015; Mangiacotti *et al.*, 2017). This underestimation may well have jeopardized our understanding of species recognition in lizards, as proteins would make excellent SSCs (Wyatt, 2010, 2014). Indeed, the very first attempt to compare FG proteins among related lizard species revealed strong support for the species-specificity of the protein profiles (Alberts, 1991). Unfortunately, Albert (1991) did not consider within-species variability and

121 the difference among species was almost hidden. Moreover, it was not made under a
122 phylogenetic comparative analysis framework, which would have allowed ruling out protein
123 specificity to be a predictable consequence of interspecific genetic differences. Recently, the
124 interest in the protein fraction has revived (e.g., (Mangiacotti *et al.*, 2017)), and supported an
125 active role of FG secretions' proteins in lizard communication, allowing, for example, self-
126 recognition (Mangiacotti *et al.*, 2019b, 2020). Furthermore, FG secretions' proteins carry
127 different badge-like information as the sender's population, the specific clade of origin
128 (Mangiacotti *et al.*, 2017), and the colour morph identity (Mangiacotti *et al.*, 2019a). Here, we
129 investigate the interspecific diversity in FG protein profiles across a family of lizards. For
130 this, we analysed the pattern of phenotypic variability in one-dimensional electrophoretic
131 profiles (hereafter EPGs) to test the SSC hypothesis. We expect: (1) larger among-species
132 than within-species EPGs variation; (2) increased EPG divergence in sympatric, closely
133 related species; (3) high evolution rate of EPGs compared to other non-signal traits.

134 Lacertid lizards (Lacertidae) constitute an excellent model system for the study of
135 vertebrate chemical communication in general (Baeckens, 2019) and to test our hypothesis in
136 particular, for a number of reasons. Firstly, lacertids are strongly chemical-oriented (Baeckens
137 *et al.*, 2017b; García-Roa *et al.*, 2017a), as they use FG secretions to send and gain different
138 information about conspecifics (individual identity, species identity, female reproductive
139 status, health and condition, fighting ability), which are used in make-decision processes
140 (female choice, rival assessment, territory defence; for details, see (Martín & López, 2014).
141 Secondly, based on different phylogenetic analyses (Mendes *et al.*, 2016; Zheng & Wiens,
142 2016; Garcia-Porta *et al.*, 2019), lacertids constitute a relatively young and species-rich lizard
143 clade with a well-supported classification. This allows testing species different evolutionary
144 approach on traits as for example their evolutionary rate of change. Thirdly, many lacertid
145 species have (partially) overlapping distributional ranges (Sillero *et al.*, 2014; Roll *et al.*,

2017) and it is not unusual that locally, species occur in the same or adjacent microhabitats (Arnold, 1987), allowing us to test the effect of sympatry on signal design.

MATERIAL AND METHODS

Femoral gland secretions: collection and profiling

We analysed samples of FG secretions of 135 male lizards belonging to 36 species (2-4 samples per species), and 12 genera of the Lacertidae family (Table S1). Samples from single populations were collected between 2002 and 2014, and stored in glass vials fitted with Teflon-lined stoppers, and kept at −20 °C until analysis. Collection procedures and permits were described in detail in previous works (Baeckens *et al.*, 2017a, 2018a,b). Briefly: (i) for all species, secretions were collected during the breeding seasons, i.e., when glandular activity is at its maximum (Cole, 1966; Alberts, Pratt, & Phillips, 1992; Mangiacotti *et al.*, 2019c); (ii) secretions were collected immediately after capture; (iii) all samples underwent the same lab protocols (notably, lipids extraction) which did not alter subsequent protein analysis (Mangiacotti *et al.*, 2019c). No lizards were killed or injured during the study, and sampling collection was not invasive and did not cause damage to any animal tissues.

The protein fraction were analysed following the procedures implemented in (Mangiacotti *et al.*, 2017, 2019c), which allow us to fingerprint the protein components of the femoral gland secretions of each specimen using Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After complete defatting (using *n*-hexane), proteins were dissolved in phosphate-buffered saline (PBS; 10 mM, pH 7.4) solution, and their concentration assessed by the bicinchoninic acid assay (Smith *et al.*, 1985), using bovine serum albumin as the standard for calibration curve. From each sample, 10 μg of proteins were added to 10 μL of loading buffer solution (50 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulphate SDS, 0.1% bromophenol blue, and 10% glycerol), and incubated at 95 °C for five minutes, before the electrophoresis run. Electrophoresis was performed in a discontinuous

mode (5% stacking gel and 15% running gel) with constant voltage (180 V for 2 h (Garfin, 2009)). Gels were stained with a 0.12% (w/v) Coomassie Blue G-250 solution, containing 10% (v/v) orthophosphoric acid, 10% (w/v) ammonium sulphate and 20% (v/v) methanol. After removing exceeding coloration with acetic acid (5% v/v), a high quality image of each gels was obtained (1200 dpi).

A standardized and comparable electrophoretogram (EPG) for each sample was extracted from each gel image, and used as proxy for the protein composition (Mangiacotti *et al.*, 2017, 2019c). Images were first converted into grayscale, by applying the luma formula (Poynton, 2012). Along each lane, the luma approximates the protein concentration at a given molecular weight. So, we extracted the luma profiles along vertical lines through the middle of each lane, and obtained the sample EPGs. To make EPGs comparable across gels, they were: (i) aligned, by fitting a cubic spline on the positions of the standard molecular weights of each gel; (ii) "de-noised", by applying a baseline detection algorithm (Gan, Ruan, & Mo, 2006); (iii) divided into 300 equal bins each bearing the mean luma of the pixels falling within each bin (about ten); (iv) normalized, dividing by the sum of the 300 values composing each EPG. This way, each EPG consisted of a sequence of 300 normalized luma values which represent the protein profile and were comparable across samples and gels. All operations were implemented in R v3.5.2 (R Core Team, 2018) adapting the functions available in (Mangiacotti *et al.*, 2019c).

Intra- vs interspecific variation of the protein profiles

To assessed the variability in the protein composition attributable to the species level, we transformed the normalized EPGs using centred-log-ratio to account for their compositional nature (Aitchison, 1982; van den Boogaart & Tolosana-Delgado, 2013) and computed the Euclidean distance matrix among all EPGs pairs. Then, we performed a distance-based ANOVA (Anderson, 2001) on the resulting matrix, using the species as the grouping factor

198 and protein concentration as a covariate (Mangiacotti *et al.*, 2019b). Significance was
199 assessed by 999 permutations of the data, which were stratified within gel, to address the
200 possible issue of non-independence of EPGs coming from the same electrophoretic run. We
201 excluded from this analysis *Gallotia stehlini*, because we only accepted as a minimum three
202 (see Table S1). A test for the homogeneity of group dispersion was previously conducted
203 (Anderson, 2006), failing to detect any significant difference (pseudo-F=1.195; P=0.087).
204 We then reversed the question to assess the ability of EPGs to predict species
205 membership. Given the high-dimensionality of the EPG data we used a shrinkage-based
206 diagonal discriminant analysis (Pang, Tong, & Zhao, 2009), where all but one EPGs for each
207 species were used to train the model, and the remnant one to test it. One-hundred replicates of
208 the so-built training and testing datasets were randomly chosen, a model was obtained, and its
209 performance evaluated by the percentage of correctly classified test data (accuracy) (Raschka,
210 2018). To highlight the most and least important molecular weight regions in discrimination
211 (i.e., the ones showing the highest or lowest among-species variability, respectively), we
212 computed a summary scores for each EPG interval, starting from the correlation-adjusted t-
213 scores (CAT scores; (Zuber & Strimmer, 2009; Ahdesmäki & Strimmer, 2012)). We then
214 classified the obtained scores into three relevance categories: high (scores above the 3rd
215 quartile); intermediate (scores between 1st and 3rd quartile); low (scores below the 1st quartile).
216 For all the above-mentioned analyses we used R v3.5.2 (R Core Team, 2018) and the
217 following packages: `compositions` (van den Boogaart, Tolosana-Delgado, & Bren, 2020);
218 `permute` (Simpson, 2019); `vegan` (Oksanen *et al.*, 2019); `sda` (Ahdesmaki *et al.*, 2015).
219
220 *Divergence of the protein signal in sympatry*
221 To test the effect of sympatry on SSC divergence, we used multivariate distance matrix
222 regressions (Zapala & Schork, 2012). Notably, we regressed the pairwise distance matrix of
223 species average EPG, against the pairwise geographic distribution overlap (proxy for the level

of sympatry between two species), adding the pairwise phylogenetic distance (i.e., the pairwise distance matrix between the tips of the phylogenetic tree) as a control factor. The geographic overlap may be a raw proxy of the real sympatry, since two geographically overlapping species may inhabit different environments, never coming into actual contact. To account for this issue, we first ran the analysis considering the whole set of species (n = 36), then we repeated the analysis focusing on *Podarcis* alone, as this genus was the most represented (11 spp.) in our dataset and included lizards with quite similar ecological traits and needs (Böhme, 1986). By restricting the analysis to a single genus, we also narrowed the evolutionary timeframe, reducing the blurred effect of the simple phylogenetic separation on the protein signatures. In both analyses, the general procedures to compute the three distances, and run the regression were the same.

We obtained species EPGs as the geometric mean of conspecific EPGs (Aitchison, 1982; van den Boogaart & Tolosana-Delgado, 2013), and calculated the distances matrix as in the previous analysis. We normalized distances dividing by the maximum observed value (Legendre & Legendre, 1998).

The matrix of geographic overlap was obtained basing on the distribution maps available in (Roll *et al.*, 2017), re-projected into an equal area projection (Europe Equal Area 2001http://www.ec-gis.org). We computed the overlap index (s_{ij}) between species i and j as follows:

$$s_{ij} = \frac{A_i \cap A_j}{\min(A_i, A_j)}$$

where $A_i \cap A_j$ is the geographic overlap (shared area) between the two distributions A_i and A_j. We bounded s_{ij} between 0 and 1, dividing by the minimum between the A_i and A_j, both to emphasize the overlap and reduce the inflation toward zero due to the wide distribution of some species. We converted s_{ij} into a distance using the formula: $d_{ij} = \sqrt{1 - s_{ij}^2}$ (Legendre & Legendre, 1998).

The matrix of phylogenetic distances was extracted from the ultrametric, calibrated phylogenetic tree accompanying the most recent reconstruction of lacertid phylogeny (Garcia-Porta *et al.*, 2019)..

For all the above-mentioned analyses we used R v3.5.2 (R Core Team, 2018) and the following packages: `compositions` (van den Boogaart *et al.*, 2020); `raster` (Hijmans, 2020); `rgeos` (Bivand & Rundel, 2019); `phytools` (Revell, 2012).

Phylogenetic comparative analysis

The third block of analyses used a phylogenetic comparative approach (Adams & Collyer, 2019) on the full species set utilized in the analysis of signal divergence in sympatry.

To track the non-signal evolutionary pattern, for all the 36 species, we compiled a morphometric dataset (Table S2) including: snout-to-vent length (SVL), head length (HL), head maximum width (HW), forelimb length (FLL), and hindlimb length (HLL). These measures are expected to respond to environmental adaptation in lizards (Vanhooydonck & Van Damme, 1999; Kohlsdorf, Garland Jr., & Navas, 2001; Herrel, Meyers, & Vanhooydonck, 2002; Herrel, Vanhooydonck, & Van Damme, 2004; Verwaijen, Van Damme, & Herrel, 2002; Goodman, Miles, & Schwarzkopf, 2008), and they should not show a signal-like pattern of evolution (Harmon *et al.*, 2003; Arnegard *et al.*, 2010; Weber *et al.*, 2016). We disentangled size and shape information by using the log-transformed SVL as size proxy, and the residuals of a standardized major axis regression of log-transformed head size (HS), FLL and HLL against size as shape variables (Kaliontzopoulou, Carretero, & Llorente, 2008); HS was the geometric mean of the head measures (Kaliontzopoulou *et al.*, 2008). All the shape variables were bound together to constitute the shape matrix.

We first estimated the strength of the phylogenetic signal (K;(Blomberg, Garland, & Ives, 2003)) on lizard EPGs, size and shape. Being EPGs and shape considering as multivariate traits, we adopted a distance-based K estimation (Adams, 2014b; Adams &

Collyer, 2019), which equally applies to univariate traits (Adams, 2014b). As in its original formulation, under a Brownian motion, K has an expected value of 1; so, K < 1 indicates a low phylogenetic signal, K near or above 1 means the phylogenetic signal is strong. The non-randomness of K was assessed via 999 permutations (Adams & Collyer, 2015). For interpretational purposes, we also calculated the univariate phylogenetic signal (K_{uni}) along the scores of the first principal components (PCs) of the transformed EPGs. We considered PCs accounting for at least 95% of total variation, and selected the axes which retained significant K_{uni} values after Holm correction (Holm, 1979).

Secondly, we estimated the evolutionary rate (σ^2) of EPGs and morphometric data, and tested whether the former was larger than the latter. We followed the distance-based method proposed by Adams (Adams, 2014a), as modified for non-modular datasets (Denton & Adams, 2015). Together with a σ^2 estimation for each multivariate or univariate trait, the pairwise ratios are computed, and tested against the distribution of simulated ratios obtained under the assumption of no difference in evolutionary rate among the three subsets (Adams, 2014a; Denton & Adams, 2015).

Thirdly, we compared the divergence pattern of EPGs, size and shape, along the phylogeny, using a disparity-true-time (DTT) analysis (Harmon *et al.*, 2003; Guillerme *et al.*, 2020). Disparity is an index of the among-group morphological difference, evaluated at each node of the phylogenetic tree (Foote, 1997; Harmon *et al.*, 2003): small values indicate that trait variation most occurs among clades, and closely related species share similar phenotypes; on the opposite, large values imply variation is partitioned within subclades, and distant species may overlap in the morphospace (Harmon *et al.*, 2003). The observed DTT profile was compared to that obtained by simulating trait evolution under a null model (Brownian motion; 999 simulations; (Harmon *et al.*, 2003)). The direction and significance of the difference between the observed and simulated trajectories were tested by the Morphological Disparity Index test (MDI) and the rank-envelope test (Murrell, 2018). MDI is

an overall measure of the difference between observed- and null-trajectory: positive values indicate disparity is mainly held within-clades, whereas negative values imply that differences occur among-clades (Harmon *et al.*, 2003; Slater *et al.*, 2010). The rank-envelope test compares the whole DTT curve, and identifies the time-points along the trajectory where the curve deviates from the null model predictions (Murrell, 2018). For both tests we used the R functions `dtt1`, `getMDIp2t`, `rank_env_dtt`, available in Murrell (Murrell, 2018).

All the analyses were conducted in R v3.5.2 (R Core Team, 2018) using the following packages: `compositions` (van den Boogaart *et al.*, 2020); `ape` (Paradis & Schliep, 2019); `smatr` (Warton *et al.*, 2012); `geomorph` (Adams, Collyer, & Kaliontzopoulou, 2020).

RESULTS

All samples provided useful EPGs, and a species-specific pattern was notably apparent: the samples belonging to the same species showed highly similar banding schemes, consistently sharing the main peaks (Fig 1, grey lines in each species panel); on the opposite, different species (even congeneric) were characterized by a distinct pattern, both in the position and intensity of the bands (Fig 2). The distance-based ANOVA found EPGs to be significantly affected by the "species" factor (pseudo-$F=5.013$; $P \leq 0.001$), which accounted for 63.5% of the total variation, while the protein concentration did not affect electrophoretic runs (pseudo-$F=0.999$; $P \leq 0.616$). The strong relation between EPGs and species membership was confirmed by the discriminant analysis, which correctly matched samples and species in 86.5% of cases (accuracy range: 74.3%-100.0%; IRQ=5.71%). CAT scores identified two main EPGs' regions (HRR1, HRR2, Fig. 1, bottom panel) contributing most to species discrimination: a low molecular weight zone, between 9 and 18 kDa, and a middle zone between 38 and 48 kDa. These regions showed the highest interspecific variability. On the opposite, the most preserved EPG region was between 19 and 25 kDa (Fig. 1), where all the species showed at least one highly expressed band (Fig. 2).

The sampled lizards differed in geographic overlap, ranging between zero (allopatry) and one (complete overlap; Table S3). Regarding the *Podarcis* set, the pairwise overlap varied between zero and 0.98. The multivariate distance matrix regression on the complete species dataset revealed a significant effect only for the phylogenetic distance (pseudo-t=15.119; P≤0.001), the geographic overlap being irrelevant (distance-transformed geographic overlap; pseudo-t=-0.470; P≤0.765). The SSC divergence increased with increasing phylogenetic distance (β=0.317) supporting the occurrence of a phylogenetic signal. The same model applied to the *Podarcis* group reported an importantly different outcome: the phylogenetic distance still kept a significant effect (β=0.218; pseudo-t=1.872; P≤0.037), but also the geographic overlap did (pseudo-t=-2.123; P≤0.049), showing a negative trend (β=-0.302; Fig. 3): more specifically, signal divergence (as measured by the distance between EPGs), was greater between species with more overlapping distributional areas.

The occurrence of a phylogenetic signal in EPGs, suggested by the previous analysis, may be coupled with a K value of 0.501 associated to protein profiles (P<0.001; Table 1). Notably, the *Gallotia* and *Acanthodactylus* groups occupied distinct areas of the EPG morphospace (Fig. 4), the former having a typical three-bands scheme in the high-molecular weight EPG (less expressed than the mid-part), the latter showing a simplified single-band pattern in the same EPG region (Figs. 1 and 2). The species from the other genera were dispersed without a clear specific pattern, but with a slight tendency for congeners to aggregate with each other (Fig. 4). The EPGs' region of low variability (19-25 kDa), where all species showed an intense peak (Figs. 1 and 2) may be responsible for this effect and for the overall weak phylogenetic signal.

The phylogenetic signal of the reference morphological traits was significantly larger than zero and very strong for body size (K = 1.372; P<0.001; Table 1), small and not significant for body shape (Table 1). Particularly, body size remains consistently large in the genus *Gallotia*, medium in *Lacerta* and small in the remaining taxa (Fig. 4). No clear pattern

352 emerged from the analysis of body shape morphospace, but the lower than 1 and not
353 significant K value (0.398; P=0.081; Table 1) indicated a poor phylogenetic effect (Fig. 4).
354 With regard to the results of the evolutionary diversification tests, the evolutionary rate
355 of EPGs (σ^2 = 11.599; Table 1) was much higher than those of body size (σ^2 = 0.002; Table 1)
356 and shape (σ^2 = 0.0003; Table 1), with both the ratios $\sigma^2_{EPG}/\sigma^2_{size}$ and $\sigma^2_{EPG}/\sigma^2_{shape}$ being
357 significantly larger than one (P≤0.001). Further, MDI of EPGs was significantly higher than
358 expected under a Brownian motion model (Table 1), and the relative disparity index stayed
359 above the predicted range from about 50 Mya on, peaking near the crown of the tree (Fig. 5).
360 In comparison, though also MDI of body shape showed a marginally significant larger-than-
361 zero value (Table 1), the relative disparity index followed a completely different trajectory
362 (Fig. 5), with values above the prediction only between 32 and 15 Mya. The disparity of body
363 size did not vary more than expected (Table 1; Fig. 5), supporting the phylogenetic effect on
364 it.
365
366 DISCUSSION
367 Species-specific components (SSCs) have been identified in signals of various sensory
368 modalities and in a wide variety of animal lineages. They have been implicated in
369 mechanisms of reproductive isolation and speciation (Mayr, 1963; West-Eberhard, 1984;
370 Smadja & Butlin, 2009; Sobel *et al.*, 2010; Schaefer & Ruxton, 2015; Rabosky, 2016). Here,
371 we provide comprehensive, albeit indirect, evidence that proteinaceous secretions from the
372 femoral glands of lacertid lizards might carry SSCs.
373 The FG protein profiles show a noticeable species-specific pattern, which is a necessary
374 prerequisite for a signal to bear SSC (Wiley, 1983; West-Eberhard, 1984; Pillay & Rymer,
375 2012; Schaefer & Ruxton, 2015; Weber *et al.*, 2016). Despite a certain degree of variability
376 (Fig. 1), within-species EPGs clearly share the same overall silhouette, and can be effectively

discriminated from heterospecific profiles. The intraspecific variability is of the same magnitude as that observed in the common wall lizard (*Podarcis muralis*; (Mangiacotti *et al.*, 2017, 2019c)), the desert iguana (*Dipsosaurus dorsalis*; (Alberts, 1991)), and the green iguana (*Iguana iguana*; (Alberts, Phillips, & Werner, 1993)), suggesting that we can reasonably exclude the bias due to small within-species sample size used to assess both intra- and inter-specific variation.

Most of the interspecific variability is loaded by two disjoint EPG regions (Fig. 1), where both the number and intensity of the peaks are species-dependent. The intermediate weight range, which often represents the most intense EPG part, shows a more stable pattern. However, the level of interspecific variability in EPGs we observed in this study seems large enough to allow lizards to discriminate species identity using protein SSC alone. Indeed, lizards are not only able to detect proteins as an independent chemical class (Cooper, 1991; Mangiacotti *et al.*, 2020), but they can also recognize the occurrence of very slight differences, e.g., among conspecifics (Alberts & Werner, 1993; Mangiacotti *et al.*, 2019b, 2020), suggesting a very fine chemosensory ability (Cooper, 1994; Schwenk, 1995; Baeckens *et al.*, 2017b).

Although it may be argued that the specificity of FG proteins may simply be the consequence of the genetic difference among-species, a further result supporting their possible SSC function is the tendency of the protein signature to diverge more as the current geographic overlap increases, at least when congeneric species (i.e., *Podarcis* group) were considered. Probably, this tendency did not emerge when non-congeneric species were included due to the noise added by the accumulated ecological and phylogenetic distance on the species signature. Inflated divergence between the signals of closely related sympatric species suggests the occurrence of reproductive character displacement as it is in line with the idea that SSCs may help in pre-mating isolation and hybridization avoidance (Smadja & Butlin, 2009; Edwards *et al.*, 2015; Grether *et al.*, 2017). As such, by increasing the distance

between two SSCs, the accuracy of conspecifics recognition improves (Wiley, 1983; Johnstone, 1997b), contributing to the coexistence of sympatric species. Sympatry of closely related species may impose high cost in term of fitness to one or both species because of interspecific aggression (Tynkkynen *et al.*, 2005), competition for resources or reproductive interactions (e.g., hybridization). Indeed, both current and past hybridization are well-known in the genus *Podarcis* (Capula, 1993, 2002; Pinho *et al.*, 2009; Ficetola *et al.*, 2021). While the first is quantitatively limited, genetic evidence suggests that its effectiveness is not 100% (Pinho *et al.*, 2009; Caeiro-Dias *et al.*, 2021; Yang *et al.*, 2021). Consequently, selective pressures are expected to promote character displacement in species traits involved in species recognition, to reduce detrimental interactions, but only where they occur in sympatry. Thus, in sympatry, SSCs should rapidly diverge when compared to allopatric populations, as direct response to the presence of the other species (Pfennig & Pfennig, 2009). Examples of SSC displacement in sympatry are not rare in animals. In orchid bees (*Euglossa* sp.), sympatric species were found to diverge more than allopatric ones in their chemical signals, but only for a relatively small subset of compounds, which are probably involved in species recognition (Weber *et al.*, 2016). In two European Odonates of the genus *Calopteryx* males from populations of *C. splendens* living in sympatry with *C. virgo* have significantly smaller wing spots than male conspecifics living in allopatric populations (Tynkkynen, Rantala, & Suhonen, 2004; Cigognini *et al.*, 2014). Wing spot works as SSC in these species and size reduction in *C. splendens* males improves recognition by *C. virgo* males, significantly decreasing the risk of inter-specific aggression (Tynkkynen *et al.*, 2004). We do acknowledge that our survey sampled just one population per species, precluding the explicit analysis of the effect of sympatric congeners at the within-species level (Collyer & Adams, 2007; Wheatcroft, 2015). Nonetheless, our comparison of species within the ecologically homogeneous group of wall lizards revealed that the protein signal diverged more in those species pairs with higher geographic overlap. The amount of geographic overlap can be

viewed as a proxy for the probability of interference, which, in turn, may have favoured the SSC differentiation (Curé *et al.*, 2012). Indeed, sympatric *Podarcis* lizards hybridize in natural conditions (Gorman *et al.*, 1975; Capula, 1993, 2002; Pinho *et al.*, 2009; Jančúchová-Lásková, Landová, & Frynta, 2015), and males engage in interspecific aggressive interactions (Böhme, 1986; Corti & Lo Cascio, 2002; Downes & Bauwens, 2002; Lailvaux, Huyghe, & Van Damme, 2012). In this scenario, a mechanism promoting SSC character displacement in sympatry may reflect the need for a more accurate species recognition mechanism in mating and male-male contest. In many lacertids, males scent mark the area in which they claim exclusive rights over females (Edsman, 1986); signals with clear SSCs would aid in avoiding misguided aggression towards non-conspecifics (López & Martín, 2001, 2002; López, Martín, & Cuadrado, 2002; Carazo, Font, & Desfilis, 2008; Font *et al.*, 2012). The SSCs in the peptide fraction of FG secretions may accordingly explain the well-established ability of lacertid males to distinguish conspecific from heterospecific individuals on the basis of chemical cues (Barbosa *et al.*, 2005, 2006; Martín & López, 2006; Gabirot *et al.*, 2010b,a; Labra, 2011; Font *et al.*, 2012). Alternatively or additionally, an enhanced SSC in male scent may allow females to accurately recognize the species identity of the territory owner, providing the basis for a pre-mating reproductive barrier (Smadja & Butlin, 2009; Runemark, Gabirot, & Svensson, 2011; García-Roa *et al.*, 2016). Indeed, previous studies have established that lacertid females can also recognize conspecifics through chemoreception (Gabirot *et al.*, 2010b; Labra, 2011), although not in all species (Martín & López, 2006; Font *et al.*, 2012; Gabirot, Lopez, & Martín, 2013; Martín *et al.*, 2016). Because the role of female choice in lacertid lizards has been questioned repeatedly (Olsson *et al.*, 2003; Font *et al.*, 2012; Gabirot *et al.*, 2013; Sacchi *et al.*, 2015, 2018; MacGregor *et al.*, 2017), we are inclined to prefer the scenario in which SSCs evolved to minimize misguided male-male conflict. However, an (additional) role in avoiding hybridization cannot be excluded.

A third support to the prediction for an SSC-bearing signal come from the macroevolutionary pattern emerging from the phylogenetic comparative analysis of EPGs. Firstly, the phylogenetic signal for protein profiles is weak, indicating that EPGs are evolutionary labile and their variability cannot be explained by classic Brownian motion along the current tree. Indeed, EPGs evolved much faster than indexes of body size and shape in the same clade (Table 1). Secondly, much of the EPGs variability has been maintained within clades, and their disparity boosted towards the tips of the phylogeny, i.e., at most recent speciation events, highlighting a rapid divergence between sister taxa. Taken together, the above findings support the SSC-hypothesis. Indeed, the morphological traits used as reference, and supposed not to bear SSC, did not show any combination of evolutionary patterns, being characterized by a stronger phylogenetic signal (body size), a slow evolutionary rate (body size and shape), and a punctual, increased disparity far from the tips of the tree (body shape).

Other hypotheses, alternative to SSC, may explain the low EPGs phylogenetic signal. For instance, an equally low K for the lipophilic profiles in FG secretions of lacertid lizards (K = 0.45) has been attributed to adaptive evolution, driven by environmental conditions (Baeckens *et al.*, 2017a, 2018a,b; García-Roa *et al.*, 2017b). Such hypothesis may apply also to the FG proteins, where the species-specific pattern may reflect an environmental adaptation to increase signal efficiency (Endler, 1992, 1993). Additionally, since proteins are homogeneously associated to lipids, and may serve as chemical matrix supporting the more volatile counterpart (Alberts, 1990; Alberts & Werner, 1993), they may show a phylogenetic pattern of variation correlating with the one observed for lipid composition. However, the disparity in DDT trajectories of the lipophilic fractions (García-Roa *et al.*, 2017b) and protein fractions (this study) strongly suggests different drivers. This does not exclude the environment may have influenced the evolution of some components of the FG proteinaceous secretions (Symonds & Elgar, 2008; Edwards *et al.*, 2015; Schaefer & Ruxton, 2015), or that

some proteins may associate to lipids (Alberts, 1990; Wyatt, 2014). But rather, it suggests that the design of the protein signal could be mainly driven by other selective forces. Identifying whether and which EPG fractions have been shaped by environmental variables or by lipid composition, on the other hand, open an interesting question which requires specific studies.

In conclusion, using lacertids as model group, we demonstrated that the FG protein secretions include SSC, which may allow for interspecific recognition on a chemical basis. Proteins are well-suited to work as elements of species signature in terrestrial vertebrates, being highly specific, genetically determined, and long-lasting on substrates (Wyatt, 2010, 2014). Lizards are able to detect and respond to protein signals (Alberts & Werner, 1993; Mangiacotti *et al.*, 2019b, 2020), but additional behavioural studies are needed to confirm that they actually use protein SSC to modulate interspecific interactions, including perhaps to avoid interspecific hybridization. Another obvious next step is the identification of the proteins involved in species recognition. Is species identity coded by the amino acid sequence of one or more proteins, or does it involve changes in the relative abundance of molecules within a protein cocktail? Can concomitant changes be found in the vomeronasal receptors? How fast does this proteinaceous SSC system evolve – in the presence and absence of congeneric species, and which evolutionary mechanisms are involved? The current finding that the protein fraction in lizard femoral secretions acts like a species-badge opens a promising avenue for further investigation.

ACKNOWLEDGEMENTS

We are in debt with Erez Maza, Shai Meri, Panayiotis Pafilis, Katarina Ljubisavljević, and Oscar Arribas for their kind and valuable help in providing the morphological data for those species lacking bibliographic information. We thank three anonymous reviewers for their useful comments on an early draft of the manuscript. M.M. and R.S. were supported by FRG 2016 (Ministry of Education, University and Research - MIUR); S.B. was supported by a

FWO-Flanders postdoctoral fellowship (12I8819N). The experimental design and procedures complied the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). This work was conducted under permits for Croatia (UP/I-612-07/14-48/111 & UP/I-612-07/14-48/33), The Netherlands (FF/74A/2015/009), Israel (2014/40323), SA Free State Province (S54C-515022511060), SA Eastern Cape Province (CRO 45/15CR & 46/15CR), SA Western Cape Province (0056-AAA041-00093), SA Northern Cape Province (FAUNA 229/2015 & 230/2015) and SA Limpopo Province (0092-MKT001-00004), and was in accordance with University of Antwerp (Belgium) animal welfare standards and protocols (ECD 2014-32). Captures of lizards and sampling procedures were performed under different licenses for the Environmental Agencies of the different Regional Governments of Spain where lizards were studied. All Greek species were collected in accordance with the Hellenic National Legislation (Presidential Decree 67/81).

Data availability Statement. The data underlying this article are available in Zenodo, at https://dx.doi.org/10.5281/zenodo.4116401 (DOI will be activated upon acceptance).

REFERENCES

Ache BW & Young JM. **2005**. Olfaction: Diverse species, conserved principles. *Neuron* **48**: 417–430.

Adams DC. **2014a**. Quantifying and comparing phylogenetic evolutionary rates for shape and other high-dimensional phenotypic data. *Systematic Biology* **63**: 166–177.

Adams DC. **2014b**. A generalized K statistic for estimating phylogenetic signal from shape and other high-dimensional multivariate data. *Systematic Biology* **63**: 685–697.

Adams DC & Collyer ML. **2015**. Permutation tests for phylogenetic comparative analyses of high-dimensional shape data: What you shuffle matters. *Evolution* **69**: 823–829.

Adams DC & Collyer ML. **2019**. Phylogenetic Comparative Methods and the Evolution of

Multivariate Phenotypes. *Annual Review of Ecology, Evolution, and Systematics* **50**: 405–425.

Adams DC, Collyer ML & Kaliontzopoulou A. **2020**. Geomorph: Software for geometric morphometric analyses.

Ahdesmäki M & Strimmer K. **2012**. Feature selection in omics prediction problems using cat scores and false nondiscovery rate control. *Annals of Applied Statistics* **6**: 503–519.

Ahdesmaki M, Zuber V, Gibb S, *et al.* 2015. sda: Shrinkage Discriminant Analysis and CAT Score Variable Selection.

Aitchison J. **1982**. The Statistical Analysis of Compositional Data. *Journal of the Royal Statistical Society. Series B (Methodological)* **44**: 139–177.

Alberts AC. **1990**. Chemical properties of femoral gland secretions in the desert iguana, *Dipsosaurus dorsalis*. *Journal of chemical ecology* **16**: 13–25.

Alberts AC. **1991**. Phylogenetic and adaptive variation in lizard femoral gland secretions. *Copeia* **1991**: 69–79.

Alberts AC. **1992**. Constraints on the design of chemical communication systems in terrestrial vertebrates. *The American Naturalist* **139**: S62–S89.

Alberts AC, Phillips JA & Werner DI. **1993**. Sources of intraspecific variability in the protein composition of lizard femoral gland secretions. *Copeia* **1993**: 775–781.

Alberts AC, Pratt NC & Phillips JA. **1992**. Seasonal productivity of lizard femoral glands: Relationship to social dominance and androgen levels. *Physiology & Behavior* **51**: 729–733.

Alberts AC & Werner DI. **1993**. Chemical recognition of unfamiliar conspecifics by green iguanas: functional significance of different signal components. *Animal Behaviour* **46**: 197–199.

Anderson MJ. **2001**. A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26**: 32–46.

Anderson MJ. **2006**. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **62**: 245–53.

557 **Arnegard ME, McIntyre PB, Harmon LJ, *et al.* 2010**. Sexual signal evolution outpaces
558 ecological divergence during electric fish species radiation. *American Naturalist* **176**: 335–
559 356.
560 **Arnold EN**. **1987**. Resource partition among lacertid lizards in southern Europe. *Journal of*
561 *Zoology* **1**: 739–782.
562 **Baeckens S**. **2019**. Evolution of animal chemical communication : Insights from non-model
563 species and phylogenetic comparative methods. *Belgian Journal of Zoology* **149**: 63–93.
564 **Baeckens S, García-Roa R, Martín J, *et al.* 2017a**. The Role of Diet in Shaping the
565 Chemical Signal Design of Lacertid Lizards. *Journal of Chemical Ecology* **43**: 902–910.
566 **Baeckens S, Herrel A, Broeckhoven C, *et al.* 2017b**. Evolutionary morphology of the lizard
567 chemosensory system. *Scientific Reports* **7**: 10141.
568 **Baeckens S, Martín J, García-Roa R, *et al.* 2018a**. Environmental conditions shape the
569 chemical signal design of lizards. *Functional Ecology* **32**: 566–580.
570 **Baeckens S, Martín J, García-Roa R, *et al.* 2018b**. Sexual selection and the chemical signal
571 design of lacertid lizards. *Zoological Journal of the Linnean Society* **183**: 445–457.
572 **Barbosa D, Desfilis E, Carretero MA, *et al.* 2005**. Chemical stimuli mediate species
573 recognition in Podarcis wall lizards. *Amphibia Reptilia* **26**: 257–263.
574 **Barbosa D, Font E, Desfilis E, *et al.* 2006**. Chemically mediated species recognition in
575 closely related Podarcis wall lizards. *Journal of Chemical Ecology* **32**: 1587–1598.
576 **Becker PH**. **1982**. The coding of species-specific caracteristicsin bird sounds. In: Kroodsma
577 DE, Miller EH, Ouellet H, eds. *Acoustic Communication in Birds: Production, Perception,*
578 *and Design Features of Sounds*. New York: Academic Press, 213–252.
579 **Bivand R & Rundel C**. **2019**. rgeos: Interface to Geometry Engine - Open Source ('GEOS').
580 **Blomberg SP, Garland T & Ives AR**. **2003**. Testing for phylogenetic signal in comparative
581 data: behavioral traits are more labile. *Evolution* **57**: 717.
582 **Böhme W**. **1986**. *Handbuch der Reptilien und Amphibien Europas, Band 2/II., Echsen III*

583 *(Podarcis)* (W Böhme, Ed.). Wiesbaden: Aula Verlag.
584 **van den Boogaart KG & Tolosana-Delgado R**. **2013**. *Analyzing compositional data with R*.
585 Berlin: Springer.
586 **van den Boogaart KG, Tolosana-Delgado R & Bren M**. **2020**. compositions:
587 Compositional Data Analysis.
588 **Caeiro-Dias G, Brelsford A, Kaliontzopoulou A, *et al.* 2021**. Variable levels of
589 introgression between the endangered Podarcis carbonelli and highly divergent congeneric
590 species. *Heredity* **126**: 463–476.
591 **Campos SM, Pruett JA, Soini HA, *et al.* 2020**. Volatile fatty acid and aldehyde abundances
592 evolve with behavior and habitat temperature in Sceloporus lizards. *Behavioral Ecology* **31**:
593 978–991.
594 **Capula M**. **1993**. Natural hybridization in *Podarcis sicula* and *P. wagleriana* (Reptilia:
595 Lacertidae). *Biochemical Systematics and Ecology* **21**: 373–380.
596 **Capula M**. **2002**. Genetic evidence of natural hybridization between *Podarcis sicula* and
597 *Podarcis tiliguerta* (Reptilia: Lacertidae). *Amphibia Reptilia* **23**: 313–321.
598 **Carazo P, Font E & Desfilis E**. **2008**. Beyond 'nasty neighbours' and 'dear enemies'?
599 Individual recognition by scent marks in a lizard (*Podarcis hispanica*). *Animal Behaviour* **76**:
600 1953–1963.
601 **Cigognini R, Gallesi MM, Mobili S, *et al.* 2014**. Does character displacement demonstrate
602 density-dependent expression in females? A test on the wing shape of two species of
603 European damselflies. *Evolutionary Ecology* **28**: 941–956.
604 **Cole CJ**. **1966**. Femoral glands in lizards: a review. *Herpetologica* **22**: 199–206.
605 **Collyer ML & Adams DC**. **2007**. Analysis of two-state multivariate phenotypic change in
606 ecological studies. *Ecology* **88**: 683–692.
607 **Cooper WE**. **1991**. Responses to prey chemicals by a lacertid lizard, *Podarcis muralis*: Prey
608 chemical discrimination and poststrike elevation in tongue-flick rate. *Journal of Chemical*

609 *Ecology* **17**: 849–863.
610 **Cooper WE**. **1994**. Chemical discrimination by tongue-flicking in lizards: A review with
611 hypotheses on its origin and its ecological and phylogenetic relationships. *Journal of chemical*
612 *ecology* **20**: 439–87.
613 **Corti C & Lo Cascio P**. **2002**. *The lizards of Italy and adjacent areas*. Frankfurt am Mein:
614 Edition Chimaira.
615 **Curé C, Mathevon N, Mundry R, *et al.* 2012**. Acoustic cues used for species recognition
616 can differ between sexes and sibling species: Evidence in shearwaters. *Animal Behaviour* **84**:
617 239–250.
618 **Denton JSS & Adams DC**. **2015**. A new phylogenetic test for comparing multiple high-
619 dimensional evolutionary rates suggests interplay of evolutionary rates and modularity in
620 lanternfishes (Myctophiformes; Myctophidae). *Evolution* **69**: 2425–2440.
621 **Dobzhansky TG**. **1937**. *Genetics and the origin of species*. New York: Columbia University
622 Press.
623 **Downes S & Bauwens D**. **2002**. An experimental demonstration of direct behavioural
624 interference in two Mediterranean lacertid lizard species. *Animal Behaviour* **63**: 1037–1046.
625 **Edsman L**. **1986**. Territoriality and resource defence in Wall Lizards (*Podarcis muralis*).
626 *Studies in Herpetology* **1985**: 601–604.
627 **Edwards DL, Melville J, Joseph L, *et al.* 2015**. Ecological divergence, adaptive
628 diversification, and the evolution of social signaling traits: An empirical study in arid
629 Australian lizards. *American Naturalist* **186**: E144–E161.
630 **Endler JA**. **1992**. Signals, Signal Conditions, and the Direction of Evolution. *The American*
631 *Naturalist* **139**: S125–S153.
632 **Endler JA**. **1993**. Some general comments on the evolution and design of animal
633 communication systems. *Philosophical transactions of the Royal Society of London. Series B,*
634 *Biological sciences* **340**: 215–25.

635 **Ficetola GF, Silva-Rocha I, Carretero MA, *et al.* 2021**. Status of the largest extant
636 population of the critically endangered Aeolian lizard *Podarcis raffonei* (Capo Grosso,
637 Vulcano island). *PLoS ONE* **16**: 1–15.
638 **Font E, Barbosa D, Sampedro C, *et al.* 2012**. Social behavior, chemical communication,
639 and adult neurogenesis: studies of scent mark function in *Podarcis* wall lizards. *General and*
640 *comparative endocrinology* **177**: 9–17.
641 **Foote M**. **1997**. The evolution of morphological diversity. *Annual Review of Ecology and*
642 *Systematics* **28**: 129–152.
643 **Gabirot M, Castilla AM, López P, *et al.* 2010a**. Differences in chemical signals may explain
644 species recognition between an island lizard, *Podarcis atrata*, and related mainland lizards, *P.*
645 *hispanica*. *Biochemical Systematics and Ecology* **38**: 521–528.
646 **Gabirot M, Castilla AM, López P, *et al.* 2010b**. Chemosensory species recognition may
647 reduce the frequency of hybridization between native and introduced lizards. *Canadian*
648 *Journal of Zoology* **88**: 73–80.
649 **Gabirot M, Lopez P & Martín J**. **2013**. Female mate choice based on pheromone content
650 may inhibit reproductive isolation between distinct populations of Iberian wall lizards.
651 *Current Zoology* **59**: 210–220.
652 **Gabirot M, López P & Martín J**. **2012**. Interpopulational variation in chemosensory
653 responses to selected steroids from femoral secretions of male lizards, *Podarcis hispanica*,
654 mirrors population differences in chemical signals. *Chemoecology* **22**: 65–73.
655 **Gan F, Ruan G & Mo J**. **2006**. Baseline correction by improved iterative polynomial fitting
656 with automatic threshold. *Chemometrics and Intelligent Laboratory Systems* **82**: 59–65.
657 **Garcia-Porta J, Irisarri I, Kirchner M, *et al.* 2019**. Environmental temperatures shape
658 thermal physiology as well as diversification and genome-wide substitution rates in lizards.
659 *Nature Communications* **10**: 4077.
660 **García-Roa R, Cabido C, López P, *et al.* 2016**. Interspecific differences in chemical

composition of femoral gland secretions between two closely related wall lizard species, *Podarcis bocagei* and *Podarcis carbonelli*. *Biochemical Systematics and Ecology* **64**: 105–110.

García-Roa R, Jara M, Baeckens S, *et al.* 2017a. Macroevolutionary diversification of glands for chemical communication in squamate reptiles. *Scientific Reports* **7**: 9288.

García-Roa R, Jara M, López P, *et al.* 2017b. Heterogeneous tempo and mode of evolutionary diversification of compounds in lizard chemical signals. *Ecology and Evolution* **7**: 1286–1296.

Garfin DE. **2009**. One-Dimensional Gel Electrophoresis. In: Burgess RR, Deutscher MPBTM in E, eds. *Methods in Enzymology*. Academic Press, 497–513.

Goodman BA, Miles DB & Schwarzkopf L. **2008**. Life on the rocks: Habitat use drives morphological and performance evolution in lizards. *Ecology* **89**: 3462–3471.

Gorman GC, Soulé M, Yang SY, *et al.* 1975. Evolutionary Genetics of Insular Adriatic Lizards. *Evolution* **29**: 52–71.

Grether GF, Peiman KS, Tobias JA, *et al.* 2017. Causes and Consequences of Behavioral Interference between Species. *Trends in Ecology and Evolution* **32**: 760–772.

Gröning J & Hochkirch A. **2008**. Reproductive interference between animal species. *Quarterly Review of Biology* **83**: 257–282.

Guilford T & Dawkins MS. **1991**. Receiver psychology and the evolution of animal signals. *Animal Behaviour* **42**: 1–14.

Guillerme T, Cooper N, Brusatte SL, *et al.* 2020. Disparities in the analysis of morphological disparity. *Biology letters* **16**: 20200199.

Hankison SJ & Morris MR. **2003**. Avoiding a compromise between sexual selection and species recognition: Female swordtail fish assess multiple species-specific cues. *Behavioral Ecology* **14**: 282–287.

Harmon LJ, Schulte JA, Larson A, *et al.* 2003. Tempo and mode of evolutionary radiation

687 in iguanian lizards. *Science* **301**: 961–964.
688 **Heathcote RJP, Bell E, d'Ettorre P, *et al.* 2014**. The scent of sun worship: basking
689 experience alters scent mark composition in male lizards. *Behavioral Ecology and*
690 *Sociobiology* **68**: 861–870.
691 **Herrel A, Meyers JJ & Vanhooydonck B**. **2002**. Relations between microhabitat use and
692 limb shape in phrynosomatid lizards. *Biological Journal of the Linnean Society* **77**: 149–163.
693 **Herrel A, Vanhooydonck B & Van Damme R**. **2004**. Omnivory in lacertid lizards:
694 Adaptive evolution or constraint? *Journal of Evolutionary Biology* **17**: 974–984.
695 **Hijmans RJ**. **2020**. raster: Geographic Data Analysis and Modeling.
696 **Holm S**. **1979**. A Simple Sequentially Rejective Multiple Test Procedure. *Scandinavian*
697 *Journal of Statistics* **6**: 65–70.
698 **Jančúchová-Lásková J, Landová E & Frynta D**. **2015**. Are genetically distinct lizard
699 species able to hybridize? A review. *Current Zoology* **61**: 155–180.
700 **Johnstone RA**. **1997a**. The evolution of animal signals. In: Krebs JR, Davies NB, eds.
701 *Behavioural ecology: An evolutionary approach*. Malden, USA: Blackwell Publishing Ltd,
702 155–178.
703 **Johnstone RA**. **1997b**. Recognition and the evolution of distinctive signatures: When does it
704 pay to reveal identity? *Proceedings of the Royal Society B: Biological Sciences* **264**: 1547–
705 1553.
706 **Kaliontzopoulou A, Carretero MA & Llorente GA**. **2008**. Head shape allometry and
707 proximate causes of head sexual dimorphism in Podarcis lizards: Joining linear and geometric
708 morphometrics. *Biological Journal of the Linnean Society* **93**: 111–124.
709 **Kohlsdorf T, Garland Jr. T & Navas CA**. **2001**. Limb and tail lengths in relation to
710 substrate usage in Tropidurus lizards. *Journal of Morphology* **248**: 151–164.
711 **Labra A**. **2011**. Chemical stimuli and species recognition in Liolaemus lizards. *Journal of*
712 *Zoology* **285**: 215–221.

713 **Laidre ME & Johnstone RA**. **2013**. Animal signals. *Current Biology* **23**: R829–R833.
714 **Lailvaux SP, Huyghe K & Van Damme R**. **2012**. Why can't we all just get along?
715 Interspecific aggression in resident and non-resident Podarcis melisellensis lizards. *Journal of*
716 *Zoology* **288**: 207–213.
717 **Legendre P & Legendre L**. **1998**. *Numerical ecology*. Amsterdam: Elsevier Science B.V.
718 **López P & Martín J**. **2001**. Fighting roles and rival recognition reduce costs of aggression in
719 male lizards, *Podarcis hispanica*. *Behavioral Ecology and Sociobiology* **49**: 111–116.
720 **López P & Martín J**. **2002**. Chemical rival recognition decreases aggression levels in male
721 Iberian wall lizards, Podarcis hispanica. *Behavioral Ecology and Sociobiology* **51**: 461–465.
722 **López P, Martín J & Cuadrado M**. **2002**. Pheromone-Mediated Intrasexual Aggression in
723 Male Lizards, *Podarcis hispanicus*. *Aggressive Behavior* **28**: 154–163.
724 **MacGregor HEA, Lewandowsky RAM, D'Ettorre P, *et al.* 2017**. Chemical
725 communication, sexual selection, and introgression in wall lizards. *Evolution* **71**: 2327–2343.
726 **Mangiacotti M, Fumagalli M, Cagnone M, *et al.* 2019a**. Morph-specific protein patterns in
727 the femoral gland secretions of a colour polymorphic lizard. *Scientific Reports* **9**: 8412.
728 **Mangiacotti M, Fumagalli M, Scali S, *et al.* 2017**. Inter- and intra-population variability of
729 the protein content of femoral gland secretions from a lacertid lizard. *Current Zoology* **63**:
730 657–665.
731 **Mangiacotti M, Gaggiani S, Coladonato AJ, *et al.* 2019b**. First experimental evidence that
732 proteins from femoral glands convey identity related information in a lizard. *Acta Ethologica*
733 **22**: 57–65.
734 **Mangiacotti M, Martín J, López P, *et al.* 2020**. Proteins from femoral gland secretions of
735 male rock lizards Iberolacerta cyreni allow self—but not individual—recognition of
736 unfamiliar males. *Behavioral Ecology and Sociobiology* **74**: 68.
737 **Mangiacotti M, Pezzi S, Fumagalli M, *et al.* 2019c**. Seasonal Variations in Femoral Gland
738 Secretions Reveals some Unexpected Correlations Between Protein and Lipid Components in

739 a Lacertid Lizard. *Journal of Chemical Ecology* **45**: 673–683.
740 **Martin SJ, Helanterä H & Drijfhout FP**. **2008**. Evolution of species-specific cuticular
741 hydrocarbon patterns in *Formica* ants. *Biological Journal of the Linnean Society* **95**: 131–140.
742 **Martín J & López P**. **2006**. Interpopulational differences in chemical composition and
743 chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*:
744 Implications for sexual isolation in a species complex. *Chemoecology* **16**: 31–38.
745 **Martín J & López P**. **2011**. Pheromones and reproduction in reptiles. In: Lopez KH, Norris
746 DO, eds. *Hormones and Reproduction of Vertebrates*. London: Academic Press, 141–167.
747 **Martín J & López P**. **2014**. Pheromones and Chemical Communication in Lizards. In:
748 Rheubert JL, Siegel DS, Trauth SE, eds. *Reproductive Biology and Phylogeny of Lizards and*
749 *Tuatara*. New York, NY: Taylor and Francis Group USA, 54–88.
750 **Martín J & López P**. **2015**. Condition-dependent chemosignals in reproductive behavior of
751 lizards. *Hormones and behavior* **68**: 14–24.
752 **Martín J, López P, Iraeta P, *et al.* 2016**. Differences in males' chemical signals between
753 genetic lineages of the lizard *Psammodromus algirus* promote male intrasexual recognition
754 and aggression but not female mate preferences. *Behavioral Ecology and Sociobiology* **70**:
755 1657–1668.
756 **Martín J, Zamora-Camacho FJ, Reguera S, *et al.* 2017**. Variations in chemical sexual
757 signals of *Psammodromus algirus* lizards along an elevation gradient may reflect altitudinal
758 variation in microclimatic conditions. *Science of Nature* **104**: 16.
759 **Mayerl C, Baeckens S & Van Damme R**. **2015**. Evolution and role of the follicular
760 epidermal gland system in non-ophidian squamates. *Amphibia-Reptilia* **36**: 185–206.
761 **Mayr E**. **1942**. *Systematics and the origin of species*. New York: Columbia University Press.
762 **Mayr E**. **1963**. Isolating mechanism. In: *Animal Species and Evolution*. Harvard University
763 Press, 89–109.
764 **Mendes J, Harris DJ, Carranza S, *et al.* 2016**. Evaluating the phylogenetic signal limit from

765 mitogenomes, slow evolving nuclear genes, and the concatenation approach. New insights
766 into the Lacertini radiation using fast evolving nuclear genes and species trees. *Molecular*
767 *Phylogenetics and Evolution* **100**: 254–267.
768 **Murrell DJ**. **2018**. A global envelope test to detect non-random bursts of trait evolution.
769 *Methods in Ecology and Evolution* **9**: 1739–1748.
770 **Oksanen J, Blanchet FG, Friendly M, *et al.* 2019**. vegan: Community Ecology Package.
771 **Olsson M, Madsen T, Nordby J, *et al.* 2003**. Major histocompatibility complex and mate
772 choice in sand lizards. *Proceedings of the Royal Society of London. Series B: Biological*
773 *Sciences* **270**: S254–S256.
774 **Ord TJ & Martins EP**. **2006**. Tracing the origins of signal diversity in anole lizards:
775 phylogenetic approaches to inferring the evolution of complex behaviour. *Animal Behaviour*
776 **71**: 1411–1429.
777 **Ord TJ & Stamps JA**. **2009**. Species Identity Cues in Animal Communication. *The*
778 *American Naturalist* **174**: 585–593.
779 **Padoa E**. **1933**. Ricerche sperimentali sui pori femorali e sull'epididimo della lucertola
780 (*Lacerta muralis* Laur.) considerati come caratteri sessuali secondari. *Arch. ital. anat. embriol*
781 **31**: 205–252.
782 **Pang H, Tong T & Zhao H**. **2009**. Shrinkage-based diagonal discriminant analysis and its
783 applications in high-dimensional data. *Biometrics* **65**: 1021–1029.
784 **Paradis E & Schliep K**. **2019**. Ape 5.0: An environment for modern phylogenetics and
785 evolutionary analyses in R. *Bioinformatics* **35**: 526–528.
786 **Partan SR & Marler P**. **1999**. Communication goes multimodal. *Science* **283**: 1272–1273.
787 **Partan SR & Marler P**. **2005**. Issues in the Classification of Multimodal Communication
788 Signals. *The American Naturalist* **166**: 231–245.
789 **Percy DM, Taylor GS & Kennedy M**. **2006**. Psyllid communication: Acoustic diversity,
790 mate recognition and phylogenetic signal. *Invertebrate Systematics* **20**: 431–445.

Pfennig KS & Pfennig DW. **2009**. Character Displacement: Ecological and Reproductive Responses to a Common Evolutionary Problem. *The Quarterly Review of Biology* **84**: 253–276.

Pillay N & Rymer TL. **2012**. Behavioural divergence, interfertility and speciation: A review. *Behavioural Processes* **91**: 223–235.

Pinho C, Kaliontzopoulou A, Carretero MA, *et al.* 2009. Genetic admixture between the Iberian endemic lizards *Podarcis bocagei* and *Podarcis carbonelli*: Evidence for limited natural hybridization and a bimodal hybrid zone. *Journal of Zoological Systematics and Evolutionary Research* **47**: 368–377.

Poynton C. **2012**. *Digital video and HD: algorithms and interfaces*. Elsevier Science.

Quipildor AM, Ruiz-Monachesi MR, Ruiz S, *et al.* 2021. Male genitalia's evolutionary rate is higher than those of body traits: the case of two Liolaemus lizards' group. *Journal of Zoology* **313**: 54–65.

R Core Team. **2018**. R: A Language and Environment for Statistical Computing.

Rabosky DL. **2016**. Reproductive isolation and the causes of speciation rate variation in nature. *Biological Journal of the Linnean Society* **118**: 13–25.

Raschka S. **2018**. Model Evaluation, Model Selection, and Algorithm Selection in Machine Learning.

Ratcliffe LM & Grant PR. **1983**. Species recognition in Darwin's finches (Geospiza, Gould) I. Discrimination by morphological cues. *Animal Behaviour* **31**: 1139–1153.

Revell LJ. **2012**. phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* **3**: 217–223.

Ritchie MG. **2007**. Sexual Selection and Speciation. *Annual Review of Ecology, Evolution, and Systematics* **38**: 79–102.

Roll U, Feldman A, Novosolov M, *et al.* 2017. The global distribution of tetrapods reveals a need for targeted reptile conservation. *Nature Ecology and Evolution* **1**: 1677–1682.

817 **Rundle HD & Nosil P**. **2005**. Ecological speciation. *Ecology Letters* **8**: 336–352.
818 **Runemark A, Gabirot M & Svensson EI**. **2011**. Population divergence in chemical signals
819 and the potential for premating isolation between islet- and mainland populations of the
820 Skyros wall lizard (Podarcis gaigeae). *Journal of Evolutionary Biology* **24**: 795–809.
821 **Ryan MJ**. **1983**. Frequency modulated calls and species recognition in a neotropical frog.
822 *Journal of Comparative Physiology □ A* **150**: 217–221.
823 **Sacchi R, Coladonato AJ, Ghitti M, *et al.* 2018**. Morph-specific assortative mating in
824 common wall lizard females. *Current Zoology* **64**: 449–453.
825 **Sacchi R, Ghitti M, Scali S, *et al.* 2015**. Common Wall Lizard Females (*Podarcis muralis*)
826 do not Actively Choose Males Based on their Colour Morph (T Tregenza, Ed.). *Ethology* **121**:
827 1145–1153.
828 **Schaefer HM & Ruxton GD**. **2015**. Signal Diversity, Sexual Selection, and Speciation.
829 *Annual Review of Ecology, Evolution, and Systematics* **46**: 573–592.
830 **Schwenk K**. **1995**. Of tongues and noses: chemoreception in lizards and snakes. *Trends in*
831 *Ecology & Evolution* **10**: 7–12.
832 **Sillero N, Campos J, Bonardi A, *et al.* 2014**. Updated distribution and biogeography of
833 amphibians and reptiles of Europe. *Amphibia Reptilia* **35**: 1–31.
834 **Simpson GL**. **2019**. permute: Functions for Generating Restricted Permutations of Data.
835 **Slater GJ, Price SA, Santini F, *et al.* 2010**. Diversity versus disparity and the radiation of
836 modern cetaceans. *Proceedings of the Royal Society B: Biological Sciences* **277**: 3097–3104.
837 **Smadja C & Butlin R**. **2009**. On the scent of speciation: the chemosensory system and its
838 role in premating isolation. *Heredity* **102**: 77–97.
839 **Smith PK, Krohn RI, Hermanson GT, *et al.* 1985**. Measurement of protein using
840 bicinchoninic acid [published erratum appears in Anal Biochem 1987 May 15;163(1):279].
841 *Anal Biochem* **150**: 76–85.
842 **Sobel JM, Chen GF, Watt LR, *et al.* 2010**. The biology of speciation. *Evolution* **64**: 295–

315.

Stamps JA & Barlow GW. **1973**. Variation and Stereotypy in the Displays of Anolis aeneus (Sauria: Iguanidae). *Behaviour* **47**: 67–94.

Symonds MRE & Elgar MA. **2004**. Species overlap, speciation and the evolution of aggregation pheromones in bark beetles. *Ecology Letters* **7**: 202–212.

Symonds MRE & Elgar MA. **2008**. The evolution of pheromone diversity. *Trends in Ecology and Evolution* **23**: 220–228.

Tibbetts EA, Mullen SP & Dale J. **2017**. Signal function drives phenotypic and genetic diversity: The effects of signalling individual identity, quality or behavioural strategy. *Philosophical Transactions of the Royal Society B: Biological Sciences* **372**: 20160347.

Tyler F, Fisher D, D'Ettorre P, *et al.* 2015. Chemical cues mediate species recognition in field crickets. *Frontiers in Ecology and Evolution* **3**: 1–9.

Tynkkynen K, Kotiaho JS, Luojumäki M, *et al.* 2005. Interspecific aggression causes negative selection on sexual characters. *Evolution* **59**: 1838–1843.

Tynkkynen K, Rantala MJ & Suhonen J. **2004**. Interspecific aggression and character displacement in the damselfly *Calopteryx splendens*. *Journal of Evolutionary Biology* **17**: 759–767.

Valdecantos S & Labra A. **2017**. Testing the functionality of precloacal secretions from both sexes in the South American lizard, *Liolaemus chiliensis*. *Amphibia-Reptilia* **38**: 209–216.

Vanhooydonck B & Van Damme R. **1999**. Evolutionary relationships between body shape and habitat use in lacertid lizards. *Evolutionary Ecology Research* **1**: 785–803.

Verwaijen D, Van Damme R & Herrel A. **2002**. Relationships between head size, bite force, prey handling efficiency and diet in two sympatric lacertid lizards. *Functional Ecology* **16**: 842–850.

Warton DI, Duursma RA, Falster DS, *et al.* 2012. smatr 3- an R package for estimation and inference about allometric lines. *Methods in Ecology and Evolution* **3**: 257–259.

869 **Weber MG, Mitko L, Eltz T, *et al.* 2016**. Macroevolution of perfume signalling in orchid
870 bees. *Ecology Letters* **19**: 1314–1323.
871 **Weiss I, Hofferberth J, Ruther J, *et al.* 2015**. Varying importance of cuticular hydrocarbons
872 and iridoids in the species-specific mate recognition pheromones of three closely related
873 *Leptopilina* species. *Frontiers in Ecology and Evolution* **3**: 1–12.
874 **West-Eberhard MJ**. **1983**. Sexual selection, Social competition, and speciation. *The*
875 *Quarterly Review of Biology* **58**: 155–183.
876 **West-Eberhard MJ**. **1984**. Sexual selection, competitive communication and species specific
877 signals in insects. In: Lewis T, ed. *Insect Communication*. New York, NY: Academic Press,
878 283–324.
879 **Wheatcroft D**. **2015**. Reproductive interference via display signals: the challenge of multiple
880 receivers. *Population Ecology* **57**: 333–337.
881 **Wiley RH**. **1983**. The evolution of communication: information and manipulation. In:
882 Halliday TR, Slater PJB, eds. *Animal Behaviour, Vol. 2, Communication*. Oxford: Blackwell
883 Scientific Publications, 156–189.
884 **Wyatt TD**. **2003**. *Pheromones and Animal Behaviour*. New York, NY: Cambridge University
885 Press.
886 **Wyatt TD**. **2010**. Pheromones and signature mixtures: defining species-wide signals and
887 variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative*
888 *Physiology A* **196**: 685–700.
889 **Wyatt TD**. **2014**. Proteins and peptides as pheromone signals and chemical signatures.
890 *Animal Behaviour* **97**: 273–280.
891 **Yang W, Feiner N, Pinho C, *et al.* 2021**. Extensive introgression and mosaic genomes of
892 Mediterranean endemic lizards. *Nature Communications* **12**: 2762.
893 **Zapala MA & Schork NJ**. **2012**. Statistical properties of multivariate distance matrix
894 regression for high-dimensional data analysis. *Frontiers in Genetics* **3**: 1–10.

Zheng Y & Wiens JJ. **2016**. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Molecular phylogenetics and evolution* **94**: 537–47.

Zozaya SM, Higgie M, Moritz C, *et al.* 2019. Are pheromones key to unlocking cryptic lizard diversity? *American Naturalist* **194**: 168–182.

Zuber V & Strimmer K. **2009**. Gene ranking and biomarker discovery under correlation. *Bioinformatics* **25**: 2700–2707.

905 **Table 1**. Phylogenetic signal (K), mean evolutionary rate (σ^2), and morphological disparity
906 index (MDI) of the protein profiles (EPG) and the morphological traits (body size and shape).
907 The P value associated to K was obtained by permutation; the one coupled to MDI by
908 simulating DTT curves under a Brownian motion model (see methods for detail).

Trait	K		σ^2	MDI		
	value	P		value	P	$P_{\text{rank-envelop test}}$
EPG	0.501	≤ 0.001	11.599	0.284	<0.001	0.009
Body size	1.372	≤ 0.001	0.002	0.062	0.416	0.372
Body shape	0.398	0.081	0.0003	0.229	0.068	0.012

909

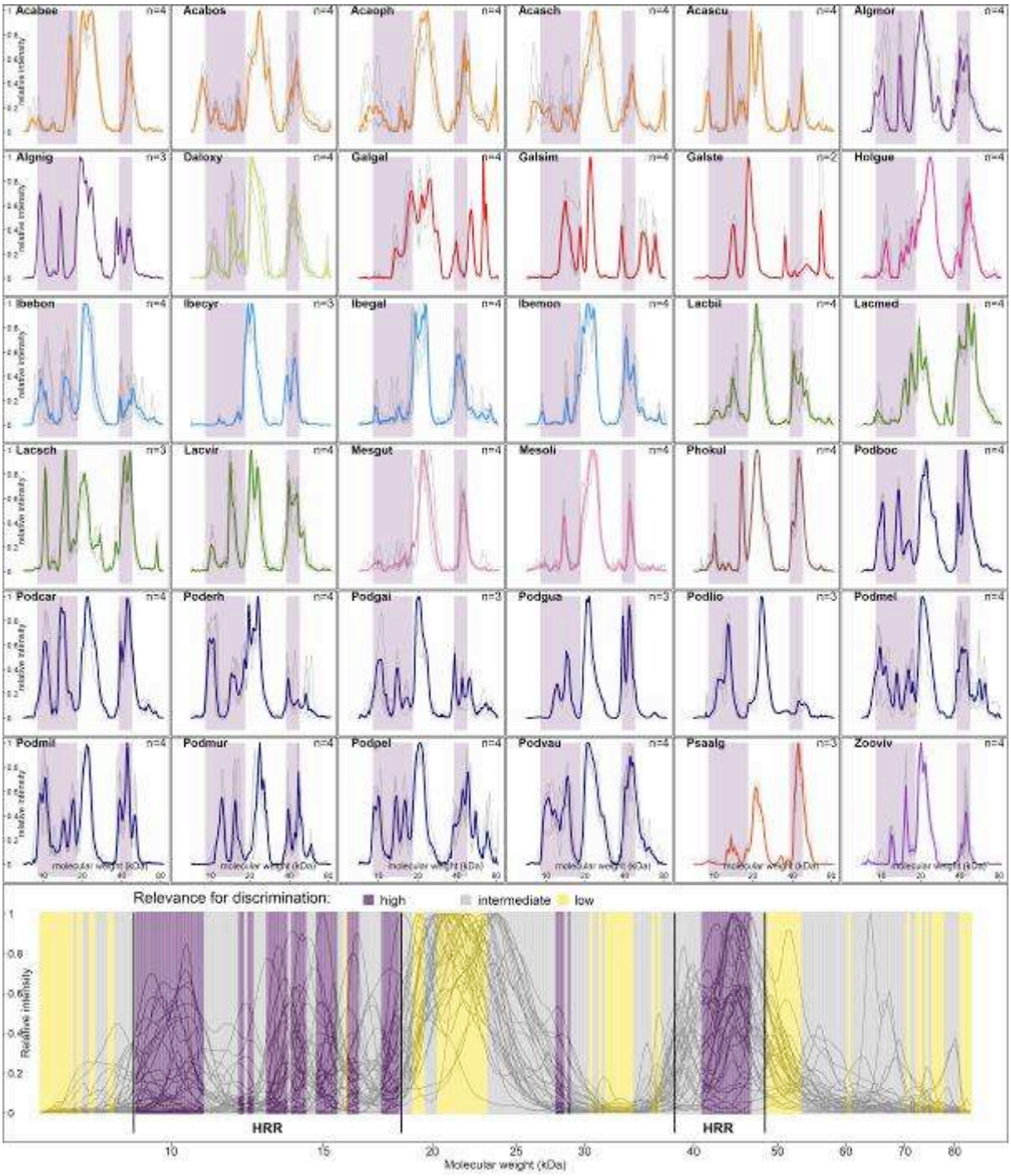
910

911 FIGURE LEGENDS

912 **Figure 1.** *Top six rows*: EPGs for each species group; in each plot: the abbreviation of the
913 species Latin name is reported at top-left corner (see below for the legend); grey lines =
914 individual samples; colour line = average profile (the same colour is used for species of the
915 same genus); sample size is reported at top-right corner; y-axis reports relative intensity of the
916 electrophoretic profiles, x-axis the molecular weight (kDa); light-purple shaded areas = HRR
917 (see below). *Bottom panel*: ranking of the EPG regions according to the CAT scores analysis:
918 purple shaded = high relevance zones (i.e., the most important zone for discrimination); grey
919 shaded = intermediate relevance zones; yellow shaded = low relevance zones (i.e., the least
920 useful for classification); grey lines = species average EPGs; HRR = High Relevance Region,
921 i.e., the overall areas of high relevance corresponding to the same shaded areas in the single
922 species plots. Species names legend: Acabee = *Acanthodactylus beershebensis*; Acabos = *A.*
923 *boskianus*; Acaoph = *A. opheodurus*; Acasch = *A. schreiberi*; Acascu = A. *scutellatus*;
924 Algmor = Algyroides moreoticus; Algnig = *A. nigropunctatus*; Daloxy = *Dalmatolacerta*
925 *oxycephala*; Galgal = *Gallotia galloti*; Galsim = *G. simonyi*; Galste = *G. stehlini*; Holgue =
926 *Holaspis guentheri*; Ibebon = *Iberolacerta bonnali*; Ibecyr = *I. cyreni*; Ibegal = *I. galani*;
927 Ibemon = *I. monticola*; Lacbil = *Lacerta bilineata*; Lacmed = *L. media*; Lacsch = *L.*
928 *schreiberi*; Lacvir = *L. viridis*; Mesgut = *Mesalina guttulata*; Mesoli = *M. olivieri*; Phokul =
929 *Phoenicolacerta kulzeri*; Podboc = *Podarcis bocagei*; Podcar = *P. carbonelli*; Poderh = *P.*
930 *erhardii*; Podgai = *P. gaigeae*; Podgua = *P. guadarramae*; Podlio = *P. liolepis*; Podmel = *P.*
931 *melisellensis*; Podmil = *P. milensis*; Podmur = *P. muralis*; Podpel = *P. peloponnesiacus*;
932 Podvau = *P. vaucheri*; Psaalg = *Psammodromus algirus* ; Zooviv = *Zootoca vivipara*.
933
934 **Figure 2.** Phylogenetic tree of the lacertid lizards included in the comparative analyses.
935 Below each tip, a "virtual lane" representing the average EPG for that species has been added:

936 blue intensity is proportional to the relative expression of protein of a given molecular weight.
937 Tips are coloured according to genus; tip labels are the abbreviation of the species Latin name
938 (see caption to Fig. 1 for details).
939
940 **Figure 3.** Divergence of the protein signal and geographic overlap in the *Podarcis* species of
941 our dataset. Top panel: geographic distribution of the ten *Podarcis* species considered in the
942 analysis; bottom-left panel: phylogeny of the same *Podarcis* species ensemble (from (Garcia-
943 Porta *et al.*, 2019)); bottom-right panel: regression of the distance matrix of *Podarcis* EPGs
944 corrected for phylogeny against the geographic distribution overlap (converted to distance in a
945 way that larger overlap corresponds to lower distance; see methods for details); solid line
946 represents the fitted regression, dashed line the 95% confidence interval, grey crosses =
947 phylogenetically corrected pairwise distances.
948
949 **Figure 4.** Phylomorphospace representation of the analised multivariate traits (EPGs, left
950 panel; body shape, bottom-right panel), together with the body size phenogram (top-right
951 panel).. The intensity of the phylogenetic signal (K) is reported for each trait in each panel.
952 Points in the space are coloured according to the genus. When principal components (PC) are
953 used to represent the morphospace, their percentage contributions are reported along the axes.
954 For EPGs (left panel), it was also reported the value of the univariate phylogenetic signal
955 (K_{uni}) of each PC, while the associated phenotypic variability is represented by a "virtual
956 lane" simulating an electrophoretic run: the greater the intensity of blue, the greater the
957 expression of the band.
958
959

960 **Figure 5.** Disparity-through-time plots of EPGs (top), body size (left-bottom) and shape
961 (right-bottom): solid line = observed trajectory; dashed line = predicted trajectory (median)
962 after 1000 runs of a Brownian motion model; grey area = 95% confidence interval according
963 to the rank envelope test. MDI and rank envelope tests results are also reported for each trait.
964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Acabos n=4
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Acasch n=4
Acascu n=4
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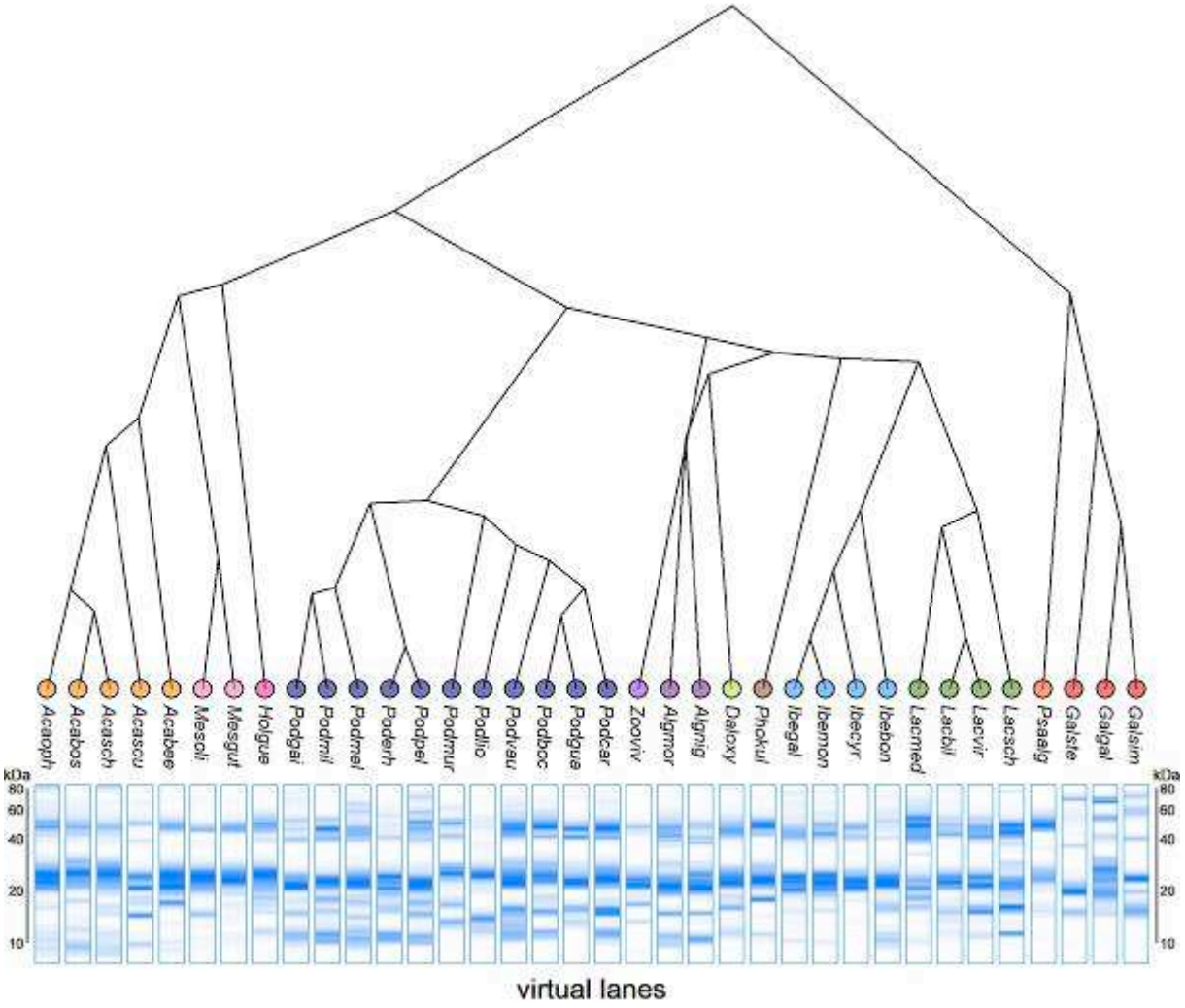
Galgal n=4
Galsim n=4
Galste n=2
Holgue n=4
Ibebon n=4
Ibecyr n=3
Ibegal n=4
Ibemon n=4
Lacbil n=4
Lacmed n=4
Lacsch n=3
Lacvir n=4
Mesgut n=4
Mesoli n=4
Phokul n=4
Podboc n=4
Podcar n=4
Poderh n=4
Podgal n=3
Podgua n=3
Podlio n=3
Podmel n=4
Podmil n=4
Podmur n=4
Podpel n=4
Podvau n=4
Psaalg n=3
Zooviv n=4
relative intensity
molecular weight (kDa)
Relevance for discrimination:
high
intermediate
low
HRR
HRR
Relative intensity
Molecular weight (kDa)

Fig. 1


Acaoph
Acabos
Acasch
Acascu
Acabee
Mesoli
Mesgut
Holgue
Podgai
Podmil
Podmel
Poderh
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Podlio
Podriau
Podboc
Podgua
Podcar
Zooviv
Algmor
Algnig
Daloxy
Phokul
Ibegal
Ibemon
Ibecyr
Ibebon
Lacmed
Lacbil
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Psaalg
Galste
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kDa
80
60
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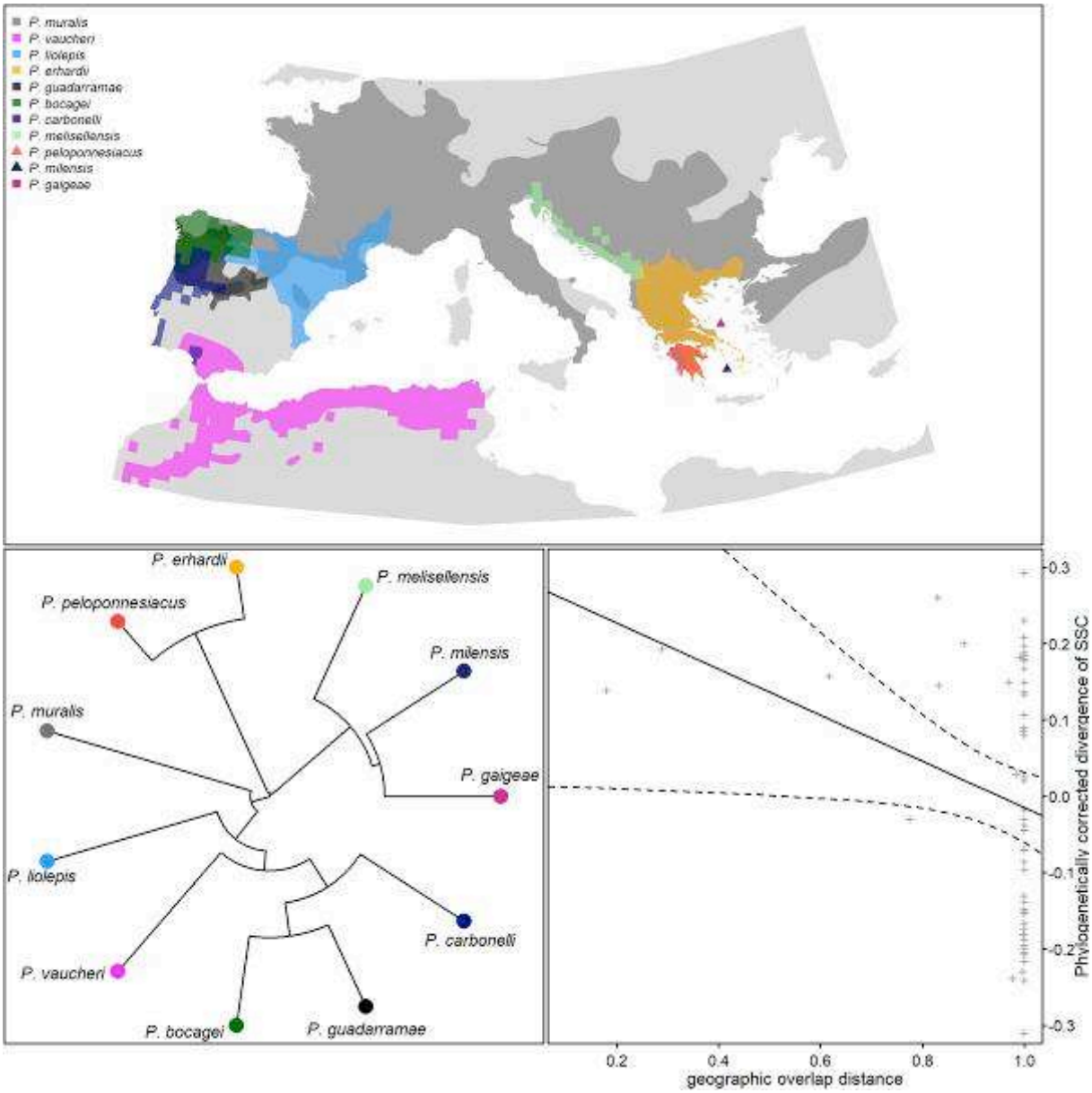
virtual lanes

967

968 Fig. 2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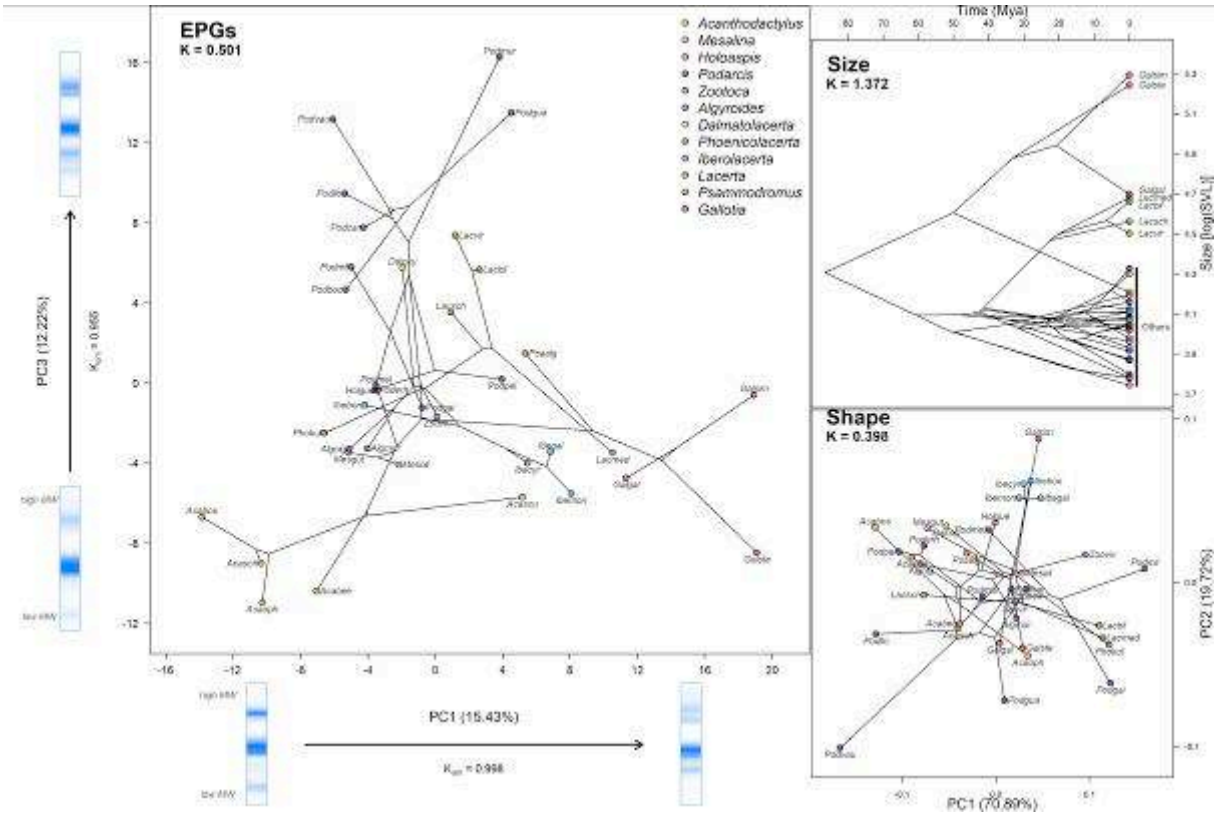
P. vaucheri
P. lilolepis
P. erhardii
P. guadarramae
P. bocagei
P. carbonelli
P. melisellensis
P. peloponnesiacus
P. milensis
P. gaigeae
P. erhardii
P. melisellensis
P. peloponnesiacus
P. milensis
P. muralis
P. gaigeae
P. lilolepis
P. carbonelli
P. vaucheri
P. bocagei
P. guadarramae
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
Phylogenetically corrected divergence of SSC
0.2
0.4
0.6
0.8
1.0
geographic overlap distance

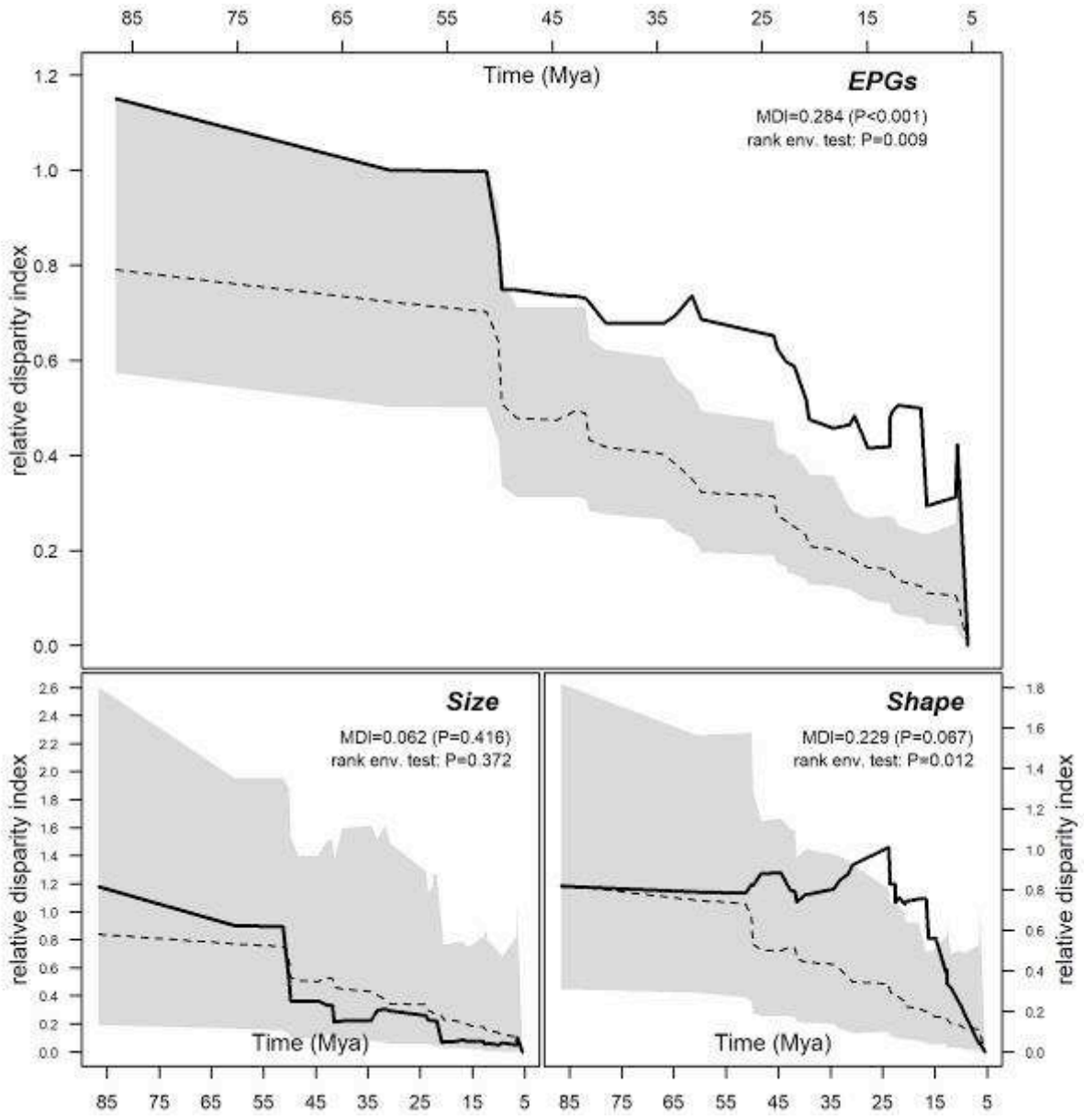
969

970 Fig. 3


EPGs
K = 0.501
Acanthodactylus
Mesalina
Holaspis
Podarcis
Zootoca
Algyroides
Dalmatolacerta
Phoenicolacerta
Iberolacerta
Lacerta
Psammodromus
Gallotia
PC1 (15.43%)
PC3 (12.22%)
Size
K = 1.372
Time (Mya)
Shape
K = 0.398
PC1 (70.89%)
PC2 (19.72%)

971

972 Fig. 4


Time (Mya)
EPGs
MDI=0.284 (P<0.001)
rank env. test: P=0.009
relative disparity index
Size
MDI=0.062 (P=0.416)
rank env. test: P=0.372
Shape
MDI=0.229 (P=0.067)
rank env. test: P=0.012

973

974 Fig. 5