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Evolutionary and biogeographical support for species-specific proteins in lizard chemical signals

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The species-specific components of animal signals can facilitate species recognition and reduce the risks of mismatching and interbreeding. Nonetheless, empirical evidence for species-specific components in chemical signals is scarce and mostly limited to insect pheromones. Based on the proteinaceous femoral gland secretions of 36 lizard species (Lacertidae), we examine the species-specific component potential of proteins in lizard chemical signals. By quantitative comparison of the one-dimensional electrophoretic patterns of the protein fraction from femoral gland secretions, we first reveal that the 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 with non-specifically signal traits (i.e. body size and shape). Together, these findings provide support for the species specificity of proteins in the chemical signals of a vertebrate lineage.

ADDITIONAL KEYWORDS: chemical communication – interspecific interference – lizards – proteins – signal evolution – species recognition.

INTRODUCTION

The spectacular diversity of animal signals and displays has long been a source of wonder (Guilford & Dawkins, 1991; Laidre & Johnstone, 2013). Species-specific components (SSCs), i.e. those signal 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' introduction in the advertisement calls of some Leiuperinae frogs (Ryan, 1983) and the specific cuticular hydrocarbons of *Formica* ants (Martin *et al.*, 2008), all of which exhibit striking species specificity.

Acting as a type of species-identity badge, SSCs have been implicated in species recognition mechanisms (Wiley, 1983; Ord & Stamps, 2009) and might therefore play a role in speciation and the maintenance of reproductive isolation (Dobzhansky, 1937; Mayr, 1942, 1963; West-Eberhard, 1983; Sobel *et al.*, 2010; Rabosky, 2016). The 'badge' might 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 multicomponent and multimodal communication (Partan & Marler, 1999). Although the evolution of a simple or complex badge might 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 patterns of variability (Weber *et al.*, 2016; Tibbetts *et al.*, 2017). Indeed, in order to ensure the accurate detection

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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 (sympatric and synthopic) species (West-Eberhard, 1984; Percy *et al.*, 2006; Schaefer & Ruxton, 2015; Grether *et al.*, 2017), enhancing accuracy in species recognition and avoiding 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 would exhibit a weaker phylogenetic signal and, possibly, higher evolutionary rates than other, non-specifically signal traits, such as morphology (especially non-genital traits) or trophic ecology (Ritchie, 2007; Arnegard *et al.*, 2010; Weber *et al.*, 2016; Zozaya *et al.*, 2019; Quipildor *et al.*, 2021). Indeed, SSCs are expected to diverge as speciation occurs, contributing to increase intraclade variability (Symonds & Elgar, 2004; Weber *et al.*, 2016; García-Roa *et al.*, 2017a).

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, exhibit strong chemical orientation and are equipped with both a nasal system and a well-developed vomeronasal–lingual system that allow them to sample and process chemicals from the environment efficiently (Schwenk, 1995; Baeckens *et al.*, 2017b). Furthermore, most lizard species have epidermal glands [precloacal or femoral glands (FGs)] that produce chemical signals (Martín & López, 2011, 2014; Mayerl *et al.*, 2015; Zozaya *et al.*, 2019). The FG secretions consist of a protein–lipid mixture (Alberts, 1990; Mangiacotti *et al.*, 2019a, c) used to convey a wide range of different messages (Martín & López, 2011, 2015; Baeckens, 2019), including species identity (Gabirot *et al.*, 2010a; Labra, 2011; García-Roa *et al.*, 2016; Valdecantos & Labra, 2017).

Most 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 by Martín & López, 2015), but in at least some taxa, the composition of the lipid fraction varies greatly among closely related groups and therefore might also function in species recognition (Martín & López, 2006; Zozaya *et al.*, 2019). Interestingly, phylogenetic comparative analyses revealed that the lipid fraction has a weak phylogenetic signal (Baeckens *et al.*, 2018a), with specific compounds following different evolutionary patterns (García-Roa *et al.*, 2017a; Campos *et al.*, 2020). Maximizing signal efficacy is considered to be the main evolutionary driver of both the variability and the complexity of the lipid signal (Baeckens *et al.*, 2017a, 2018a, b), because chemical signals respond to different environmental constraints (Gabirot *et al.*, 2012; Heathcote *et al.*, 2014; Martín *et al.*, 2017; Baeckens *et al.*, 2018a).

In contrast to the lipophilic component of FG secretions, hardly anything is known about the protein fraction. Although it has been recognized for a long time that FG contains proteins with a possible function in communication (Padoa, 1933; Cole, 1966; Alberts, 1990; Alberts & Werner, 1993), studies of lizard chemical communication have almost ignored them (Font *et al.*, 2012; Mayerl *et al.*, 2015; Mangiacotti *et al.*, 2017). This oversight might have biased our understanding of species recognition in lizards, because proteins would make excellent SSCs (Wyatt, 2010, 2014). The 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 the difference among species was almost hidden. Moreover, the comparison was not made under a phylogenetic comparative analysis framework, which would have allowed protein specificity to be ruled out as a predictable consequence of interspecific genetic differences. Recently, the interest in the protein fraction has revived (e.g. Mangiacotti *et al.*, 2017), and an active role of the protein component of FG secretions in lizard communication has been suggested to allow, for example, self-recognition (Mangiacotti *et al.*, 2019b, 2020). Furthermore, the proteins in FG secretions carry different badge-like information, such as the specific clade of origin (Mangiacotti *et al.*, 2017) and the colour morph identity (Mangiacotti *et al.*, 2019a).

Here, we investigate the interspecific diversity in FG protein profiles across a family of lizards. For this, we analysed the pattern of phenotypic variability in one-dimensional electrophoretic profiles (EPGs) to test the SSC hypothesis. We expect: (1) larger among-species than within-species variation of EPGs; (2) increased EPG divergence in sympatric, closely related species; and (3) a high rate of evolution of EPGs compared with other non-signal traits.

Lacertid lizards (Lacertidae) constitute an excellent model system for the study of vertebrate chemical communication in general (Baeckens, 2019) and to test our hypothesis specifically, for a number of reasons. Firstly, lacertids exhibit strong chemical orientation (Baeckens *et al.*, 2017b; García-Roa *et al.*, 2017b),

because they use FG secretions to send and gain different information about conspecifics (individual identity, species identity, female reproductive status, health and condition, and fighting ability), which are used in decision-making processes (female choice, rival assessment and territorial defence; for details, see Martín & López, 2014). Secondly, based on different phylogenetic analyses (Mendes *et al.*, 2016; Garcia-Porta *et al.*, 2019), lacertids constitute a relatively young and species-rich lizard clade with a well-supported classification. This allows testing species for different evolutionary approaches to traits, such as their evolutionary rate of change. Thirdly, many lacertid species have (partly) overlapping distributional ranges (Sillero *et al.*, 2014; Roll *et al.*, 2017) and it is not unusual for species to occur locally 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 (two to four samples per species) and 12 genera of the Lacertidae family (Supporting Information, Table S1). Secretions were collected by applying agently pressure along the thighs, with the help of a steel spatula, until all the glands (both legs) were emptied. Samples from single populations were collected between 2002 and 2014 (Baeckens *et al.*, 2017a, 2018a, b) and stored in glass vials fitted with Teflon-lined stoppers at –20 °C until analysis. Notably: (1) for all species, secretions were collected during the breeding seasons, i.e. when glandular activity is at its maximum (Cole, 1966; Alberts *et al.*, 1992; Mangiacotti *et al.*, 2019c); (2) secretions were collected immediately after capture; and (3) all samples underwent the same laboratory protocols (notably, lipid 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. Considering the timespan (2002–2014) of sample collection, we checked whether sites had a temperature increase associated with global warming during the 13-year period (see Supporting Information, Fig. S1). Despite a significant trend, the temperature increase was negligible owing to its low effect size and compared with other source of variations (e.g. season; Mangiacotti *et al.*, 2019c).

The protein fraction was analysed following the procedures implemented by Mangiacotti *et al.* (2017, 2019c), which allowed us to fingerprint the protein components of the FG secretions of each specimen using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE procedure followed exactly the one described by Mangiacotti *et al.* (2019c) and allowed us to obtain a high-quality image of each gel (1200 dpi). After greyscale conversion of the image, individual electrophoretograms (EPGs) were obtained along vertical lines through the middle of each lane. Raw EPGs were subsequently aligned, de-noised, binned into equal intervals and normalized, in order to obtain standardized and comparable protein profiles across samples and gels to be used as a proxy for the protein composition (Mangiacotti *et al.*, 2019c). All operations were implemented in R v.3.5.2 (R Core Team, 2018), adapting the functions available in the paper by Mangiacotti *et al.* (2019c).

Intra- vs. interspecific variation of the protein profiles

To assess the variability in the protein composition attributable to the species level, we transformed EPGs using the centred log-ratio to account for their compositional nature (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 and protein concentration as a covariate (Mangiacotti *et al.*, 2019c). Significance was assessed by 999 permutations of the data, which were stratified within gel, to address the non-independence of EPGs originating from the same electrophoretic run. We excluded *Gallotia stehlini* from this analysis, because we accepted a minimum of three samples per species (see Supporting Information, Table S1). A test for the homogeneity of group dispersion was previously conducted (Anderson, 2006), failing to detect any significant difference (pseudo-F = 1.195; P = 0.087).

We assessed the ability of EPGs to predict species membership using a shrinkage-based diagonal discriminant analysis (Pang *et al.*, 2009), given the high dimensionality of the data. All but one EPG for each species were used to train the model, and the remnant one to test it. One hundred replicates of the these training and testing datasets were chosen randomly, a model was obtained, and its performance was evaluated by the percentage of correctly classified test data (Pang *et al.*, 2009). To highlight the most and least important molecular weight regions in discrimination (i.e. the ones showing the highest or lowest among-species variability, respectively), we computed a summary scores for each EPG interval, starting from the correlation-adjusted t scores (CAT scores; Zuber & Strimmer, 2009; Ahdesmäki

& Strimmer, 2012). We then classified the scores obtained into three relevance categories: high (scores above the third quartile); intermediate (scores between first and third quartile); or low (scores below the first quartile).

For all the above-mentioned analyses, we used R v.3.5.2 (R Core Team, 2018) and the following packages: *compositions* (van den Boogaart *et al.*, 2020); *permute* (Simpson, 2019); *vegan* (Oksanen *et al.*, 2019); and *sda* (Ahdesmaki *et al.*, 2015).

Divergence of the protein signal in sympatry

To test the effect of sympatry on SSC divergence, we used multivariate distance matrix regressions (Zapala & Schork, 2012). Notably, we regressed the pairwise distance matrix of species average EPG against the pairwise geographical distribution overlap (a 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 geographical overlap might be a raw proxy of the real sympatry, because two geographically overlapping species might inhabit different environments, never coming into contact. To account for this issue, we initially ran the analysis considering the whole set of species ($N = 36$), then repeated the analysis focusing on *Podarcis* alone, because this genus was the most represented (11 spp.) in our dataset and included lizards with 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 to run the regression were the same.

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

The matrix of geographical overlap was obtained based on the distribution maps available in the paper by Roll *et al.* (2017), re-projected into an equal-area projection (Europe Equal Area, 2001; https://epsg.io/19986). 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 geographical overlap (shared area) between the two distributions A_i and A_j. We bounded s_{ij} between zero and one, dividing by the minimum between A_i and A_j, both to emphasize the overlap and to reduce the inflation toward zero attributable to the wide distribution of some species. We converted s_{ij} into a distance (d_{ij}) 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 v.3.5.2 (R Core Team, 2018) and the following packages: *compositions* (van den Boogaart *et al.*, 2020); *raster* (Hijmans, 2020); *rgeos* (Bivand & Rundel, 2019); and *phytools* (Revell, 2012).

Phylogenetic comparative analysis

The third block of analyses took a phylogenetic comparative approach (Adams & Collyer, 2019) to the full species set.

To track the non-signal evolutionary pattern, for all the 36 species we compiled a morphometric dataset (Supporting Information, Table S2) including: snout–vent length (SVL), head length, head maximum width, forelimb length (FLL) and hindlimb length (HLL). These measures are expected to respond to environmental adaptation in lizards (Vanhooydonck & Van Damme, 1999; Herrel *et al.*, 2002; Verwaijen *et al.*, 2002; Goodman *et al.*, 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 $\log_{10}$-transformed SVL as a proxy for size, with the residuals of a standardized major axis regression of $\log_{10}$-transformed head size (HS), FLL and HLL against size as shape variables (Kaliontzopoulou *et al.*, 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 *et al.*, 2003) on lizard EPGs, size and shape. Given that EPGs and shape are multivariate traits, we adopted a distance-based estimation of K (Adams, 2014b; Adams & Collyer, 2019), which applies equally to univariate traits (Adams, 2014b). As in its original formulation, under Brownian motion, K has an expected value of one; hence, $K < 1$ indicates a weak phylogenetic signal, and K near to one or above one means that the phylogenetic signal is high. The significance 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 ≥ 95% of total variation and selected

the axes that retained significant K_{uni} values after Holm correction (Holm, 1979).

Secondly, we estimated the evolutionary rate (σ^2) of EPGs and morphometric data, and we tested whether the former was larger than the latter. We followed the distance-based method proposed by 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 were 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-through-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 occurs mostly among clades and that closely related species share similar phenotypes, whereas large values imply that variation is partitioned within subclades, and distant species might overlap in the morphospace (Harmon *et al.*, 2003). The observed DTT profile was compared with 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 (MDI) test and the rank envelope test (Murrell, 2018). The MDI is an overall measure of the difference between observed and null trajectory. Positive values indicate that 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 and rank_env_dtt, available in the paper by Murrell (2018).

All the analyses were conducted in R v.3.5.2 (R Core Team, 2018) using the following packages: *compositions* (van den Boogaart *et al.*, 2020); *ape* (Paradis & Schliep, 2019); *smatr* (Warton *et al.*, 2012); and *geomorph* (Adams *et al.*, 2020).

RESULTS

All samples provided useful EPGs, and a species-specific pattern was apparent. Samples belonging to the same species showed highly similar banding schemes, consistently sharing the main peaks (Fig. 1; grey lines in each species panel); in contrast, different species (even congeneric) were characterized by a distinct pattern, in both the position and the 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, whereas the protein concentration did not affect electrophoretic runs (pseudo-F = 0.999; $P \leq 0.616$). The strong relationship between EPGs and species membership was confirmed by the discriminant analysis, which matched samples and species correctly in 86.5% of cases (range, 74.3–100.0%; interquartile range = 5.71%). The CAT scores identified two main EPG regions (HRR1 and 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. In contrast, 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 geographical overlap, ranging between zero (allopatry) and one (complete overlap) (Supporting Information, 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; $P \leq 0.001$), with the geographical overlap being irrelevant (distance-transformed geographical overlap; pseudo-t = −0.470; $P \leq 0.765$). 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 important different outcome: the phylogenetic distance retained a significant effect (β = 0.218; pseudo-t = 1.872; $P \leq 0.037$), but so too did the geographical overlap (pseudo-t = −2.123; $P \leq 0.049$), showing a negative trend (β = −0.302; Fig. 3). More specifically, EPG divergence 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 significant K value of 0.501 associated with protein profiles ($P < 0.001$; Table 1). Notably, the *Gallotia* and *Acanthodactylus* groups occupied distinct areas of the EPG morphospace (Fig. 4), with the former having a typical three-band scheme in the high molecular weight EPG (less expressed than the mid-part) and the latter showing a simplified single-band pattern in the same EPG region (Figs 1, 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 EPG region of low variability (19–25 kDa), where all species showed

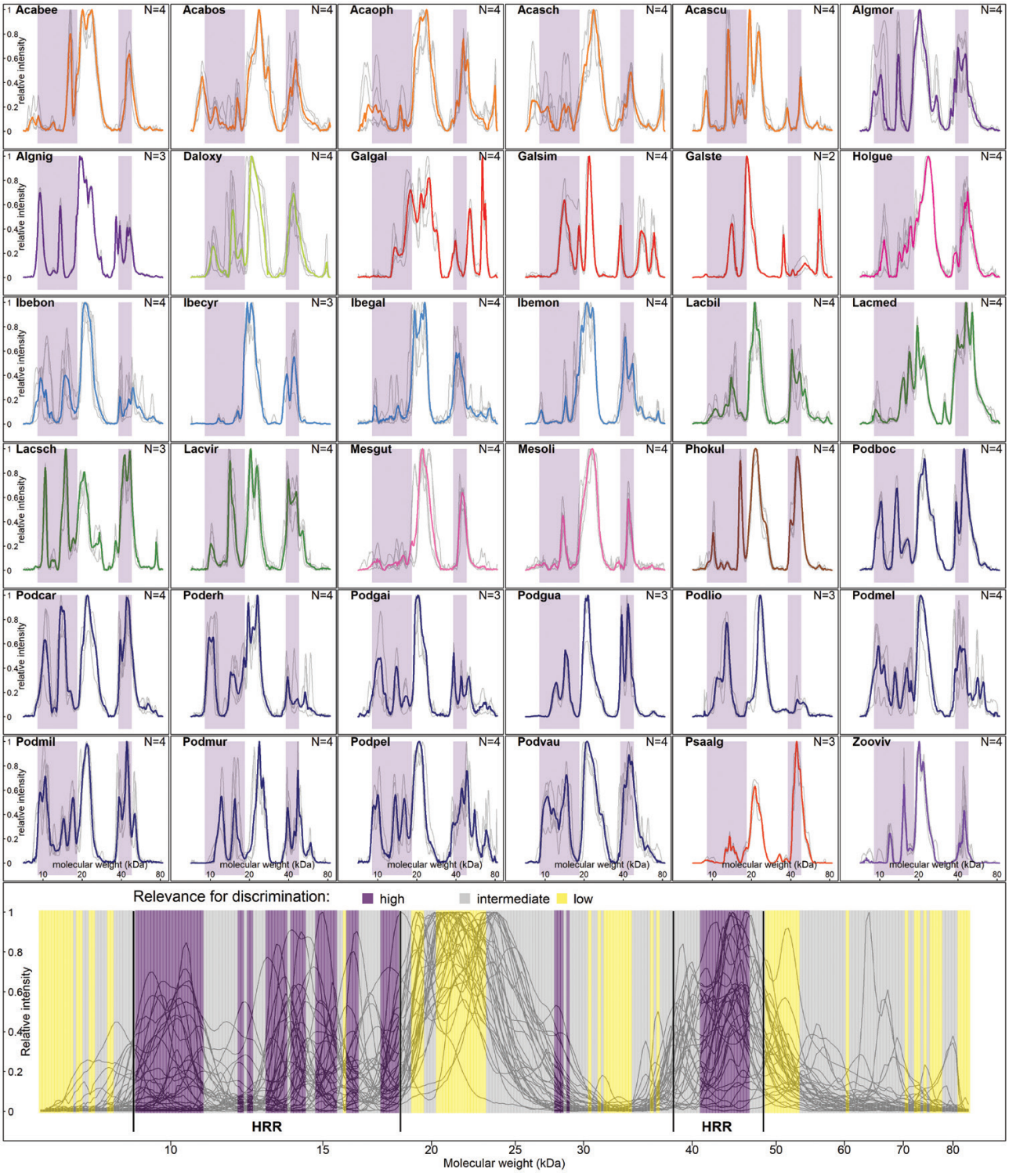

Figure 1. Top six rows: electrophoretograms (EPGs) for each species group. In each plot are shown: the abbreviation of the species name in the top left corner; grey lines, individual samples; coloured line, average profile (the same colour is used for species of the same genus); sample size in the top right corner; *y*-axis, relative intensity of the electrophoretic profiles; *x*-axis, molecular weight (in kilodaltons); light-purple shaded areas, high-relevance regions (HRRs). Bottom panel: ranking of the EPG regions according to the correlation-adjusted *t* scores (CAT scores) analysis: purple shading, HRRs (i.e. the most important zone for discrimination, corresponding to the same shaded areas in the single-species plots); grey

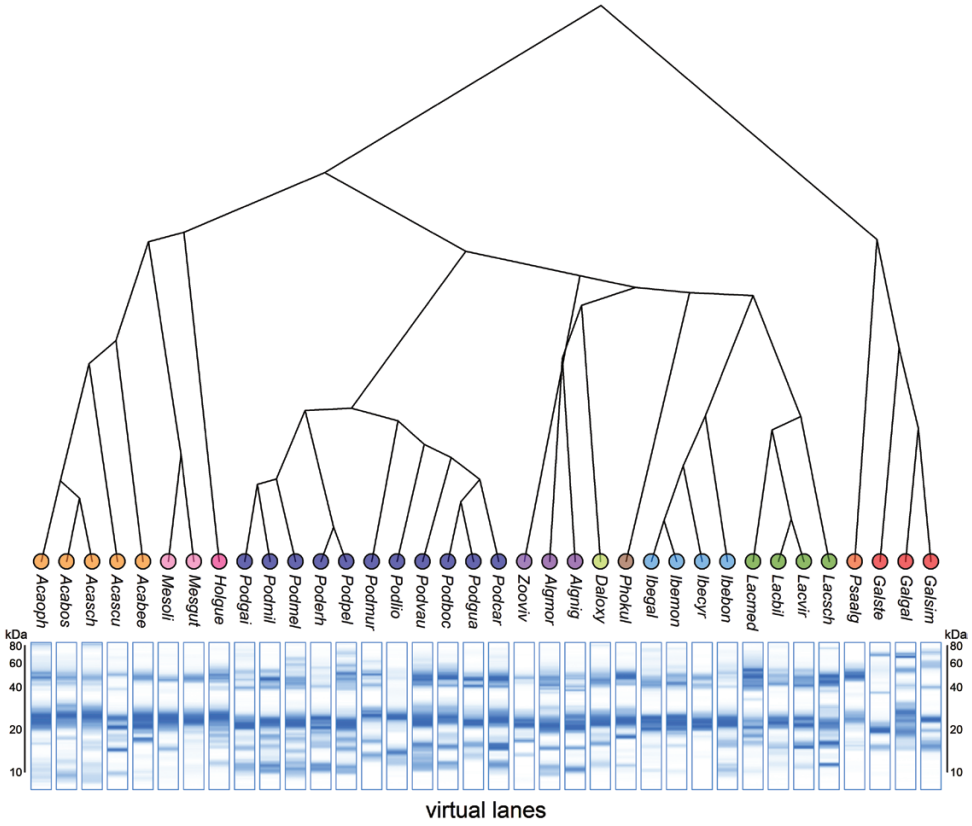

Figure 2. Phylogenetic tree of the lacertid lizards included in the comparative analyses. Below each tip is a 'virtual lane' representing the average electrophoretogram for that species, in which blue intensity is proportional to the relative expression of protein of a given molecular weight. Tips are coloured according to genus, and tip labels are the abbreviation of the species name (for details, see legend to Fig. 1).

an intense peak (Figs 1, 2), might 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), but small and not significant for body shape (Table 1). In particular, the phylogenetic signal for body size remained consistently large in the genus *Gallotia*, medium in *Lacerta* and small in the remaining taxa (Fig. 4). No clear pattern emerged from the analysis of body shape morphospace, but the lower than one and non-significant K value (0.398; P = 0.081; Table 1) indicated a poor phylogenetic effect (Fig. 4).

With regard to the results of the evolutionary diversification tests, the evolutionary rate of EPGs (σ^2 = 11.599) was much higher than those of body size (σ^2 = 0.002) and shape (σ^2 = 0.0003), with both the ratios, $\sigma^2_{EPG} : \sigma^2_{size}$ and $\sigma^2_{EPG} : \sigma^2_{shape}$, being significantly larger than one ($P \leq 0.001$); the ratio $\sigma^2_{size} : \sigma^2_{shape}$ was statistically equivalent to one (P = 1.000). Furthermore, the MDI of EPGs was significantly higher than expected under a Brownian

shading, intermediate-relevance zones; yellow shading, low-relevance zones (i.e. the least useful for classification); grey lines, species average EPGs. Species names: Acabee, *Acanthodactylus beershebensis*; Acabos, *Acanthodactylus boskianus*; Acaoph, *Acanthodactylus opheodurus*; Acasch, *Acanthodactylus schreiberi*; Acascu, *Acanthodactylus scutellatus*; Algmor, *Algyroides moreoticus*; Algnig, *Algyroides nigropunctatus*; Daloxy, *Dalmatolacerta oxycephala*; Galgal, *Gallotia galloti*; Galsim, *Gallotia simonyi*; Galste, *Gallotia stehlini*; Holgue, *Holaspis guentheri*; Ibebon, *Iberolacerta bonnali*; Ibecyr, *Iberolacerta cyreni*; Ibegal, *Iberolacerta galani*; Ibemon, *Iberolacerta monticola*; Lacbil, *Lacerta bilineata*; Lacmed, *Lacerta media*; Lacsch, *Lacerta schreiberi*; Lacvir, *Lacerta viridis*; Mesgut, *Mesalina guttulata*; Mesoli, *Mesalina olivieri*; Phokul, *Phoenicolacerta kulzeri*; Podboc, *Podarcis bocagei*; Podcar, *Podarcis carbonelli*; Poderh, *Podarcis erhardii*; Podgai, *Podarcis gaigeae*; Podgua, *Podarcis guadarramae*; Podlio, *Podarcis liolepis*; Podmel, *Podarcis melisellensis*; Podmil, *Podarcis milensis*; Podmur, *Podarcis muralis*; Podpel, *Podarcis peloponnesiacus*; Podvau, *Podarcis vaucheri*; Psaalg, *Psammodromus algirus*; Zooviv, *Zootoca vivipara*.

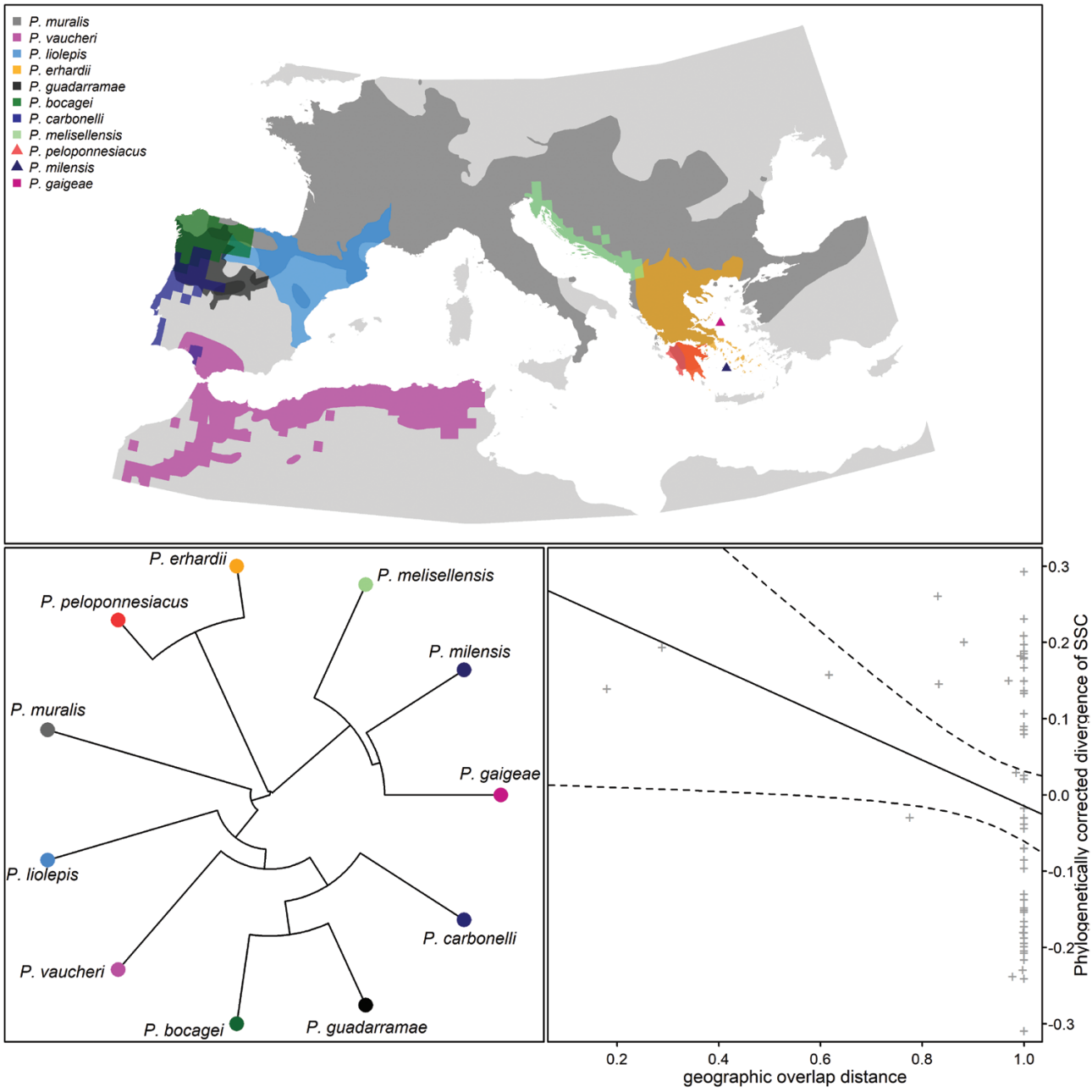

Figure 3. Divergence of the protein signal and geographical overlap in the *Podarcis* species of our dataset. Top panel: geographical distribution of the ten *Podarcis* species considered in the analysis. Bottom left panel, phylogeny of the same *Podarcis* species ensemble (from Garcia-Porta *et al.*, 2019). Bottom right panel, regression of the distance matrix of *Podarcis* electrophoreticograms corrected for phylogeny against the geographical distribution overlap (converted to distance such that larger overlap corresponds to lower distance; see Material and methods for details); continuous line represents the fitted regression, dashed line the 95% confidence interval, and grey crosses the phylogenetically corrected pairwise distances. Abbreviation: SSC, species-specific component.

motion model (Table 1), and the relative disparity index stayed above the predicted range from ~50 Mya on, peaking near the crown of the tree (Fig. 5). In comparison, although MDI of body shape also showed a marginally significant value larger than zero (Table 1), the relative disparity index followed a completely different trajectory (Fig. 5), with values above the prediction only between 32 and 15 Mya. The disparity of body size did not vary more than expected (Table 1; Fig. 5), supporting the phylogenetic effect on it.

Table 1. Multivariate phylogenetic signal (*K*) and morphological disparity index of the electrophoretograms and the morphological traits (body size and shape)

Trait	K		Morphological disparity index		
	Value	P-value	Value	P-value	$P_{\text{rank envelope test}}$
EPG	0.501	≤ 0.001	0.284	< 0.001	0.009
Body size	1.372	≤ 0.001	0.062	0.416	0.372
Body shape	0.398	0.081	0.229	0.068	0.012

The *P*-value associated with *K* was obtained by permutation. The *P*-value coupled to morphological disparity index was obtained by simulating disparity-through-time (DTT) curves under a Brownian motion model (for details, see Materials and methods). Abbreviations: EPG, electrophoretogram.

DISCUSSION

Species-specific components have been identified in signals of various sensory modalities and in a wide variety of animal lineages. They have been implicated in mechanisms of reproductive isolation and speciation (Mayr, 1963; West-Eberhard, 1984; Smadja & Butlin, 2009; Sobel *et al.*, 2010; Schaefer & Ruxton, 2015; Rabosky, 2016). Here, we provide comprehensive, albeit indirect, evidence that proteinaceous secretions from the femoral glands of lacertid lizards might carry SSCs.

The FG protein profiles show a noticeable species-specific pattern, which is a necessary prerequisite for a signal to bear an SSC (Wiley, 1983; West-Eberhard, 1984; Pillay & Rymer, 2012; Schaefer & Ruxton, 2015; Weber *et al.*, 2016). Despite a certain

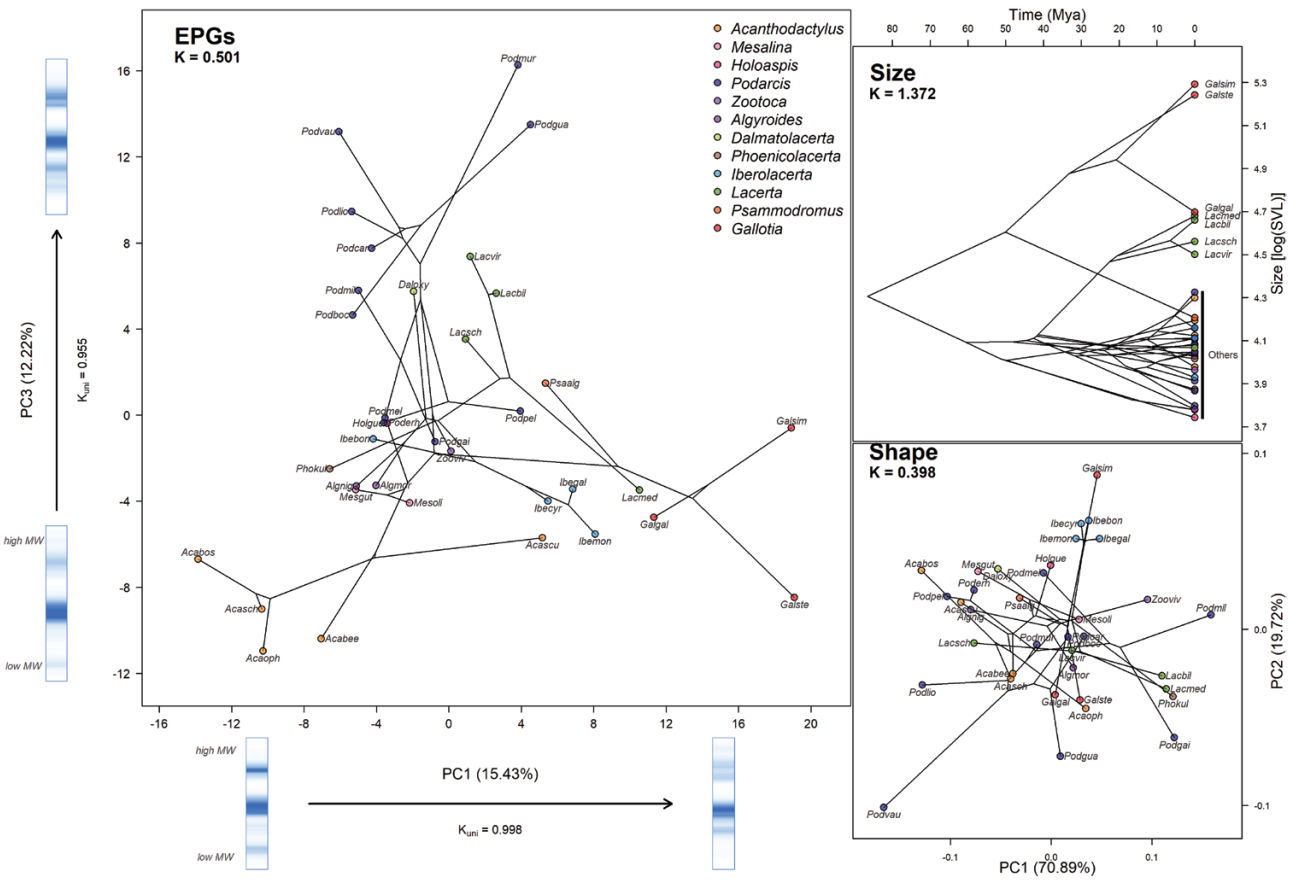

Figure 4. Phylomorphospace representation of the analysed multivariate traits [left panel, electrophoretograms (EPGs); bottom right panel, body shape], together with the body size phenogram (top right panel). The intensity of the phylogenetic signal (*K*) is reported for each trait in each panel. Points in the space are coloured according to the genus. When principal components (PCs) are used to represent the morphospace, their percentage contributions are reported along the axes. For EPGs (left panel), the value of the univariate phylogenetic signal (K_{uni}) of each PC is also reported, and the associated phenotypic variability is represented by a 'virtual lane' simulating an electrophoretic run; the greater the intensity of blue, the greater the expression of the band. Abbreviation: SVL, snout–vent length.

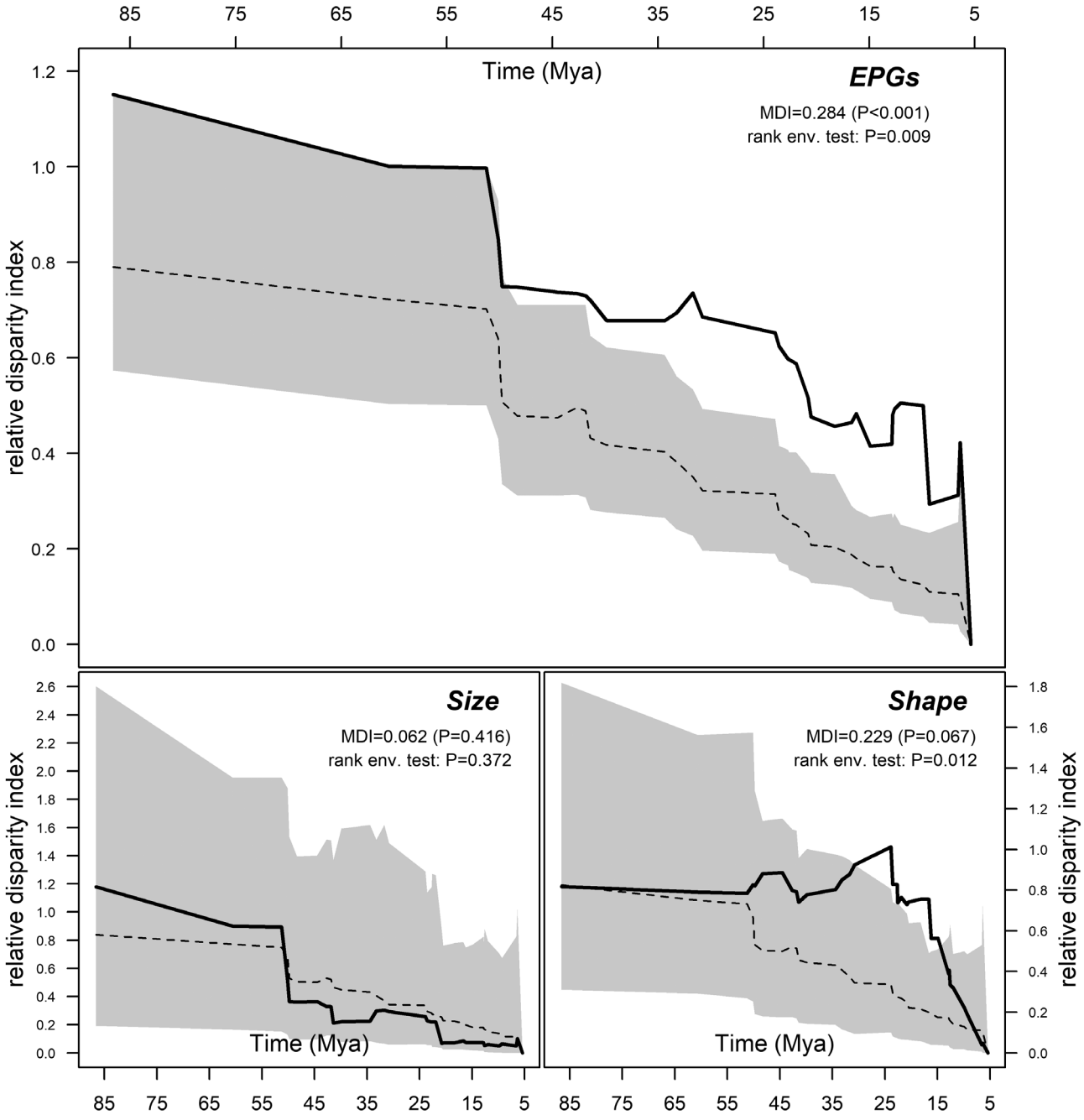

Figure 5. Disparity-through-time plots of electrophoretograms (EPGs; top), body size (bottom left) and shape (bottom right): continuous line, observed trajectory; dashed line, predicted trajectory (median) after 1000 runs of a Brownian motion model; grey area, 95% confidence interval according to the rank envelope test. The morphological disparity index (MDI) and rank envelope test results are also reported for each trait.

degree of variability (Fig. 1), within-species EPGs clearly share the same overall silhouette and can be discriminated effectively 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, 2019b), the desert iguana (*Dipsosaurus dorsalis*; Alberts, 1991) and the green iguana (*Iguana iguana*; Alberts *et al.*, 1993), suggesting that we can reasonably exclude the bias attributable to small within-species sample size used to assess both intra- and interspecific variation.

Most of the interspecific variability is attributable to two disjoint EPG regions (Fig. 1), where both the number and the 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. 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 can be argued that the specificity of FG proteins might 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 geographical overlap increases, at least when congeneric species (i.e. *Podarcis* group) were considered. It is likely that this tendency did not emerge when non-congeneric species were included owing 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 is a possible outcome of reproductive character displacement (Grether *et al.*, 2017), because it is in line with the idea that SSCs might help in pre-mating isolation and avoidance of hybridization (Smadja & Butlin, 2009; Edwards *et al.*, 2015; Grether *et al.*, 2017). As such, by increasing the distance between two SSCs, the accuracy of recognition of conspecifics improves (Wiley, 1983; Johnstone, 1997b), contributing to the coexistence of sympatric species. Sympatry of closely related species can impose a high cost in terms of fitness to one or both species because of interspecific aggression, 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; Jančúchová-Lásková *et al.*, 2015), and genetic evidence of reticulation 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 divergence in species traits involved in species recognition, to reduce detrimental interactions, but only where they occur in sympatry. We acknowledge that our survey sampled only 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 geographical overlap. The amount of geographical overlap can be viewed as a proxy for the probability of interference, which, in turn, might have favoured the SSC differentiation (Curé *et al.*, 2012).

Sympatric *Podarcis* lizards can hybridize in natural conditions (Gorman *et al.*, 1975; Capula, 1993, 2002; Pinho *et al.*, 2009; Jančúchová-Lásková *et al.*, 2015), and males engage in interspecific aggressive interactions (Böhme, 1986; Corti & Lo Cascio, 2002; Downes & Bauwens, 2002; Lailvaux *et al.*, 2012). In this scenario, a mechanism promoting SSC divergence in sympatry might reflect the need for a more accurate species recognition mechanism in mating and male–male contests. 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 avoidance of misguided aggression toward non-conspecifics (López & Martín, 2001, 2002; López *et al.*, 2002; Carazo *et al.*, 2008; Font *et al.*, 2012). The SSCs in the peptide fraction of FG secretions might, 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.*, 2010a, b; Font *et al.*, 2012). Alternatively or additionally, an enhanced SSC in male scent might allow females to recognize the species identity of the territory owner accurately, providing the basis for a pre-mating reproductive barrier (Smadja & Butlin, 2009; Runemark *et al.*, 2011; García-Roa *et al.*, 2016). Indeed, previous studies have established that lacertid females can also recognize conspecifics through chemoreception (Gabirot *et al.*, 2010b), albeit not in all species (Martín & López, 2006; Font *et al.*, 2012; Gabirot *et al.*, 2012; Martín *et al.*, 2016).

A third support to the prediction for an SSC-bearing signal comes from the macroevolutionary pattern emerging from the phylogenetic comparative analysis of EPGs. Firstly, the phylogenetic signal for protein profiles is weak, indicating that the EPGs of species are dependent to only a minimal extent upon their relatedness and that most of their variability cannot be explained by classic Brownian motion along the current tree. Electrophoretograms evolved much faster than indexes of body size and shape in the same clade (Table 1). Secondly, much of the variability in EPGs has been maintained within clades and their disparity has been 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. We found that the morphological traits used as reference, and supposed not to bear SSCs, 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, might explain the low phylogenetic signal of EPGs. 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.*, 2017a). This hypothesis might also apply to the FG proteins, where the species-specific pattern might reflect an environmental adaptation to increase signal efficiency (Endler, 1992, 1993). Additionally, given that proteins are homogeneously associated with lipids and might serve as a chemical matrix supporting the more volatile components (Alberts, 1990; Alberts & Werner, 1993), they might 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.*, 2017a) and protein fractions (present study) strongly suggests different drivers. This does not exclude the possibility that the environment might 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 might be associated with lipids (Alberts, 1990; Wyatt, 2014); instead, it suggests that the design of the protein signal could be driven mainly by other selective forces. Identifying whether and which EPG fractions have been shaped by environmental variables or by lipid composition is an interesting puzzle that requires specific studies.

In conclusion, using lacertids as a model group, we demonstrated that the FG protein secretions include SSCs, which might allow for interspecific recognition on a chemical basis. Proteins are well suited to function as elements of the 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 use protein SSCs in species recognition. Another obvious next step is the identification of the proteins involved in the recognition process. 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 present finding that the protein fraction in lizard femoral secretions might act as a species-badge opens a promising avenue for further investigation.

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DATA AVAILABILITY

The data underlying this article are available at: https://dx.doi.org/10.5281/zenodo.4116401

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Plots of the maximum (left panel) and minimum (right panel) monthly temperatures in the 36 sites (grey dots) by sampling year (2002–2014). The blue line represents the predicted trend line, grey shading its 95% confidence interval.

Table S1. List of the species considered in the analysis of the protein from the femoral gland secretions. Species name, abbreviation, number of samples (*N*), geographical coordinates and sampling period of the sampled populations are given for each species.

Table S2. Morphometric measures for the species used in the phylogenetic comparative analyses: FLL, forelimb length; HL, head length; HLL, hindlimb length; HW, maximal head width; *N*, sample size; SVL, average snout–vent length. All measures are expressed in millimetres, and only adult males were considered.

Table S3. Matrix of pairwise geographical overlap between the distribution areas of the lacertid species considered in this study.