




Identifying potential cues of species identity in complex animal signals



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Communication signals are often key for encoding information on species identity, but determining the features important in conveying those cues is challenging. This is especially the case when attempting to compare across closely related sympatric taxa, where the need for accurate species recognition is critical. We developed an analytical framework to comprehensively quantify the complex movement displays used by male lizards to advertise territory ownership in 11 taxa of Puerto Rican *Anolis*, many of which were sympatric. Our analyses were able to assign the majority of individuals to the correct population of a given species based on only a handful of displays, showing ample information exists in these displays for species recognition. Instances where lizards were misassigned appeared to have occurred because of similarities in display design resulting from local adaptation to similar environments or recent shared evolutionary history. Our analyses also revealed there was no common ('magic') display characteristic for recognition across the 11 taxa. Instead lizards likely assess the entire display or a combination of different display cues. Taken together, we illustrate a powerful approach that offers a rigorous statistical and holistic evaluation of complex animal signals for elucidating features likely to be important in species recognition. We provide a guide for implementing this analytical framework in R, with associated code and worked examples. Information obtained from these analyses can then be used to design experiments testing the utility of identify cues or comparative studies investigating how those cues contribute to reproductive isolation among populations and ultimately speciation.

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Identifying the selective forces that drive signal differentiation among species is important for understanding signal evolution and how sexual communication promotes and maintains reproductive isolation among closely related taxa, which can ultimately facilitate the formation of new species (Coyné & Orr, 2004). Investigating these forces can be challenging because the design and diversification of animal signals are often shaped by antagonistic selection pressures, including the need to convey species identity (Coultridge & Alexander, 2002; Krause et al., 2014; Ng et al., 2013; Seddon, 2005), provide reliable cues for opponent assessment (Chen et al., 2012; Pitcher et al., 2014) or mate attraction (Baker & Baker, 1990; Bastiaans et al., 2014), while also being adequately designed for effective transmission through signal environments (Derryberry, 2009; Fialko, 2018; Goutte et al., 2016; Ord et al., 2010). For example, signal characteristics that encode information on species identity are expected to diverge when species frequently

encounter sympatric congeners, while similarities in signal design among closely related taxa can evolve because of shared environmental constraints on signal propagation or be retained because of recent shared evolutionary history (Ord, 2012a; Seddon, 2005; Stanger-Hall & Lloyd, 2015).

Conveying reliable information on species identity is especially important for sympatric species (e.g. Garcia et al., 2020). Mistaking a congener as a potential mate wastes reproductive investment in energy expenditure (e.g. through courtship) and time diverted from other activities such as mating opportunities with conspecifics (Gerhardt, 1982; Macedonia & Stamps, 1994). Therefore, signals that allow animals to recognize, obtain access to and attract compatible mates through conveying reliable cues on species identity should experience considerable selective advantage (Krause et al., 2014; Ryan & Rand, 1993). To be effective for recognition, such cues should differ among taxa and exhibit low intra-specific variation.

By extension, any factor that promotes differences in the design of signals among populations and is used in territorial defence or the attraction of mates could become an important driver of the

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formation of new species (Boughman, 2001; Ng et al., 2013; Seehausen et al., 1997; Williams & Rand, 1977). One factor that can prompt populations to diverge in signal design is the environment (Marchetti, 1993). The detectability and consequently the design of signals are influenced by the level of noise, ambient light (in the case of visual signals), or physical obstructions that decrease the effective transmission of signals (Brumm, 2004; Fialko, 2018; Ord et al., 2007, 2010; Rosenthal et al., 2018). However, environmental constraints can also inhibit changes in attributes that might be useful for species recognition. For example, sympatric species are typically exposed to the same environmental conditions, and hence, can evolve similarities in signal design in order to maintain effective transmission. This can inhibit the ability of receivers to accurately assess species identity (e.g. Seehausen et al., 1997).

Similarities in signal design among closely related taxa can also occur because of shared evolutionary history (Garcia et al., 2020; Hunter & Krebs, 1979; Podos, 2010; Stafford et al., 2001). This is because closely related species often retain similarities in signal design because they are inherited characteristics from a common ancestor. On the other hand, more distantly related species are likely to differ in signal design because of various evolutionary stochastic and adaptive reasons. Because of this, species recognition is likely to be more challenging among closely related than distantly related species. Studies on closely related species or populations of the same species in different habitats therefore provide opportunities to understand the selection pressures that might drive signal differentiation and ultimately speciation.

For this reason, the study of animal signals in the context of species recognition among closely related taxa has been a popular area of research (meta-analysis by Ord et al., 2011). Most of this work has relied on playback-style experiments to determine whether receivers discriminate between conspecific and hetero-specific signals (e.g. Baker, 1991; Braune et al., 2008; Derlink et al., 2014; Macedonia et al., 2015; Martins et al., 2005; Rakotonirina et al., 2016; Uy et al., 2009). While this approach can show that animals use signals to identify conspecifics, it is limited in the extent to which it can reveal the specific cues or combinations of cues used in recognition, or the relative importance of those cues in making discriminative decisions (e.g. Derlink et al., 2014; Martins et al., 2005). This is especially problematic when multiple aspects of a signal might be used in recognition, and it is often unknown whether closely related taxa might use the same cues or a different combination of cues in making identity assessments. More generally, we have a poor understanding of how animals resolve the competing demands on signal design (e.g. remaining conspicuous and recognizing conspecifics), particularly among large groups of closely related species that are likely to be interacting in sympatry and are less likely to differ in signal design because of recent shared ancestry.

To help resolve these challenges, we developed an analytical framework to quantitatively analyse complex signal design across 11 taxa (belonging to eight species) of closely related Puerto Rican anoles (genus *Anolis*). Our approach leverages a range of statistical methods to identify salient components that might be used to delineate one species from another, and importantly one population from another, which is likely to be particularly useful for elucidating the role of signals in reproductive isolation. At the centre of our approach, we applied random forest tree classification (Breiman, 2001) to determine (1) whether male lizards could be assigned to the correct taxon using their display characteristics (specifically, various measures of their temporal display movements and 'motifs') and (2) which of those display characteristics were specifically relevant in those assignments. Motifs are formed from the combination of one or more display components (head-bobs, dewlap movements and tail displays). A random forest tree

classification (Breiman, 2001) is a machine-learning algorithm that constructs and combines multiple classification trees to predict a categorical response, using a group of explanatory variables (Breiman, 2001; Briec et al., 2018; Cutler et al., 2007). Our approach also helps to resolve the difficulties faced by playback studies by identifying all components that have the potential to encode information on species identity but makes no assumptions regarding the extent to which different taxa might use the same cues for discrimination.

The presence of multiple sympatric anole species on Puerto Rico, coupled with data previously compiled on habitat and social factors shown to be important in determining signal efficiency and function, provides an ideal opportunity to investigate how different selection pressures might shape the distinct variation in display design between populations. Male anoles defend territories through the performance of stereotyped visual displays composed of two discrete signal components that differ in their conspicuousness and motion: a core display of up-and-down movements of the head and body, known generally as headbobs (but also called push-ups), and the extension and retraction of an often colourful throat fan or dewlap (Losos, 2009). It has often been suggested that the dewlap, and specifically its pattern and colour, plays an important role in species recognition when multiple species live in sympatry (Losos, 1985; Nicholson et al., 2007; Rand & Williams, 1970; Vanhooydonck et al., 2009; Williams & Rand, 1977). However, the colour of the dewlap is only one component of a much larger signal repertoire, and it also seems to be intimately dependent on the properties of the environment (e.g. Fleishman, 2000), potentially making colour less conducive for reliable species recognition.

The extensive interspecific variation and assumed low intra-specific variation in the elaborate movement of both the headbob and dewlap aspects of the territorial display has led to the assumption that these attributes could also assist lizards in recognizing conspecifics (Macedonia & Stamps, 1994; Ord & Stamps, 2009; Rothblum & Jenssen, 1978). However, there have been few formal investigations of the extent to which the motion characteristics of anole territorial displays might convey accurate cues on species identity (e.g. Jenssen & Gladson, 1984; Ord & Stamps, 2009). We assessed the contribution of all aspects of the territorial display in potentially conveying species identity in Puerto Rican anoles, while also examining how the properties of the signal environment and the shared evolutionary history of taxa might confound that role in species recognition.

METHODS

We began our study by exploring the variation that exists among display characteristics, including headbob and dewlap display variables (Table 1; using display action pattern (DAP) graphs) and the type of display components (Table 1) and motifs (Supplementary Table S1), using a phylogenetic principal component analysis (pPCA). This allowed us to evaluate which characteristics have potentially evolved jointly or independently of one another. We then applied a random forest classification analysis to determine the combination of display characteristics – variables, components and motifs – that has the greatest potential to encode information on species identity, and whether these characteristics were consistent or different among taxa. Finally, we assessed the extent to which incorrect classifications in this analysis occurred because of similarities in signal design that might result from lizards living in similar environments – assessed via a model fitting approach of habitat characteristics known to influence signal detection in these lizards – or recent shared evolutionary history – assessed using Mantel tests and estimates of phylogenetic signal on display variables.

Table 1

Display variables and components scored from video recordings and display action pattern graphs of territorial advertisement displays performed by male Puerto Rican *Anolis* lizards

Characteristic	Description
Display variables	
Headbob number (HBn)	Total number of individual headbob movements
Headbob duration (HBd)	Duration (s) of an individual headbob movement, recorded from the start of the upward movement to the following downward movement of the head/legs
Headbob interval (HBint)	Duration (s) of each 'gap' between headbobs
Headbob amplitude (HBamp)	Proportional measure of amplitude (relative to the maximum height of the head from the substrate recorded for that display)
Headbob amplitude variation (HBvar)	Number of different headbob amplitudes per headbob movement
Dewlap number (DWN)	Total number of complete extensions and retractions of the dewlap
Dewlap duration (DWd)	Duration (s) of a dewlap display recorded from the start of a dewlap extension to the complete retraction of the dewlap
Dewlap pulse (DWpul)	Total number of times the dewlap was partially extended during a single dewlap display
Dewlap amplitude (DWamp)	Proportional distance that the dewlap was extended from the throat (relative to the maximum extension distance recorded for that display)
Dewlap interval (DWint)	Time (s) between the complete retraction of the dewlap to the start of a dewlap extension
Dewlap latency (DWlat)	Time (s) from start of the first dewlap to the start of the first headbob; negative values indicate that dewlap displays precede headbobs
Motif components	
Headbob (Hb)	Up-and-down movement of the head and neck
Two-legged push-up (Ht)	Vertical movement of the forebody
Four-legged push-up (Hf)	Flexion of all four legs
Dewlap (Dp)	Extension and complete retraction of the dewlap
Tail raise (Mtr)	Base to the tip of the tail is straight and elevated horizontally above substrate
Tail arch (Mta)	Base to the tip of the tail is arched and elevated horizontally above substrate
Tail curl (Mtc)	Base to the mid-section of tail is straight and elevated horizontally above substrate, while the tip of the tail is curled
Tail flick (Mtf)	Back-and-forth horizontal movement of the tail

Data

Display variables

Headbob and dewlap movements were quantified from 475 territorial displays filmed from 99 males (5–10 displays per male, 6–10 males per taxon, 11 taxa, 8 species; see Fig. 1). Video recordings of free-living anoles were obtained from an existing video archive collected by T.J.O. (e.g. see Ord et al., 2010, 2016). Analysed video clips were those in which the lizard was positioned perpendicular to the video camcorder and where the camcorder remained stationary for the entirety of the display sequence, both requirements critical for accurately constructing DAP graphs. Individuals were selected depending on whether they had the appropriate number of clips (at least 5). Clips in which males moved position were not included. Headbob and dewlap movements were manually tracked in videos using ImageJ v.1.50i (Schneider et al., 2012) to create the DAP graphs, which represent the change in the position of the head and dewlap over time (Carpenter et al.,

1970; Jenssen, 1977; e.g. Fig. 1). From these graphs, we measured 11 headbob and dewlap variables (Table 1) that have been previously suggested to explain much of the variability in display design among *Anolis* species (Ord & Martins, 2006) and among iguanid lizards more generally (Clark et al., 2015; Martins, 1993; Martins et al., 1998).

Display components and motifs

Previous attempts to describe species-specific visual displays in lizards have focused primarily on temporal display measurements (i.e. those described in Table 1; see previous section). However, our initial assessment of videos suggested there was additional complexity to the displays not captured by the timing of display movements.

We therefore developed an additional categorization in which all distinct behaviours were assigned to a type of display component: (1) a headbob movement, which was further refined to a headbob (sometimes referred to in the literature as a headnod),

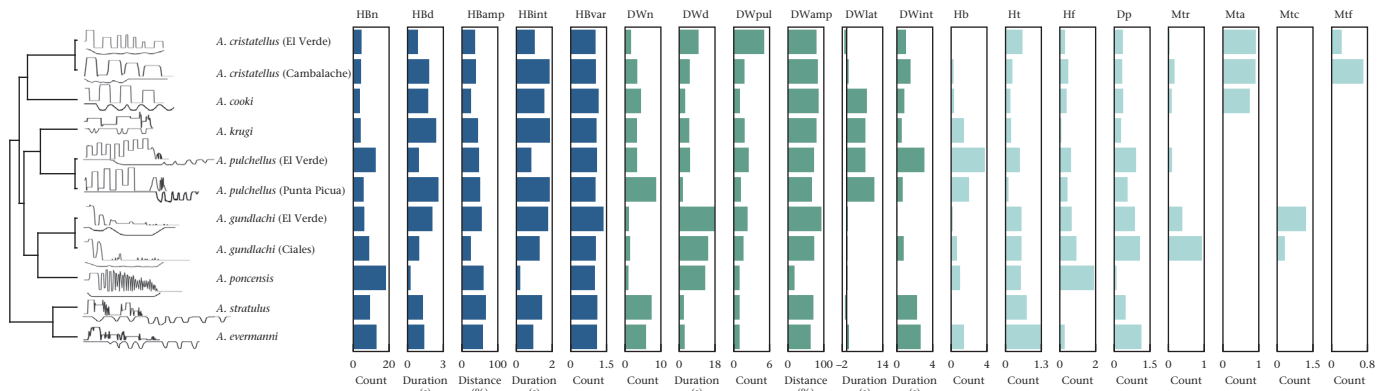


Figure 1. The territorial advertisement displays of male Puerto Rican *Anolis* lizards illustrated by representative DAP graphs of display movements presented alongside the taxon phylogeny and bar graphs of display means for each display characteristic outlined in Table 1.

two-legged push-up or four-legged push-up; (2) a tail display that was further refined to a tail raise, arch, curl or flick (Appendix, Fig. A1), or (3) a dewlap extension (Table 1). These display components could be performed independently or (more typically) in combination (with 1 or 2 other components), resulting in a total of 18 combinational groupings, termed motifs (see Supplementary Table S1; our use of motif is distinct from that of neurobiology (e.g. Sporns & Kötter, 2004) and was inspired in part by the motif categorization of Elias et al. (2012) used to quantify the complex vibrational signals of spiders). There seemed to be various grammatical rules that govern the combination of display components into motifs. Headbobs were either performed independently or combined with a dewlap display, while push-ups could be performed independently but were more often combined with dewlap extensions, a tail component (i.e. tail raise or arch), or both. Tail curls were only ever performed with push-ups and dewlap extensions, while tail flicks were always performed independently of anything else. A display is formed from the addition of multiple motifs. For example, an individual's display could consist of two motifs, one containing three components – a four-legged push-up, dewlap display and a tail arch – performed four times, followed by another motif made up of two components – a two-legged push-up and a dewlap display – performed two times (i.e. 4HfMtaDp 2HtDp; Supplementary Table S1). The online supplementary material provides additional context on motif categorizations in relation to past descriptions of *Anolis* territorial displays (e.g. Janssen, 1977, 1978).

Environmental variables

Several environment measures, taken at the time video recordings of each male were made, were extracted from the data archives of Ord et al. (2010) and Charles and Ord (2012). Full details on methodology associated with these data are provided in these publications. What follows is a brief summary to provide context for each measure.

During videorecording, the environment was monitored for male territorial neighbours to provide an estimate of the total number of neighbours surrounding the focal male. These data were then averaged across focal males to provide a mean taxon estimate of neighbour number. Ambient light was measured at the end of video recording at the perch of first display for the focal male using a LiCor LI-190SA Quantum Sensor connected to a hand-held LI-205A light meter. These data were averaged across males to provide a mean taxon estimate of habitat light. Finally, the visual background noise generated by windblown vegetation in the backgrounds of display clips was quantified using the Matlab-based program 'Analysis of Image Motion' (Peters et al., 2002). These data were summarized as the maximum speed of vegetation movement occurring behind the display lizard, which was then averaged across lizards to provide a mean taxon estimate of habitat visual noise.

Neighbour number, habitat light and visual noise have been previously shown to influence various aspects of display production in these same anoles (Ord et al., 2007, 2010) and so were included in our analyses in order to evaluate the impact that communicating in similar environments might have on display design.

Phylogeny

We used the phylogeny of *Anolis* developed by Gamble et al. (2014), with branch lengths set proportional to divergence time between taxa. The tree was pruned to the 11 taxa of interest, with populations within species set to an estimate of the likely minimum divergence time following Ord et al. (2010).

Statistical Analyses

All analyses were performed using R v.4.0.5 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Associations among characteristics making up territorial displays

We applied a phylogenetic principal components analysis (pPCA; Revell, 2009) based on Pagel's λ (Pagel, 1999) using the 'phytools' package v.0.7–80 (Revell, 2012) to 16 display characteristics (11 display variables, 5 display components; Table 1). The objective was to determine those characteristics that tended to be associated with one another, but also those loading on orthogonal axes and subsequently more likely to have evolved semi-independently of each other.

Classification of lizards based on display design

We ran a random forest tree classification (Breiman, 2001) using the 'randomForest' package v.4.6–14 (Liaw & Wiener, 2002) to determine whether male lizards could be assigned to the correct taxon using their display characteristics and which of those characteristics were especially important in those assignments. We provide a brief guide to the application of random forest analyses to behavioural data using R in the online supplementary material. All of the procedures described below (as well as methods for evaluating the impact of environmental effects outlined in the following section) are included in this guide with the associated R code.

A random forest is a machine-learning algorithm that fits a series of classification trees to predict a categorical response (here, taxon identity), using a group of explanatory variables (display variables, components and motifs), and combines the predictions to improve accuracy (Breiman, 2001; Briec et al., 2018; Cutler et al., 2007). It has many advantages over other statistical methods of classification (i.e. logistic regression and linear discriminant analysis) such as providing higher classification accuracy, an estimate of variable importance in assignments and the ability to characterize data with complex interactions among predictor variables (Briec et al., 2018; Cutler et al., 2007). It also enables a straightforward interpretation and graphical representation of otherwise complex patterns (Briec et al., 2018; Cutler et al., 2007). Random forest is widely used in bioinformatics, but in recent years, classification trees have become a popular statistical method used by ecologists to model species distributions (Guisan & Thuiller, 2005) and to map vegetation by remote sensing (Lawrence et al., 2006). However, it has yet to be widely used in behavioural ecology (but see Albornoz et al., 2017; Hoffman et al., 2017).

Of special importance for the objectives of this study, the random forest analysis makes no a priori distinction of taxon but instead uses the variation in the display variables themselves to statistically assign male lizards to specific groups of other male lizards that exhibit similar display attributes. However, the analysis does require complete data on all individuals. Our data set had 96 missing entries of the total 2871 (29 display characteristics for 99 lizards), which equates to 3.34% missing information. These missing data occurred because of difficulty in accurately obtaining a measure for a given characteristic (e.g. because of camera movement or a visual obstruction) or the absence of a characteristic altogether (e.g. the display did not include a dewlap extension). In the latter instance, this was accounted for directly in the data set with a value of 0 for the associated motif, but other variables of that behaviour could not be tabulated (e.g. duration or amplitude). Missing data were instead imputed using the 'rfImpute()' function available in the 'randomForest' package. Imputing missing data in this way is a standard protocol for ensuring that as much empirically derived information (existing data) as possible is leveraged in subsequent statistical

analyses (see [Tang & Ishwaran, 2017](#)). Nevertheless, as a sensitivity test of the influence of these imputed data on our classification outcomes, we repeated the random forest analysis on the truncated data set in which the male lizards with missing data were removed completely from the data set. This resulted in the removal of 11 of the 99 lizards and 223 empirically derived data points, reducing the data set by 8.04% (2775 down to 2648).

Following the implementation of the random forest procedure to the data, plots were produced showing the 'variable importance' of characteristics used to assign lizards to each taxon and the overall classification of male lizards to those taxa. Higher variable importance values indicate characteristics that are more important to the classification of individuals.

Assessing the impact of habitat in generating similarities in display design

Signal design is likely to be influenced by both the social and physical environment in which male lizards advertise territory ownership. For example, lizards that experience similar environmental conditions are likely to converge in aspects of their territorial display to improve signal efficiency (e.g. [Ord et al., 2013](#)). We assessed whether this type of similarity generated in display design might account for incorrect assignments in our random forest in two ways.

First, we used the 'MASS' package v.7.3–54 ([Venables & Ripley, 2002](#)) and the 'stepAIC' function specified with a ' $k=\log(\text{nrow}(\text{dat}))$ ' command to perform stepwise selection using a Bayesian information criterion to look at which social and environmental factor (male neighbours, ambient light or background noise) might be associated with display characteristics. Those factors that remained in final models were considered potential environmental confounders on display design and were used to compute environment 'free' residuals of the associated display characteristic. This was done by extracting the residuals from the model of the display characteristic regressed on the environmental variable. These residuals were then substituted into the data set for that display characteristic (other characteristics not found to be correlated with environmental variables were left as is in this new data set). We then ran a new random forest classification. If incorrect assignments in the first analysis were due to male lizards using displays of similar design because of adaptations for efficacy in similar environments, then this second random forest on the display residuals would lead to a tangible reduction in misclassification.

Second, we applied an alternative model fitting approach to the original (untransformed) display characteristics to examine various combinations of the social and environmental factors to provide a more explicit adaptive investigation into the extent to which environmental factors were associated with similarities in display design. These analyses were focused on the subset of display characteristics that had the highest contribution to taxa assignments (i.e. variable importance >0.02) and that were also responsible for most of the incorrect assignments (i.e. taxa with error rates $>40\%$). Once these display characteristics were identified, models testing various combinations of male neighbour number, ambient light or background noise were applied on taxon mean data for all taxa for which male lizards had been misclassified (10 of the 11 taxa). This was done using Ornstein–Uhlenbeck evolutionary regressions ([Hansen et al., 2008](#)) implemented in the 'phylolm' package v.2.6.2 ([Ho & Ane, 2014](#)). We used Akaike's information criterion with a correction for sample size (AIC_c) to rank models to identify factors that might have influenced similarities in display design.

Assessing the impact of shared evolutionary history on similarities in display design

We used a [Mantel \(1967\)](#) test to determine whether recent shared evolutionary history among taxa might have contributed to similarities in display design and the misclassification of some lizards in the random forest models. To do so, a measure of display similarity had to be first derived.

First, display characteristics were combined into a single estimate of signal complexity for each taxon. This involved converting each display characteristic to a common scale by splitting data into equal-sized range bins, calculating the proportion of times a certain display characteristic occurred in each bin, and then computing a Shannon–Wiener value of 'actual entropy', H , across all characteristics ([Shannon & Weaver, 1949](#)). Details and a worked example of how these conversions and calculations were made are provided in the online supplementary material. Actual entropy was estimated for each taxon separately as follows:

$$H_j = - \sum P_i (\ln P_i)$$

where P_i is the proportional occurrence of i th display characteristic within a taxon, j , summed across all characteristics (display variables, motif combinations and dewlap colours). A pooled estimate of actual entropy was then estimated for all possible taxon pairs using:

$$H_{\text{pooled}(x,y)} = - \sum P_{i,(x,y)} (\ln P_{i,(x,y)})$$

where $P_{i,(x,y)}$ is the proportional occurrence of the i th display characteristic computed across both taxon x and taxon y and then summed across all characteristics.

Second, pooled entropy and taxon-specific entropy were used to calculate mutual information (MI; [Reshef et al., 2011](#)) between taxon pairs:

$$MI_{(x,y)} = H_{\text{pooled}(x,y)} - \frac{1}{2} (H_x + H_y)$$

Here, an MI of 0 indicates that the design of displays between two taxa (x and y) were identical, while increasing values reflect increasing dissimilarity in the design of displays between those taxa.

Finally, the Mantel test was applied to these data to test the extent to which the phylogenetic distance between taxa was correlated with the magnitude of MI values computed for the displays of taxa (i.e. the extent to which sharing a recent common ancestor was positively correlated with performing displays of similar design). The test was implemented using the 'ecodist' package v.2.0.7 ([Goslee & Urban, 2007](#)) and the function 'mantel' with 9999 permutations.

To evaluate which display characteristics potentially contributed to those similarities in display design, we estimated the degree of phylogenetic signal exhibited by each display characteristic using Blomberg's K statistic ([Blomberg et al., 2003](#)) implemented in the 'phytools' package. When K converges on 0, differentiation among taxa in a display characteristic has varied independently of phylogeny, while values approaching or exceeding 1 imply differentiation has closely tracked phylogeny and has therefore unlikely contributed to similarities in display design reported by the Mantel test. We used randomization tests based on 100 000 simulations of K to obtain a probability value that K could be statistically distinguishable from 0.

RESULTS

General Patterns of Display Design among Taxa

Visual inspection of representative DAP graphs (Fig. 1) suggested gross similarities in the design of territorial advertisement displays between populations of the same species – e.g. *Anolis pulchellus* – or between closely related sister species – e.g. *Anolis stratulus* and *Anolis evermanni*. Nevertheless, even among these same taxa, there were differences in display design that were apparent depending on the display characteristic considered. For example, while the DAP graphs of both populations of *A. pulchellus* look qualitatively similar, a number of quantitative measures suggested that the displays were potentially quite distinct (Fig. 1): *A. pulchellus* at El Verde performed an average of 13 short, 1 s headbob movements and three long, 5 s dewlap extensions, while the Punta Picua population typically performed six long, 3 s headbob movements and nine short, 2 s dewlap extensions. As another example, both populations of *Anolis gundlachi* had generally similar DAP graphs as well as a host of quantitative estimates of display, but the populations still differed noticeably in the type of tail component

Table 2
The first six phylogenetic principal components (pPC) recovered for the design of territorial advertisement displays performed by male Puerto Rican *Anolis* lizards, based on headbob and dewlap variables (excluding dewlap latency (DWlat), which could not be included because it exceeded the number of variables allowed in the analysis), only headbob variables, only dewlap variables and display components

Characteristic	pPC1	pPC2	pPC3	pPC4	pPC5	pPC6
<i>Display variable</i>						
HBn	0.57	0.40	0.72	0.02	0.01	–0.01
HBd	–0.07	–0.50	–0.65	0.34	–0.15	0.10
HBint	–0.28	–0.44	–0.74	0.30	–0.07	0.24
HBamp	1.00	0.00	–0.05	–0.01	0.00	0.00
HBvar	0.11	0.25	–0.57	0.14	0.00	0.45
DWn	0.34	–0.85	0.01	0.32	0.23	–0.05
DWd	–0.12	0.97	–0.20	0.06	0.04	0.01
DWpul	–0.31	0.24	–0.33	–0.72	0.38	–0.25
DWamp	–0.46	–0.27	–0.65	–0.12	0.20	0.45
DWint	0.22	–0.47	0.41	–0.39	0.38	0.51
DWlat	–					
% Variance explained	0.78	0.15	0.05	0.01	0.003	0.002
$\lambda < 0.01, N_{\text{taxa}} = 11$						
<i>Headbob variables only</i>						
HBn	0.58	0.82	–0.02	0.00	0.00	
HBd	–0.08	–0.81	–0.57	0.08	0.00	
HBint	–0.29	–0.86	–0.36	–0.24	–0.02	
HBamp	1.00	–0.05	0.00	0.00	0.00	
HBvar	0.12	–0.39	–0.10	–0.16	0.89	
% Variance explained	0.93	0.07	0.001	0.0001	0.00004	
$\lambda < 0.01, N_{\text{taxa}} = 11$						
<i>Dewlap variables only</i>						
DWn	0.82	0.26	0.47	0.18	–0.08	0.00
DWd	–0.94	–0.33	0.10	0.02	–0.04	0.00
DWpul	–0.44	–0.06	–0.48	0.75	0.07	–0.01
DWamp	0.11	–0.12	–0.13	0.38	–0.15	0.89
DWint	0.22	0.48	–0.51	–0.02	–0.68	–0.01
DWlat	0.81	–0.59	–0.02	0.00	–0.02	0.00
% Variance explained	0.73	0.19	0.05	0.02	0.01	0.001
$\lambda = 0.44, N_{\text{taxa}} = 11$						
<i>Motif components</i>						
Hb	–0.08	0.30	0.90	–0.32	0.01	
Ht	–0.54	–0.79	–0.13	–0.27	0.01	
Hf	–1.00	0.06	0.01	0.03	0.00	
Dp	0.09	–0.85	0.40	0.33	0.00	
Mtf	0.14	0.12	–0.19	0.25	0.93	
% Variance explained	0.78	0.14	0.06	0.02	0.002	
$\lambda = 0.39, N_{\text{taxa}} = 11$						

Tail raise (Mtr), arch (Mta) and curl (Mtc) were not included because these were always associated with headbob (Ht, Hf) components. Prominent axis loadings ($> 0.5, < -0.5$) are highlighted in bold.

added to the display (Fig. 1; see also Appendix, Fig. A1). Similarities among populations of the same species and sister taxa imply that the design of displays is potentially the outcome of shared evolutionary history, but there still appear to be aspects that are species specific.

Associations among Characteristics Making Up Territorial Displays

pPCA uncovered several general trends among display characteristics. In particular, an increase in the number of headbobs was associated with a decrease in the duration of headbobs and the intervals between headbobs. In other words, the more headbobs performed during a display, the shorter the headbob units and ‘gaps’ between the headbobs (Table 2). Similarly, as the number of dewlap extensions increased, there was an associated decrease in the duration of dewlaps (Table 2).

Classification of Lizards Based on Display Design

The initial random forest classification analysis correctly assigned roughly two-thirds (66%) of male lizards to their appropriate taxon (65 out of 99; Fig. 2). The analysis using only individuals with complete data achieved an even greater correct assignment rate (74%, 65 of 88 males; Appendix, Fig. A3). This is remarkable given only a subset of displays were analysed for each male lizard (5–10 displays recorded during video observations of less than 30 min). It suggests that in most cases, and despite general similarities in the design of male territorial displays among taxa evident in Fig. 1, there were enough cues of species identity that lizards have the potential to discriminate a conspecific based on only a brief assignment of a handful of advertisement displays.

Of the individuals misidentified in the initial random forest analysis (Fig. 2), nine lizards were classified to the wrong population of the same species, while 17 were classified to a closely related sister species (collectively 26 of 32 misidentified male lizards). *Anolis pulchellus* (El Verde) and *Anolis cristatellus* (Cambalache) had the highest overall error rates (57–60%). *Anolis krugi* also had a modest error rate of 50% and was either misidentified as one or the other of the *A. pulchellus* populations (a closely related sister species) or as *Anolis cooki* and *A. cristatellus* from El Verde (distantly related taxa). These outcomes were similar to those from the analysis focused only on individuals with complete data (Appendix, Fig. A3), where the top misclassified taxa were again males belonging to *A. cristatellus* (Cambalache) and *A. krugi* (50–63%; whereas *A. pulchellus* (El Verde) had an improved error rate of 29%). Misclassifications were again concentrated to other populations of the same species (6) or sister species (11; collectively 17 of 22 misidentified male lizards).

Figure 3b and Fig. 4 show the specific display characteristics used for assigning lizards to taxon. There appear to be no common characteristics consistently used in making assignments for all taxa. Instead, males were assigned using a host of characteristics, the combination of which differed from one taxon to the next. For instance, four-legged push-ups with a tail arch had the highest influence on lizards being assigned to *A. cristatellus* (Cambalache), while dewlap pulse, two-legged push-ups and dewlap extensions were important for *A. evermanni*.

Assessing the Impact of Habitat in Generating Similarities in Display Design

In the random forest analysis in which the influence of social and environmental factors had been removed from display characteristics, correct assignments increased to 77% with 76 of 99 male lizards correctly identified to their appropriate taxon (Fig. A2).

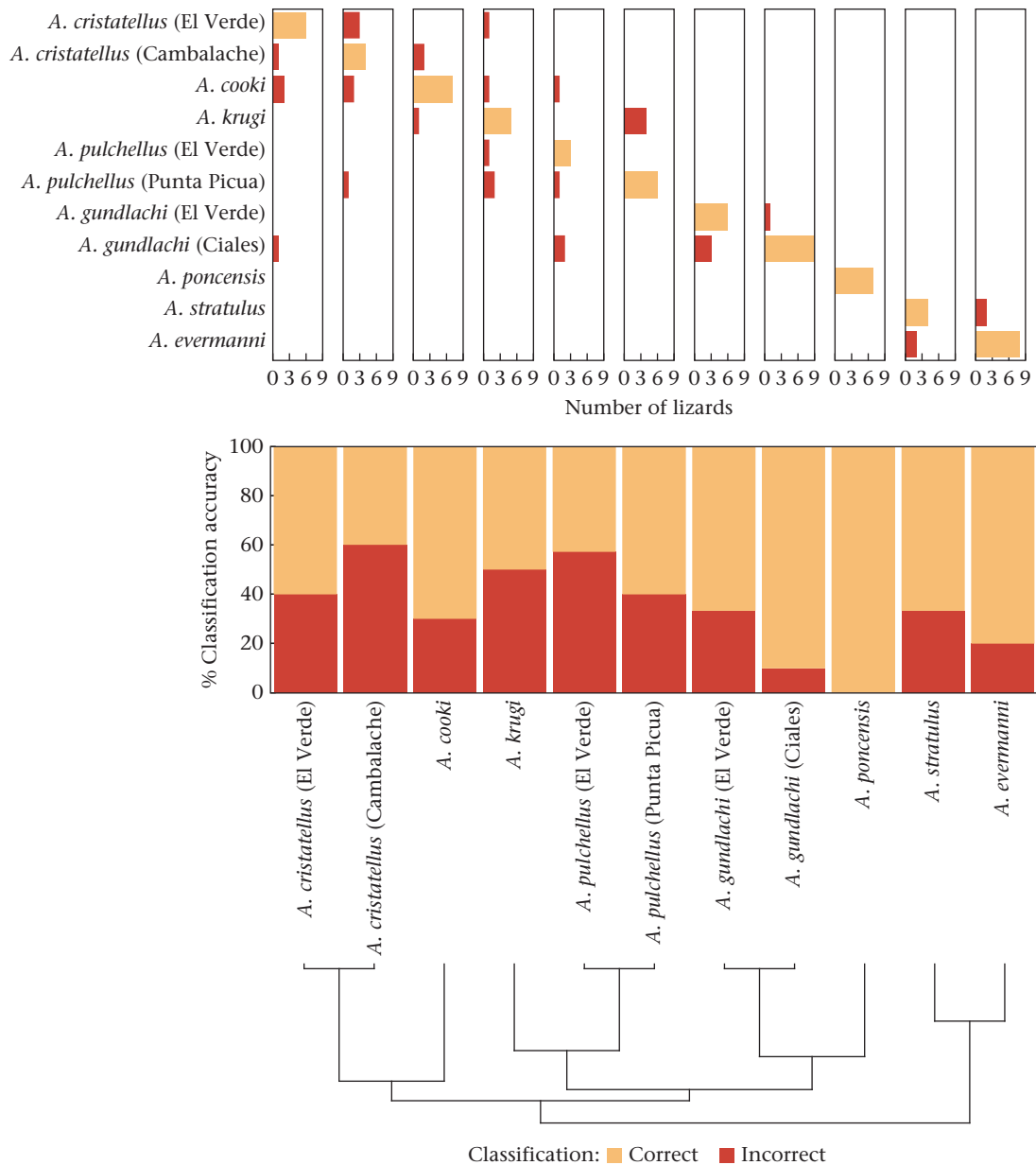


Figure 2. Random forest classifications of male Puerto Rican *Anolis* lizards based on territorial advertisement displays (described in Table 1 and in Supplementary Table S1). Upper panel: number of lizards correctly or incorrectly assigned. Lower panel: percentage of correct and incorrect classifications for each taxon. Overall accuracy of classification for all males was 66%, with individual taxon accuracies ranging from 40% to 100%.

Specifically, correct classification of previously incorrectly classified male lizards consisted of four males from *A. cristatellus* (Cambalache), two males from *A. cooki*, two males from *A. pulchellus* (El Verde), two males from *A. pulchellus* (Punta Picua) and one male from *A. evermanni*. This implied that at least some of the previous misclassifications might have reflected some of these taxa exhibiting similarities in display design because of sharing similar social and environmental conditions.

Fitting a set of evolutionary regression models that considered various combinations of social and environmental factors indicated – in all cases – the null (intercept only) model as the best-supported model (Table 3). Other credible models ($\Delta\text{AICc} \leq 3.0$) that reported statistically distinguishable effect sizes from zero (i.e. $t > 1.96$) indicated differentiation in dewlap duration and the propensity to perform four-legged push-ups with a tail arch as a negative function of ambient light (Fig. 5). That is, taxa advertising

territory ownership in poorly lit environments tended to extend their dewlaps for longer, although this trend appeared to be largely driven by two closely related taxa (*A. gundlachi* at El Verde and *A. gundlachi* at Ciales). Males that increasingly relied on four-legged push-ups with a tail arch to advertise territory ownership in poorly lit environments were limited to just three of the 10 taxa with misassignments in the initial random forest analysis (Fig. 5).

Assessing the Impact of Shared Evolutionary History on Similarities in Display Design

The Mantel test revealed a strong correlation between similarities in the design of male lizard territorial displays among taxa (quantified by MI) and their associated phylogenetic distances: $r = 0.37$ (95% confidence range: 0.25–0.49), $P = 0.003$. That is, displays tended to be more similar among closely related taxa

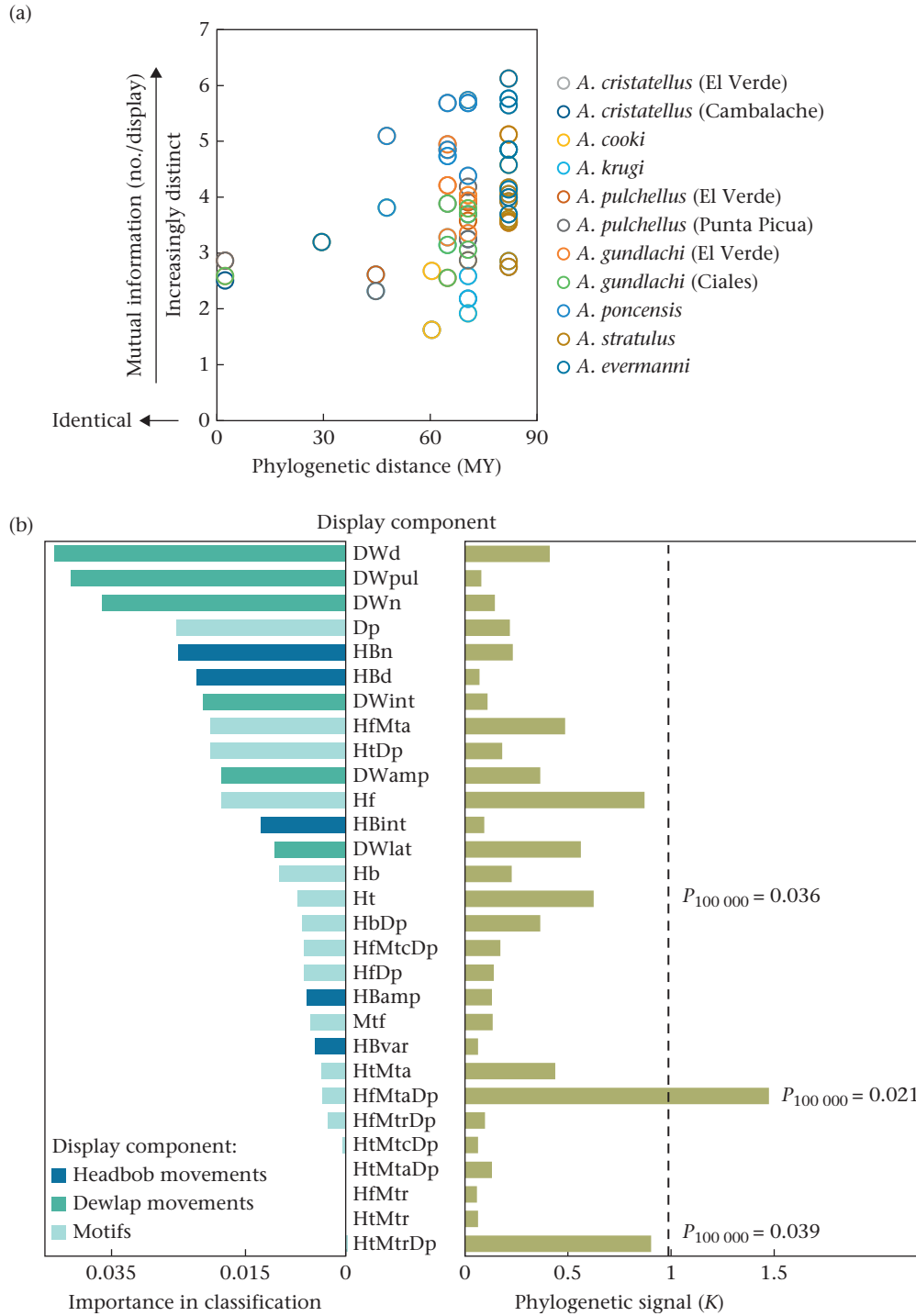


Figure 3. Impact of phylogenetic relationships among taxa on (a) shared aspects in display design (mutual information) and (b) the importance of each display characteristic in the classification of male Puerto Rican *Anolis* lizards to taxa. See Table 1 for definition of acronyms used.

(Fig. 3a), which also confirmed similarities in DAP graphs noted in Fig. 1 and suggests that the design of displays tends to be conserved among closely related taxa.

Estimates of phylogenetic signal, *K*, for display characteristics were highly variable and in general difficult to statistically distinguish from zero (Fig. 3b). Only three display characteristics – all motifs – were found to exhibit strong phylogenetic signal differentiation among taxa: the propensity to use two-legged push-ups (Ht), four-legged push-ups with both a dewlap extension and tail

arch (HfMtaDp) or tail raise (HfMtrDp). None of these motifs were particularly influential in assigning male lizards to taxa in random forest analyses (Fig. 3b).

DISCUSSION

We found considerable diversity in the design of displays among Puerto Rican male *Anolis* lizards, and this was striking given that the function of these signals appears to be the same across all taxa (i.e.

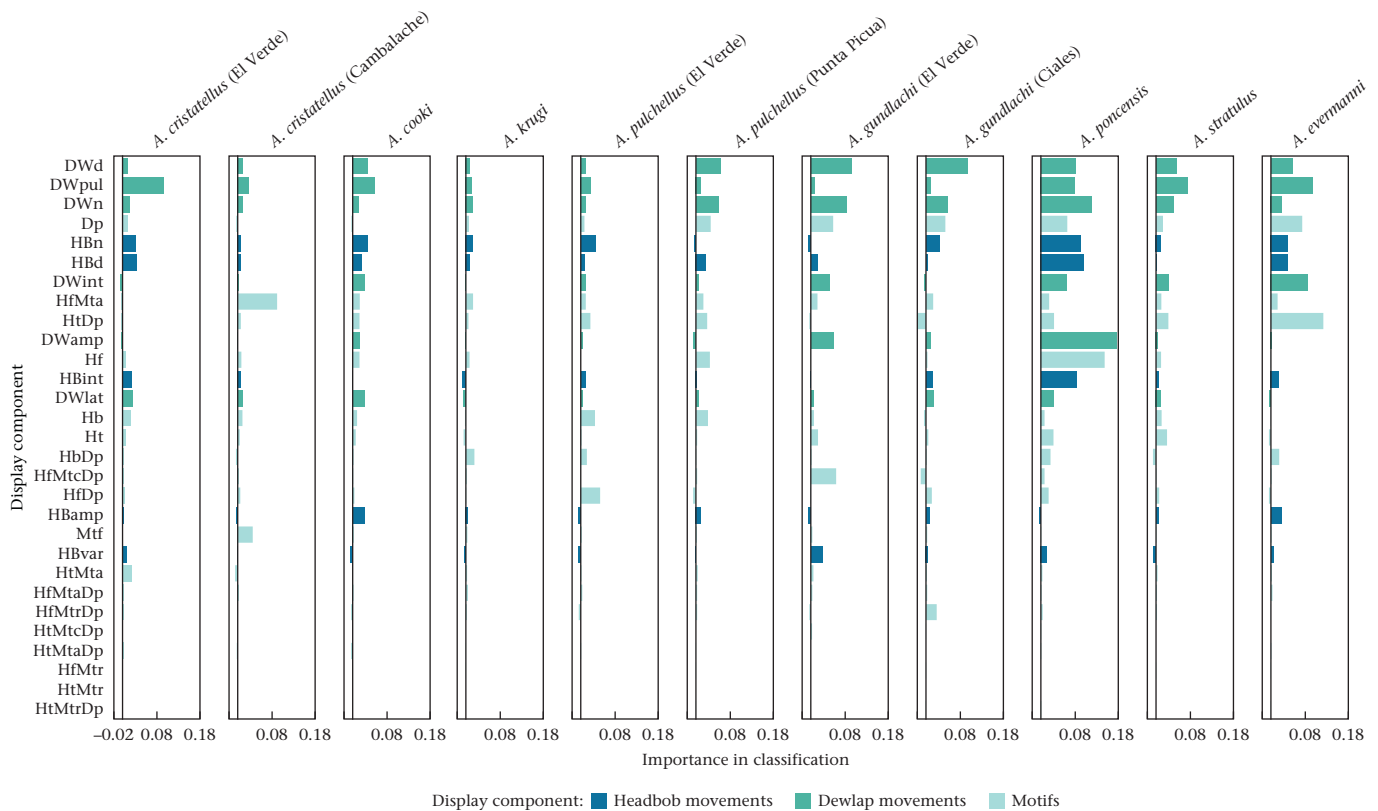


Figure 4. Importance of each display characteristic in the classification of male Puerto Rican *Anolis* lizards to taxon in random forest assignments. Higher values were more important to the classification of individuals. See Table 1 for definition of acronyms used.

to advertise and defend territories). Nevertheless, there were similarities among taxa in some aspects of design, particularly between populations of the same species or between species that were closely related to one another. This was confirmed by increasing dissimilarity in display design with increasing phylogenetic distance between taxa (Fig. 3a). But even for closely related taxa, our statistical approach was able to assign the majority of male lizards to the correct taxon, and based on the attributes of only a handful of displays per lizard. One can imagine this being comparable to a territorial holder viewing another male lizard performing a small number of displays over a period of minutes and making a judgement on taxon identity. It implies there is enough distinct variation in the design of male *Anolis* displays that lizards have the potential to quickly discriminate local rivals from lizards of other species, but also – to some extent – those from other populations of the same species. This differentiation in turn promotes reproductive isolation among populations (Losos, 1985; Rand & Williams, 1970) and ultimately drives speciation.

In particular, many aspects of the headbob and dewlap component of the display (e.g. the duration and number of bobs included in the headbob and the duration, pulse variation and number of extensions of the dewlap) appeared to have evolved independently of phylogeny (Fig. 3b) and are likely to be ideal candidates for encoding information on taxon identity (Jenssen, 1977; Macedonia & Stamps, 1994; Martins et al., 1998). In addition, tail components appeared to be unique to three of the eight species examined (i.e. *A. cooki*, *A. cristatellus* and *A. gundlachi*), and populations of the same species either used different tail components (populations of *A. gundlachi*) or varied the performance of the same tail component (populations of *A. cristatellus*; Fig. 1). This suggests that a combination of headbob, dewlap and tail

components making up advertisement displays should be enough to allow discrimination to occur among lizards (Fig. 4) without the need for additional identity cues from other factors (e.g. dewlap colour and other morphology). There also appeared to be no single ‘magic’ characteristic that could be used to discriminate male lizards by taxon (Fig. 4). Instead, it seems that lizards likely rely on a host of different cues, with the set used likely to be dependent on the taxon in question but also the community of sympatric congeners and the type of displays those lizards perform.

Reliable species recognition is likely to be undermined among sympatric species when the design of social signals has been heavily impacted by properties of the environment that prompt adaptive convergences in signal components in order to maintain communication efficiency. Sympatric species also tend to be closely related (Losos et al., 2006; Lovette & Bermingham, 1999), and closely related species often retain similarities in signal design because of shared evolutionary ancestry (Garcia et al., 2020; Podos, 2010; Stafford et al., 2001). This seems to be the case for the territorial advertisement displays of male *Anolis* lizards on Puerto Rico (Fig. 3a), and additional similarities in the design of the displays were apparent as a function of habitat. Most of the *Anolis* species examined in our study were sympatric with at least one other congener (only the populations of *A. gundlachi* at Ciales, *A. cristatellus* at Cambalache and *A. pulchellus* at Punta Picua appeared to be allopatric). Those taxa living in low light environments tended to extend the dewlap for longer (Table 3, Fig. 5), and presumably do so to facilitate signal detection (e.g. Ord & Martins, 2006; Ord et al., 2010, 2013). The propensity for some species to rely on four-legged ‘alert’ push-ups has also been previously linked to the need to maintain a conspicuous display in poor light (Ord & Stamps, 2008).

Table 3

Relative support for alternative Ornstein–Uhlenbeck evolutionary regression models of the extent to which the social (number of surrounding male territorial neighbours) and physical (background visual noise, ambient light) environment are associated with display differentiation among taxa

Model applied	AIC _c	ΔAIC	AIC _w	<i>t</i>
Dewlap duration				
Null	10.46	0.00	0.67	
Neighbours	16.18	5.72	0.04	
Light	12.52	2.05	0.24	−2.52
Visual noise	16.26	5.80	0.04	
Neighbours + light	21.35	10.89	0.00	
Neighbours + noise	24.04	13.57	0.00	
Light + noise	18.56	8.09	0.01	
Neighbours + light + noise	38.99	28.52	0.00	
Dewlap pulse				
Null	−0.60	0.00	0.83	
Neighbours	4.05	4.65	0.08	
Light	5.37	5.98	0.04	
Visual noise	5.31	5.92	0.04	
Neighbours + light	12.08	12.68	0.00	
Neighbours + noise	12.82	13.42	0.00	
Light + noise	14.27	14.88	0.00	
Neighbours + light + noise	29.72	30.32	0.00	
Headbob number				
Null	62.21	0.00	0.73	
Neighbours	67.78	5.57	0.05	
Light	67.95	5.74	0.04	
Visual noise	65.05	2.84	0.18	−0.5
Neighbours + light	76.76	14.55	0.00	
Neighbours + noise	73.00	10.79	0.00	
Light + noise	73.94	11.73	0.00	
Neighbours + light + noise	87.50	25.29	0.00	
Four-legged push-up accompanied with a tail arch (HfMta)				
Null	26.57	0.00	0.72	
Neighbours	31.95	5.39	0.05	
Light	29.50	2.94	0.17	−2.82
Visual noise	31.78	5.21	0.05	
Neighbours + light	37.53	10.96	0.00	
Neighbours + noise	40.20	13.64	0.00	
Light + noise	37.96	11.39	0.00	
Neighbours + light + noise	51.29	24.72	0.00	

Analyses were focused on the set of display characteristics found to contribute most to the incorrect assignment of males from 10 taxa (see Fig. 2). Effect sizes illustrating the magnitude and direction of effects are represented by corresponding *t* values.

The colour of the dewlap has often been argued to be important for species recognition in *Anolis* (Klomp et al., 2017; Losos, 1985; Macedonia et al., 2013; Rand & Williams, 1970; but see Leal & Fleishman, 2004), but our results also imply it is not specifically necessary for conspecific discrimination: there is enough information in the types of movements used in territory displays for lizards to discriminate a conspecific from a closely related congener. Indeed, while several aspects of the territorial display are dependent on properties of the environment (e.g. Fig. 5; see also Ord & Stamps, 2008; Ord et al., 2010, 2013, 2016), there is still much in the design of the display suitable for conveying taxon identity. Sexual selection (Mendelson & Shaw, 2012; Padian & Horner, 2013; Ryan & Rand, 1993) is one factor potentially driving the diversity in signal design across *Anolis* taxa on Puerto Rico and the genus more generally (Charles & Ord, 2012; Ord & Martins, 2006), which could lead to the evolution of taxon-specific territorial displays as well.

Identifying the selective pressures that drive diversity in functionally equivalent signals across closely related taxa is important for our general understanding of how signals evolve and adapt, but also for how reproductively important signals might promote prezygotic isolation among populations that could ultimately promote the evolution of new species (Bradbury & Vehrencamp, 2011; Coyne & Orr, 2004; West-Eberhard, 1983). Much of the interest in

this area has focused on documenting the mechanism of speciation as it relates to ecological differentiation among populations, whereby habitat-dependent differences in natural selection promote phenotypic divergence in mating signals (Losos et al., 2006; Ord et al., 2002; Slabbekoorn & Smith, 2002) that induces subsequent reproductive isolation (Nosil, 2012). While we did document environmental influences on the design of *Anolis* territorial displays, it explained only a small portion of the variation in signal design and this variation was unlikely to be useful in generating reproductive isolation among populations. This is broadly consistent with a number of other speciose taxa, such as Hawaiian fruit flies (Kaneshiro, 1988), cichlid fish (Turner et al., 2001) and birds-of-paradise (Irestedt et al., 2009).

The role of sexual selection, and in particular male–male competition, in speciation has been more controversial (Ritchie, 2007). Most studies have focused on the role of female mate choice in the speciation process, while male–male competition has been largely ignored (Grether et al., 2009; Seehausen & Schluter, 2004). However, recent studies have suggested that competition

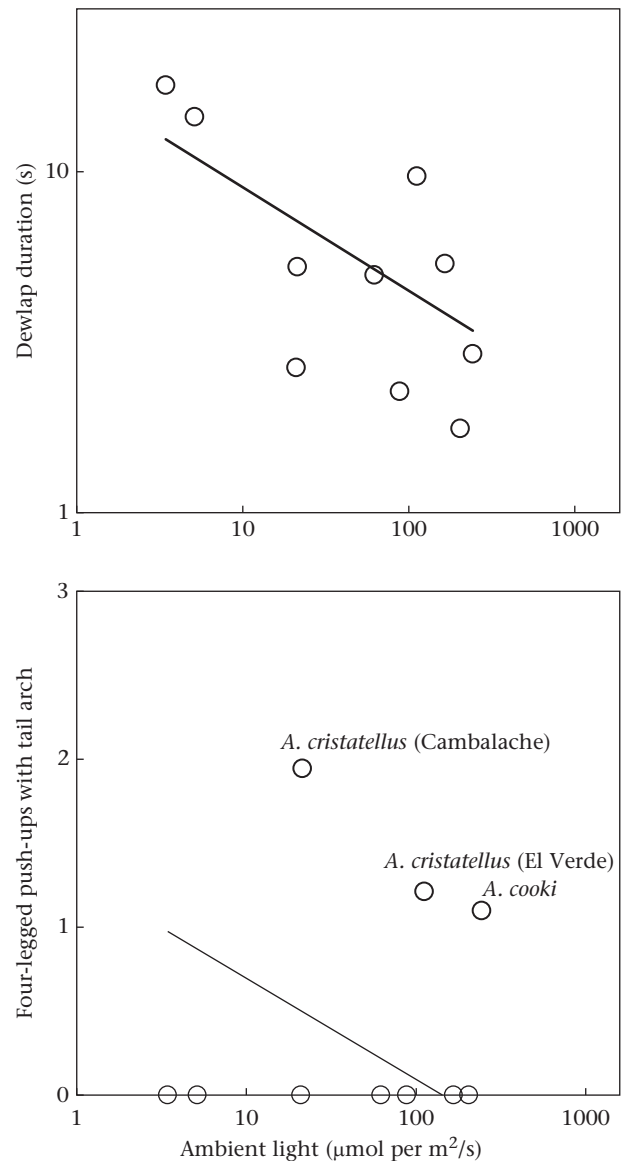


Figure 5. Relation of key display characteristics (see Table 3) with ambient habitat light in male *Anolis* lizards of 10 taxa that were found to be incorrectly assigned in random forest classifications.

among males over resources like territories has the potential to promote divergence in signal design among closely related species (Boul et al., 2007; Couldridge & Alexander, 2002; Seehausen & Schluter, 2004). If this occurs at the level of populations and results in population-specific discrimination in males, it could initiate speciation. In *Anolis* and other iguanian lizards, female mate choice seems to be weak (Jenssen & Nunez, 1998). Instead, the reproductive success of males is determined primarily by their ability to establish and defend territories against rival males in order to monopolize access to females that reside within those territories (Jenssen et al., 2001; Losos, 1985). This has led many researchers to suspect that the discrimination of territorial signals by male *Anolis* lizards should lead to behavioural reproductive isolation (Jenssen & Gladson, 1984; Losos, 1985; Macedonia & Stamps, 1994; Ng et al., 2013; Rand & Williams, 1970).

Our approach of measuring a wide range of signal variables and applying an undirected statistical approach to identify which of those variables are potentially influential in distinguishing signalers of closely related taxa offers a powerful new tool. It helps circumvent some of the challenges faced in studying species recognition using traditional methods such as playback techniques that are pragmatically restricted to single species or pairs of sympatric species. Our approach can help identify the potential salient cues used to encode information on taxon identity across many species, which can then function to focus these experimental manipulations using playback to test whether animals actually rely on those sets of cues for discrimination.

Moreover, our approach generated key insights in itself. For example, it revealed that the production of only a handful of signals are probably needed for reliable (and rapid) species recognition. This makes sense given the selection pressure that can be expected for engaging with the 'right' social partner (i.e. a member of the same species). Furthermore, our analytical framework can be used to tease apart the potential competing forces that shape the design of animal signals, whether such forces generate similarities or differences among closely related taxa and how this might impact which cues are likely to be suitable for recognition. The apparent absence of any magic signal characteristic (or set of characteristics) for conveying species identity among closely related taxa is an important biological result, but it also highlights the challenge faced by researchers interested in understanding how animals discriminate among species or foreign populations of the same species. To enable others to leverage the approaches used in our study, we provide a brief guide, R code and worked examples in the online supplementary material.

Display movement clearly has tremendous potential to convey species identity cues in anole lizards (see also Jenssen & Gladson, 1984; Macedonia & Stamps, 1994). However, the visual acuity of these lizards progressively diminishes with distance and at ranges likely to be relevant for territorial signalling (Fleishman et al., 2017; Ord, 2012b). There is therefore a remaining question of the extent to which influential aspects of the display can actually be reliably discriminated by lizards in the wild. The next logical step would be to use robot playbacks (e.g. Clark et al., 2015; Ord & Stamps, 2009) to experimentally test the response of free-living male lizards to manipulations of the display characteristics that our analyses have identified as being influential in categorizing lizards to their taxon. In doing so, it would be feasible to identify the extent to which similarities in signal design lead to recognition errors and males subsequently responding to inappropriate rivals, which would waste energy and time (Gerhardt, 1982; Macedonia & Stamps, 1994) and dilute the role of territorial advertisement displays in promoting reproductive isolation.

Data Availability

Data are available from the Dryad Digital Repository (<https://doi.org/10.5061/dryad.m905qfv31>).

Author Contributions

C.M.V.N. and T.J.O. conceived the study; T.J.O. collected the raw data and video; C.M.V.N. analysed the video and compiled the data; T.J.O. and C.M.V.N. conducted the analyses; C.M.V.N. and T.J.O. wrote the paper.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at <https://doi.org/10.1016/j.anbehav.2022.02.001>.

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Appendix



Figure A1. Examples of the display components involving the tail: tail arch in (a) *A. cooki*, (b) *A. cristatellus* (Cambalache) and (c) *A. cristatellus* (El Verde); tail raise in (d) *A. cooki*, (e) *A. gundlachi* (Ciales) and (f) *A. pulchellus* (El Verde); tail curl in (g) *A. gundlachi* (El Verde) and (h) *A. gundlachi* (Ciales); (i) tail flick in *A. cristatellus* (Cambalache) and (j) *A. cristatellus* (El Verde). Images were extracted from video records used to measure display characteristics.

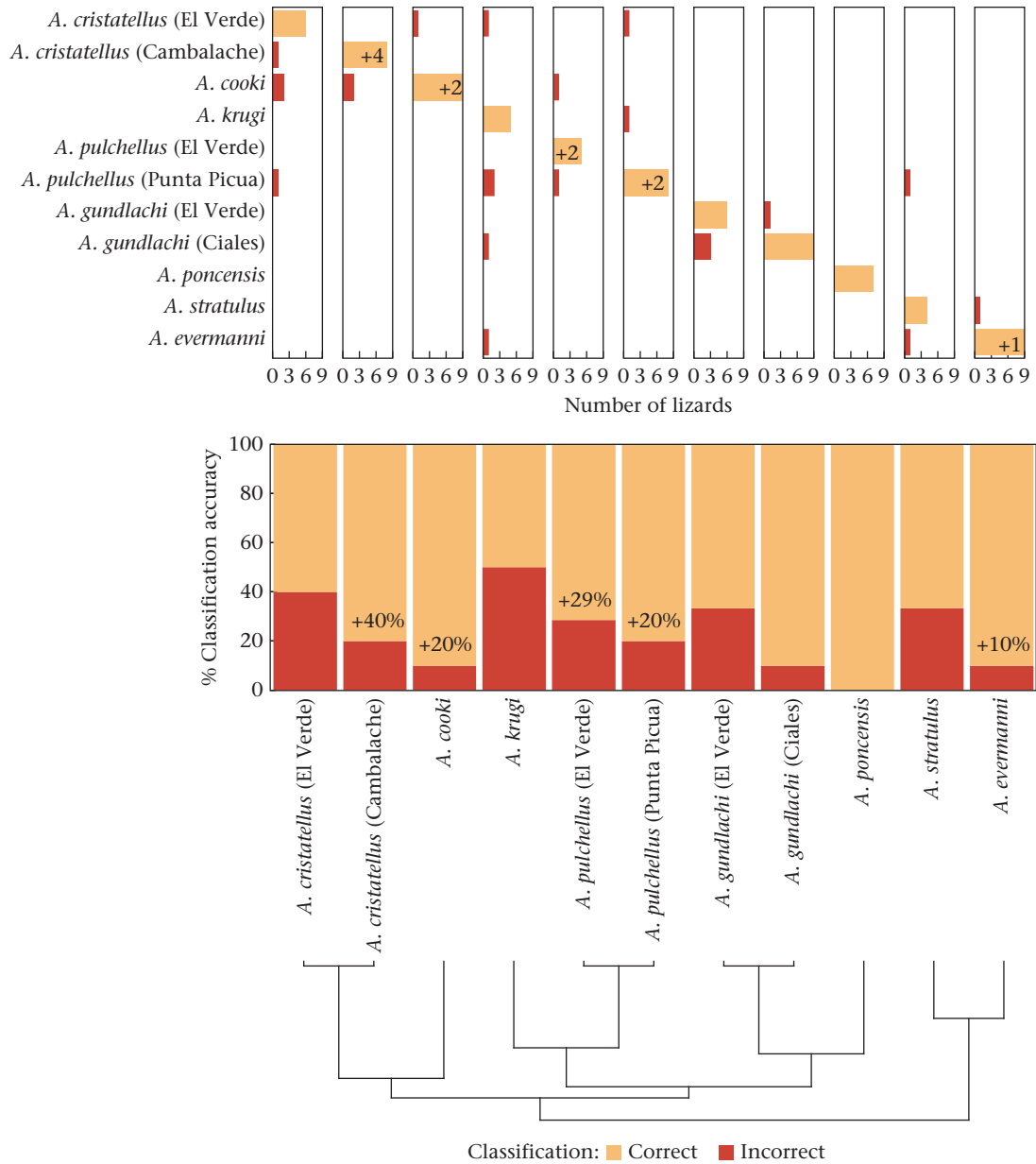


Figure A2. Random forest classifications of male Puerto Rican *Anolis* lizards based on territorial advertisement displays (described in Table 1 and in Supplementary Table S1) and the removal of potential habitat influences using residual analysis. Upper panel: number of lizards correctly or incorrectly assigned. Lower panel: percentage of correct and incorrect classifications for each taxon. Overall accuracy of classification for all males was 77%, with individual taxon accuracies ranging from 50% to 100% (improvements on classifications are highlighted over bars for relevant taxa).

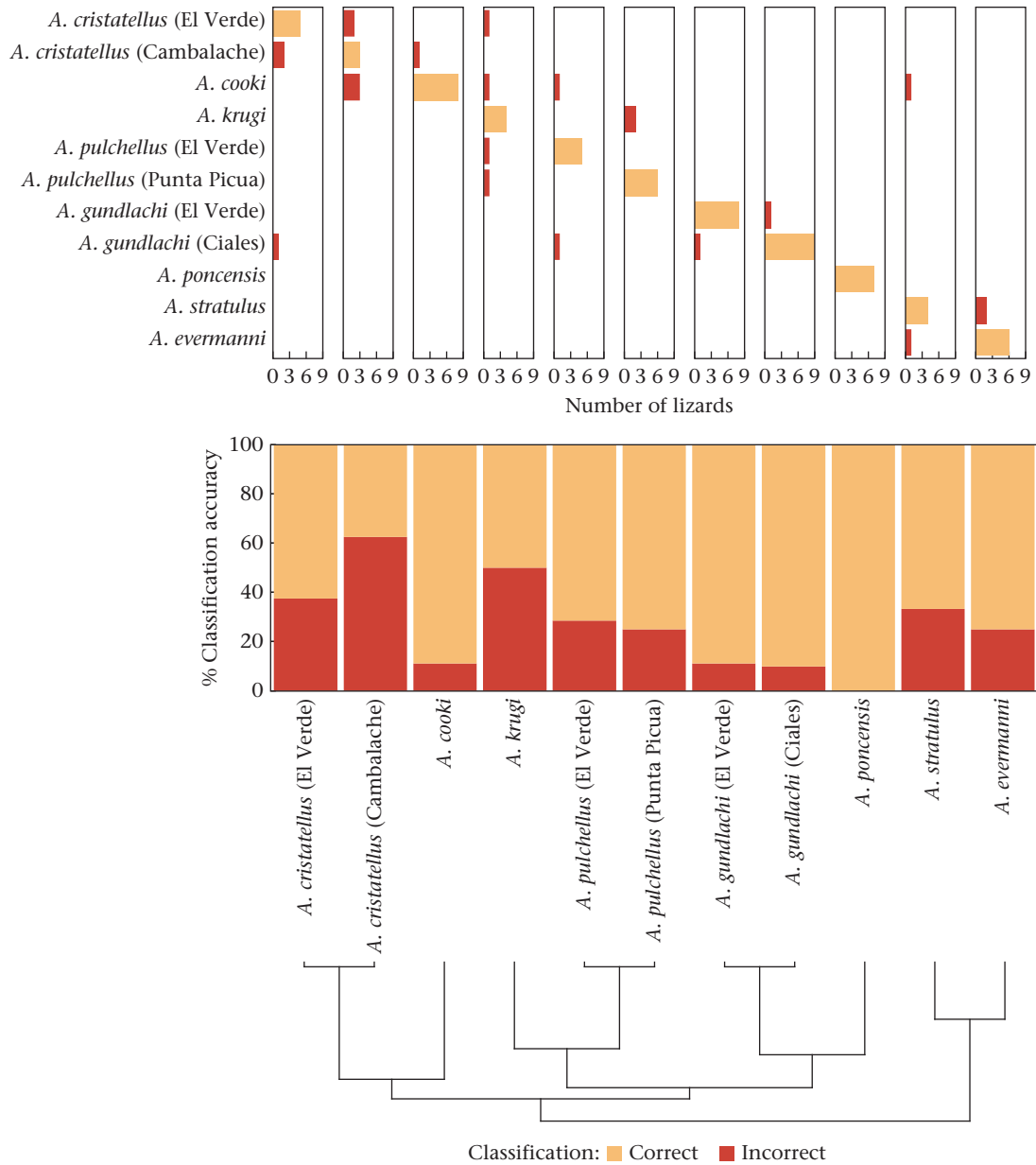


Figure A3. Random forest classifications of male Puerto Rican *Anolis* lizards based on territorial advertisement displays (described in Table 1 and in Supplementary Table S1) for which complete data was available. Upper panel: number of lizards correctly or incorrectly assigned. Lower panel: percentage of correct and incorrect classifications for each taxon. Overall accuracy of classification for all males was 74%, with individual taxon accuracies ranging from 37% to 100%.