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Effects of geographical heterogeneity in species interactions on the evolution of venom genes

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Geographical heterogeneity in the composition of biotic interactions can create a mosaic of selection regimes that may drive the differentiation of phenotypes that operate at the interface of these interactions. Nonetheless, little is known about effects of these geographical mosaics on the evolution of genes encoding traits associated with species interactions. Predatory marine snails of the family Conidae use venom, a cocktail of conotoxins, to capture prey. We characterized patterns of geographical variation at five conotoxin genes of a vermivorous species, *Conus ebraeus*, at Hawaii, Guam and American Samoa, and evaluated how these patterns of variation are associated with geographical heterogeneity in prey utilization. All populations show distinct patterns of prey utilization. Three 'highly polymorphic' conotoxin genes showed significant geographical differences in allelic frequency, and appear to be affected by different modes of selection among populations. Two genes exhibited low levels of diversity and a general lack of differentiation among populations. Levels of diversity of 'highly polymorphic' genes exhibit a positive relationship with dietary breadth. The different patterns of evolution exhibited by conotoxin genes suggest that these genes play different roles in prey capture, and that some genes are more greatly affected by differences in predator–prey interactions than others. Moreover, differences in dietary breadth appear to have a greater influence on the differentiation of venoms than differences in the species of prey.

1. Introduction

Biotic interactions shape the evolutionary trajectories of participating species [1,2]. Selection from geographical heterogeneity in the composition and strength of interactions can drive the divergence of traits that operate at the interface of these interactions [3]. Such patterns of divergence may reflect adaptive coevolutionary responses, as suggested for defences of fruitflies to parasitoid wasps [4] and resistance of garter snakes to their toxic newt prey [5]. In addition, variation in phenotypes that are directly coupled with predation or herbivory can be associated with variation in the availability or characteristics of nutritional resources. Relevant examples of such phenotypes include beaks of Darwin's finches [6], gill rakers of alewives [7] and sticklebacks [8], radular teeth and drilling behaviours of marine snails [9,10], and venoms of snakes [11–13]. Associations between phenotypes and resources may be genetically based [7,10,14], but limited knowledge of genes associated with resource acquisition limits our ability to determine the impact of geographical mosaics of species interactions on the genetic differentiation of populations [3].

Venom, widely employed as an effective tactic of predation, plays an important role at the interface of predator–prey interactions. Geographical variation of venom composition occurs in some snakes and is suggested to evolve in response to changes in diets [11,15–17]. Cone snails (of the family Conidae) are predatory gastropods that use a venom primarily to capture prey. Venom components of these snails (termed 'conotoxins' or 'conopeptides') are encoded by members of several large gene families, and target a variety of ion channels

and neuronal receptors [18]. Conotoxin genes evolve extremely rapidly, and are subject to extensive gene turnover and strong positive selection [19,20]. High variability in venom composition and potency has been found among populations [21], within populations [22], and even within a single specimen of *Conus* species through time [23]. However, the variability of conotoxin genes and their association with changes in prey specialization are unclear.

Conus ebraeus is a vermivorous species that is widely distributed and relatively common in nearshore habitats of the Indo-West Pacific. Based on the analyses of sequences of a region of the mitochondrial cytochrome oxidase I (COI) gene, this species exhibits little if any genetic population structure throughout tropical Pacific regions of the Indo-West Pacific [24,25]. Nonetheless, populations exhibit significant difference in allelic frequencies at a conotoxin gene E1, a pattern that may have resulted from selection on venom composition owing to difference in prey utilization among populations [26].

Venoms of cone snails are comprised numerous conotoxins. Do other conotoxin genes exhibit the same pattern as locus E1? Or do conotoxin loci differ in their patterns of geographical variation, suggesting different roles and functions of their gene products in species interactions? Is the evolution of conotoxin genes associated with changes in prey specialization? To address these questions, we examined the diversity of conotoxin genes of four predominant conotoxin superfamilies (A, I, M and O) that are expressed by this species, identified five loci to examine patterns of variation, genotyped individuals from three locations in the Indo-West Pacific (Hawaii, Guam and American Samoa) at the five conotoxin loci, and examined patterns of variation based on the genotype information. We also collected faecal materials of individuals from these locations, determined the identity of consumed prey items with a DNA-based approach, and quantified prey diversity within and among populations. Finally, we used statistical approaches, including canonical correspondence analysis (CCA) and regression, to evaluate the correspondence between patterns of variation of conotoxin genes and diet.

2. Material and methods

(a) Specimens and faecal samples

We collected specimens of *C. ebraeus* at Guam in June 2008, Hawaii in June/July 2009, and American Samoa in November 2000 and July/August 2009; we deposited all materials in the Mollusc Division collections at the University of Michigan Museum of Zoology. We collected faecal samples by placing individual snails into separate cups with seawater and preserved expelled faeces in 95% ethanol. We preserved venom ducts in RNAlater (Ambion, Inc.), and stored at −20°C and then −80°C.

(b) cDNA preparation and characterization of members of each gene family

We extracted messenger RNA (mRNA) from venom ducts of 31 individuals of *C. ebraeus* from Hawaii, 39 individuals from Guam and 15 individuals from American Samoa, and synthesized cDNA following the procedure described by Duda & Palumbi [20]. In short, we used streptavidin beads with biotinylated Oligo-dT to capture venom duct mRNA and synthesized cDNA by reverse-transcription. We used general primers designed in conserved regions of A-, I-, M- and O-superfamily conotoxins (electronic supplementary material, table S1) to amplify members of these gene families from venom duct transcripts of one to five individuals from each location. We ligated PCR products into vectors and transformed them into competent cells using the original TA cloning kit with top 10 competent cells (Invitrogen). We screened colonies and sequenced amplification products of expected target sizes at the University of Michigan DNA Sequencing Core.

We examined sequence chromatograms in Sequencher v. 4.8 (Gene Codes Corporation), and manually aligned sequences with Se-Al v. 2.0a11 [27] based on similarity of nucleotide and predicted amino acid sequences and consistency in the structure of the cysteine backbone of each superfamily. We constructed gene trees for members of each superfamily with maximum-likelihood methods in MEGA v. 5.05 [28], using the best models that were selected by jModelTest v. 0.1.1 [29], complete deletion of gaps, nearest-neighbour interchange (NNI) branch-swapping approach and bootstrap analyses of 100 replicates. We identified putative loci based on phylogenetic relationships of all recovered sequences and the criterion that average genetic distances within loci (i.e. among putative alleles of a single locus) are smaller than distances among loci.

(c) Individual genotyping

We designed locus-specific primers for several of the putative loci that exhibited allelic variation, and genotyped individuals from each location for five of these loci through amplifications (electronic supplementary material, table S2) and direct sequencing of products. We used GoTaq master mix (Promega) as PCR reagents, and set each PCR cycle to 30 s at 94°C for denaturation, 30 s at 50–52°C for primer annealing and 45 s at 72°C for extension. We determined allelic sequences either from sequences recovered earlier via cloning or from chromatograms of putative homozygous individuals (i.e. those that did not contain any double peaks). Sequences of new alleles that were not recovered through cloning or from homozygotes were determined by subtracting peaks of known alleles from chromatograms with double peaks. For certain alleles that could not be distinguished in these manners, we designed additional allele-specific primers (electronic supplementary material, table S3) and examined sequences obtained through amplifications with these primers.

(d) Population analyses of single locus

We aligned alleles of each locus using Sequencher (v. 4.8) with assembly parameters of 95% identity. We examined and visualized allelic divergence and patterns of variation among locations in the form of statistical parsimony networks with TCS v. 1.21 [30], after removal of the 3′ untranslated regions and stop codons. We calculated nuclear diversity and gene diversity indices with Arlequin v. 3.1 [31] using the Tamura–Nei model [32]. To verify the validity of our assumption that we were characterizing alleles of single loci, we performed exact tests of Hardy–Weinberg equilibrium in Arlequin, and determined the significance cut-off after correction for multiple tests [33]. We examined population divergence with pairwise *F*-statistics, and determined the significance of results through 10 100 random permutations from the pooled dataset. We performed hierarchical AMOVA [34] with all three possible hierarchical groupings (electronic supplementary material, table S4) for each locus, and compared levels of genetic variance among groups and within groups across the three alternative groupings.

We evaluated the neutrality of each locus at each location by estimating Tajima's *D* [35] and Fu's F_S [36] values in Arlequin, as well as Fu & Li's *D** and *F** [37] in DNASP v. 5 [38] with complete deletion of gaps in the aligned gene sequences. We also estimated the number of non-synonymous substitutions per non-synonymous site (K_a) and the number of synonymous substitutions per synonymous site (K_s) between alleles of each

locus in DNASP with the Nei–Gojobori model [39], and plotted K_a against K_s to evaluate the neutrality of each locus.

(e) Multi-locus population data analyses

We used 30 individuals from Hawaii, 29 from Guam and 14 from American Samoa that were genotyped at four loci (ED4, ED6, ED20 and EA4) for multivariate data analyses. Information from locus E1 was not included, because individuals that were genotyped for this locus are not the same as those genotyped for the other loci. We tested for linkage disequilibrium in each population with GENEPOP [40,41], and pooled genotypic data of the four genes for clustering analyses with STRUCTURE v. 2.3.3 [42]. We used an admixture model, correlated allelic frequencies model and location priors. We ran the MCMC analyses for 100 000 steps for $K = 2$, 3 and 4 (K = number of clusters), removed the first 10 000 results as burnin and examined convergence of F_{ST} values and α. We estimated posterior probabilities of different K from their log likelihood to determine the most likely clustering pattern of individuals based on these loci.

(f) Identification of prey and estimation of dietary diversity

We identified prey species from faecal samples of *C. ebraeus* individuals with a DNA-based approach that was described previously by Duda *et al.* [26]. In brief, we extracted DNA from faeces and amplified a region of the mitochondrial 16S rRNA gene with primers that should preferentially amplify annelid but not gastropod 16S rRNA gene. We directly sequenced amplification products, aligned sequences to polychaete sequences downloaded from GenBank (accession numbers labelled in the names of sequences in electronic supplementary material, figure S4) and performed model selection and phylogenetic analyses of these sequences. Because putative prey species are members of two major groups within Polychaeta (orders Eunicida and Phyllodocida), we separated the 16S rRNA gene sequences into two datasets composed exclusively of sequences of putative Eunicida species and Phyllodocida species. Bayesian consensus phylogenies were constructed in MRBAYES v. 3.1.2 [43] with each dataset (5 000 000 generations, two runs, four chains and 25% burnin) and the best models selected in JMODELTEST. We determined prey species identities based on the clustering patterns of faecal sequences with sequences of polychaetes from GenBank.

We used Shannon–Wiener's index (H') [44] and mean genetic distances to quantify dietary diversity at each location. To adjust for different sample sizes among locations, we estimated prey species richness [45] at each location by building rarefaction curves in the *vegan* package [46] in R v. 2.15.0 [47] and downsizing the sample sizes of populations to the lowest sample size (i.e. $n = 19$ for the population from American Samoa). We estimated proportional similarity indices (PS_I) [48], Pianka's overlap indices [49] and a measure of the phylogenetic disparity of prey items ('D_{ST}', a measure that is analogous to measures of Φ_{ST} and based on sequences recovered from prey) to quantify the extent of geographical differentiation in diet. We estimated mean genetic distances with the K80 model [50] in MEGA v. 5.05, and estimated D_{ST} values by F-statistics in ARLEQUIN with the Tamura–Nei distance model. We evaluated significance of PS_I and Pianka's overlap index values through a Monte Carlo simulation approach that randomizes prey items recovered for paired samples based on pooled frequencies of prey from these samples. The analysis compares observed PS_I and Pianka's overlap index values to the distribution of these values calculated from 10 000 simulated datasets, and determined p-values from the number of simulated values that are less than or equal to the values observed for the original data.

(g) Association between variation of venom genes and dietary heterogeneity

To evaluate how diversities of conotoxin genes are associated with dietary variables, we employed CCA [51]. CCA is a multivariate statistical tool for determination of correlative patterns of a set of variables [51]. We constructed two contingency tables with five conotoxin genes as column variables, three locations as row variables and the gene/nucleotide diversities as inputs of each cell; we built another contingency table with three locations as row variables, and H' and mean genetic distances of prey items as column variables. We performed canonical correspondence analyses of diversities of conotoxin genes with diversities of local prey items with the *cca* function in the package *vegan* in R v. 2.15.0. We estimated proportions of the total eigenvalues explained by each dimension and constructed biplots.

The relative positions and distances among populations in the CCA biplot represent their similarities in the gradient of dependent variables (diversities of conotoxin genes). Vectors of the two explanatory variables, H' and mean genetic distance of prey items, point to their higher values; angles of the vectors convey the relative correlations between the dietary variables; lengths of vectors represent the proportion of covariance of diversities of conotoxin genes explained by dietary variables.

As a control, we estimated the nucleotide diversity of mitochondrial COI gene sequences of populations of *C. ebraeus* at Guam, American Samoa and Hawaii presented by Duda & Lessios [24] with the Tamura–Nei model in ARLEQUIN v. 3.1. We also obtained estimates of haplotype diversities of COI from Duda & Lessios [24]. We estimated coefficients of linear regressions of haplotype and nucleotide diversities of COI with H' and genetic distances of diets at each location in R; we compared these values with values of the same coefficients estimated for the five conotoxin genes.

We also performed canonical correspondence analyses with a contingency table of pairwise Φ_{ST} values of five conotoxin genes among locations (American Samoa–Guam, American Samoa–Hawaii and Guam–Hawaii) as dependent variables, and a contingency table of pairwise PS_I and D_{ST} of prey compositions among locations as independent/explanatory variables. We converted negative Φ_{ST} values to zero. Vectors of pairwise PS_I and D_{ST} point to their higher values; lengths of vectors represent the proportion of covariance of pairwise Φ_{ST} explained by these two dietary variables. To verify the CCA results, we computed correlation coefficients of pairwise Φ_{ST} values of the highly polymorphic conotoxin genes and pairwise PS_I, Pianka's overlap index and D_{ST} of prey species among locations with Pearson [52], Spearman [53] and Kendall [54] methods in R. We did not estimate the correlation coefficients of the conserved conotoxin loci and COI because their Φ_{ST} values were not significantly different from zero.

3. Results

(a) Initial identification and genotyping of conotoxin genes

To identify putative conotoxin genes from *C. ebraeus* individuals, we recovered 30 unique sequences representing three putative A-superfamily loci out of 144 sequenced colonies, 45 sequences (which potentially encode δ-conotoxins) representing four O-superfamily loci out of 146 colonies, 22 unique sequences representing two I-superfamily loci out of 131 colonies, and 74 sequences representing at least seven M-superfamily loci out of 223 colonies (electronic supplementary material, figure S1).

We successfully determined genotypes of 24–36 individuals from Guam, 10–21 individuals from American Samoa

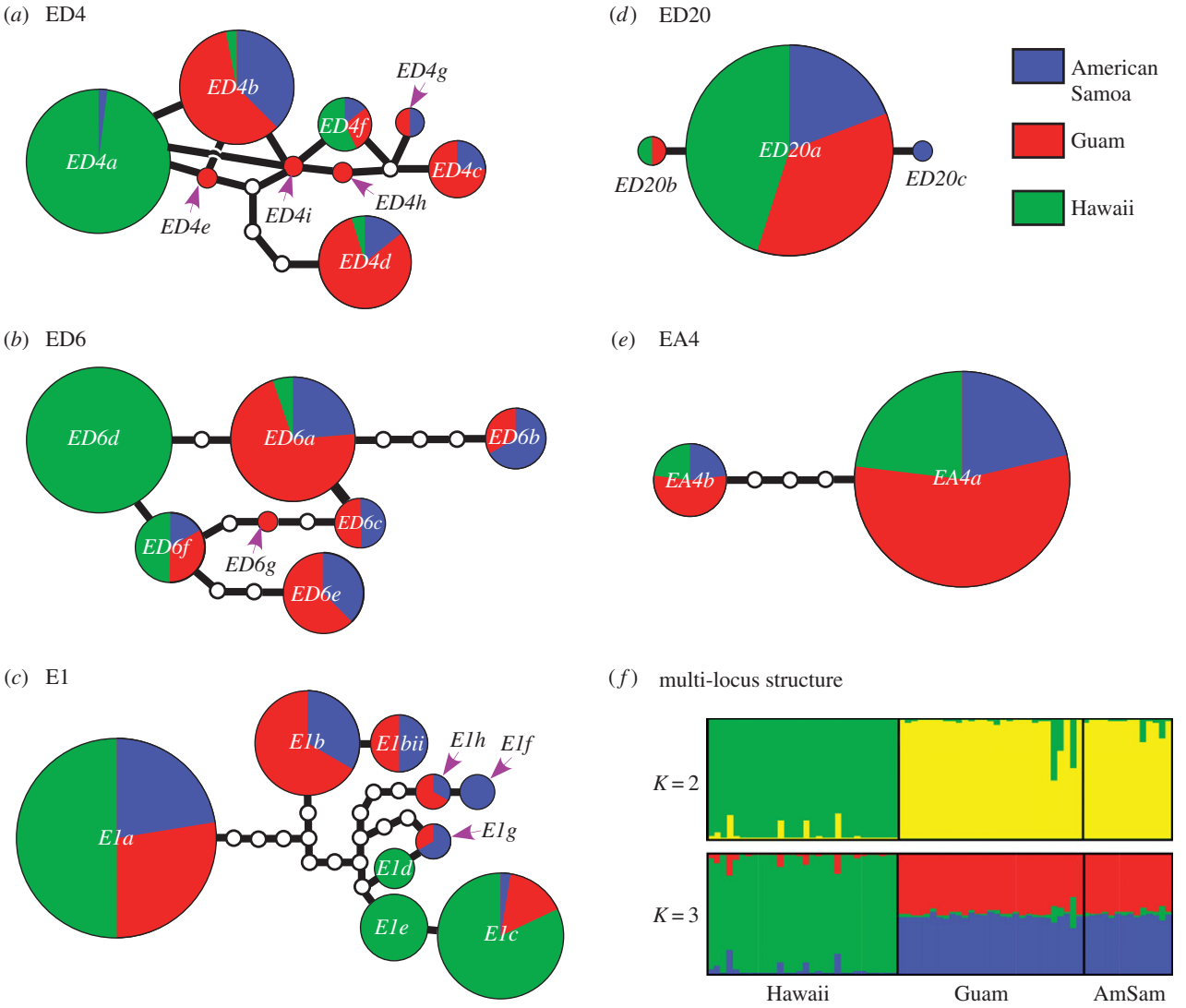

Figure 1. Haplotype networks of alleles of conotoxin loci of *C. ebraeus* at American Samoa, Guam and Hawaii, and results from multi-locus structure analyses. (*a*–*e*) Haplotype networks of (*a*) ED4, (*b*) ED6, (*c*) E1, (*d*) ED20 and (*e*) EA4. Haplotypes are illustrated as circles. Hypothetical haplotypes are shown as small white circles. Pie diagrams indicate allelic frequencies of haplotypes at each location; areas of circles are proportional to the overall frequencies of each allele combined from all three locations. (*f*) Bar plots of results of clustering analyses of four loci (ED4, ED6, ED20 and EA4) with $K = 2$ and $K = 3$ (K, number of clusters). Hypothetical clusters are illustrated with different colours in each plot. AmSam, American Samoa.

and 15–48 individuals from Hawaii at five conotoxin genes: four O-superfamily loci (ED4, ED6, ED20 and E1) and one A-superfamily locus (EA4; electronic supplementary material, table S5; figure 1). We also used genotype information for locus E1 for individuals from Hawaii and Guam that was previously reported by Duda *et al.* [26]. The five loci show no evidence of deviation from Hardy–Weinberg equilibrium or linkage disequilibrium (data not shown). We were unable to genotype a few individuals at some of these loci, a result that we interpret to have resulted from the absence of expression of these genes in these individuals (a similar phenomenon was described by Duda & Lee [55]). Interpretation of chromatograms of all individuals revealed that each individual contained at most two unique sequences of each putative locus.

(b) Genetic variation of conotoxin genes and test of neutrality

The five conotoxin genes exhibit tremendous variation in their extent of genetic diversity. Loci ED4, ED6 and E1 (designated as 'highly polymorphic loci') exhibited higher levels of allelic and nucleotide diversity than loci ED20 and EA4 (termed 'conserved loci'; electronic supplementary material, table S5 and figure S2). We observed only three and two alleles, respectively, at the 'conserved loci' (ED20 and EA4); alleles of each locus exhibit only one non-synonymous substitution in the toxin-coding region (figure 1; electronic supplementary material, figure S2). One of the alleles of locus ED20 (allele ED20c) probably represents a null allele based on the presence of a premature stop codon at the fourth *Cys* codon position (electronic supplementary material, figure S2); this allele was included in the population genetic analyses.

The conotoxin genes exhibit substantial geographical differentiation and strongly contrasting patterns of variation. The three highly polymorphic loci possess fewer alleles and lower gene and nucleotide diversities at Hawaii, whereas levels of diversity at Guam and American Samoa are equivalent (electronic supplementary material, table S5). Allelic frequencies of the highly polymorphic loci also differ substantially among locations, especially at Hawaii (figure 1), a pattern that is supported by *F*-statistics (table 1). Results from hierarchical AMOVA [34] also supported the interpretation that the

Table 1. Pairwise Φ_{ST} values and dietary overlap indices among populations of *C. ebraeus*. Values with associated *p*-values less than 0.001 are labelled with asterisks (**p*-value $<$ 0.001) and highlighted in italics. AS, American Samoa. All species in brackets represent numbers of unique species for both populations being compared.

	Φ_{ST} values of conotoxin genes					dietary overlap		
comparison	ED4	ED6	E1	ED20	EA4	PS_I	D_{ST}	shared species (all species)
AS – Guam	−0.009	0.008	−0.008	0.012	−0.025	*0.182**	*0.198**	1 (11)
Hawaii – AS	*0.270**	*0.427**	*0.177**	0.007	−0.035	*0.000**	*0.505**	0 (9)
Hawaii – Guam	*0.226**	*0.349**	*0.167**	−0.022	−0.024	*0.000**	*0.245**	0 (9)

population at Hawaii is distinct from the other two populations at these loci (electronic supplementary material, table S4). The pattern is also robust when genes are analysed jointly; results of clustering analyses showed that samples pooled from all locations are more likely to be divided into two clusters (electronic supplementary material, table S6), with the population at Hawaii completely isolated from Guam and American Samoa samples (figure 1*f*). Analysis of data from Guam and American Samoa populations alone revealed no evidence of structure (results not shown). However, the two conserved loci (ED20 and EA4) exhibited no significant differences among locations (table 1 and figure 1).

Neutrality tests of these conotoxin loci reveal different results among genes and locations. Tajima's *D* of loci ED4 and ED6 are negative for the Hawaii population, but positive at Guam and American Samoa (electronic supplementary material, table S7). Locus E1 exhibits a completely different pattern, with positive Tajima's *D* detected for all populations and significance reached for the population at Hawaii (electronic supplementary material, table S7). Negative Tajima's *D*-values of the conserved loci ED20 and EA4 were detected at all locations (electronic supplementary material, table S7). Results from other neutrality tests of each locus at each location are consistent with the pattern revealed by Tajima's *D*, though significance was not reached at some locations after correction for multiple tests. Moreover, alleles of each locus exhibit an overwhelming prevalence of non-synonymous substitutions in the toxin-coding region, with K_a/K_s ratios much larger than one (electronic supplementary material, figures S2 and S3).

(c) Geographical variation in diet

We obtained sequences of the mitochondrial 16S rRNA gene from faecal samples of 44 individuals from Guam, 19 from American Samoa and 34 from Hawaii, which represent 25 unique sequences (electronic supplementary material, figure S4). Phylogenetic analyses of these sequences revealed a total of 14 putative prey species from the annelid order Eunicida and family Nereididae (order Phyllodocida; electronic supplementary material, figure S4). The majority of prey items represent *Palola* species; others include four additional putative eunicid species and three nereidid species (table 2). Sequences of four of the putative *Palola* species were observed previously by Schulze [56] (i.e. Schulze clades A1, A3, A6 and A9). Sequences of other tip clades/putative prey species did not match any 16S sequences of polychaetes from GenBank and were considered to represent different species of eunicids and nereidids (i.e. *Palola* AX1–3; Eunicida 1–4 and Nereididae 1–3; electronic supplementary material, figure S4).

Table 2. Putative prey species, numbers of each species, total number of prey items of each higher taxonomic level, and the summary statistics of local prey diversity at the three locations. AS, American Samoa.

prey	Guam	AS	Hawaii
Eunicida (total)	(37)	(18)	(33)
Palola spp. (total)	(36)	(11)	(33)
Palola AX1	10	—	—
Palola AX2	—	—	2
Palola AX3	1	—	—
Palola A1	—	—	31
Palola A3	18	—	—
Palola A6	—	2	—
Palola A9	7	9	—
Other spp. (total)	(1)	(7)	(0)
Eunicida 1	1	—	—
Eunicida 2	—	2	—
Eunicida 3	—	4	—
Eunicida 4	—	1	—
Nereididae (total)	(7)	(1)	(1)
Nereididae 1	7	—	—
Nereididae 2	—	—	1
Nereididae 3	—	1	—
Total prey items	44	19	34
H′	1.46	1.47	0.35
mean genetic distance	0.15	0.22	0.04

Both the diversity of prey species and dietary composition differ among populations. Shannon's diversity index (*H*′) and mean genetic distances of prey are lowest at Hawaii, whereas dietary diversities at Guam and American Samoa are similar (table 2). Expected prey species richness values from rarefaction and downsizing to a common sample size of 19 are 2.37 for the population at Hawaii, 4.84 for Guam and 6.00 for American Samoa (electronic supplementary material, figure S5). Based on PS_I values and measures of the phylogenetic disparity of prey items, the three populations exhibit significant differences in diet; only one prey species was shared among any populations (tables 1 and 2).

(d) Association between conotoxin gene diversity and dietary heterogeneity

Patterns of variation of conotoxin genes are positively associated with prey heterogeneity. As revealed by CCA [51] and regression methods, gene and nucleotide diversities of conotoxin genes analysed jointly showed a positive correlation with dietary diversity at each location (figure 2; electronic supplementary material, table S8). Populations at American Samoa and Guam are completely distinct from the population at Hawaii in terms of conotoxin gene diversity in the first dimension (CCA1), which represents more than 75% of the total variance (figure 2*a*,*b*). The dietary variables point to the direction of increase in diversities of conotoxin genes in the biplots (figure 2*a*,*b*), indicating the positive correlation between the two.

Patterns of geographical differentiation of conotoxin genes, especially between Hawaii and the other two populations, are also associated with geographical heterogeneity in predator–prey interactions. The directions and lengths of vectors of dietary variables in the CCA ordination (figure 2*c*) reveal that geographical divergence of conotoxin genes (Φ_{ST}) is positively associated with prey heterogeneity (D_{ST}), but inversely related to prey similarities (PS_I), results that are also supported by regression analyses (electronic supplementary material, table S9). The strength of the association between diversities of conotoxin genes and diet originates from the three highly polymorphic loci; the two conserved loci exhibit no structure among populations. Nonetheless, examination of the mitochondrial COI locus revealed no association between patterns of differentiation and dietary diversity and heterogeneity (electronic supplementary material, table S8).

4. Discussion

Conotoxin genes of *C. ebraeus* from American Samoa, Guam and Hawaii show contrasting patterns of variation. Two genes exhibit low levels of diversity, with few alleles and no evidence of differentiation among populations. Three genes are highly polymorphic and exhibit significant differentiation between Hawaii and the other locations. While each population appears to interact with a distinct set of prey species, the population at Hawaii has the narrowest dietary breadth.

(a) Adaptive evolution of conotoxin genes

Populations of *C. ebraeus* in the Indo-West Pacific do not exhibit genetic differentiation at the mitochondrial COI locus [24]. This implies that populations experience high levels of gene flow. Moreover, high levels of haplotype diversity at COI (0.963 at Hawaii, 0.978 at Guam, 0.947 at American Samoa) suggest that this species has maintained a large population size throughout much of the Pleistocene [24]. Similar to the neutral mitochondrial COI locus, two conotoxin genes we examined, ED20 and EA4, do not show any differentiation among populations (table 1). However, the much lower levels of haplotype diversity exhibited by these two conotoxin genes (electronic supplementary material, table S5), as well as their negative Tajima's *D* (electronic supplementary material, table S7), imply that purifying selection may be operating at these loci, or that advantageous alleles at these loci recently swept through the populations. The putative null allele of locus ED20, allele ED20c, was inferred from the chromatogram of a single individual, and thus could be an artefact owing to an amplification error. Nonetheless, exclusion of this allele does not change the pattern observed

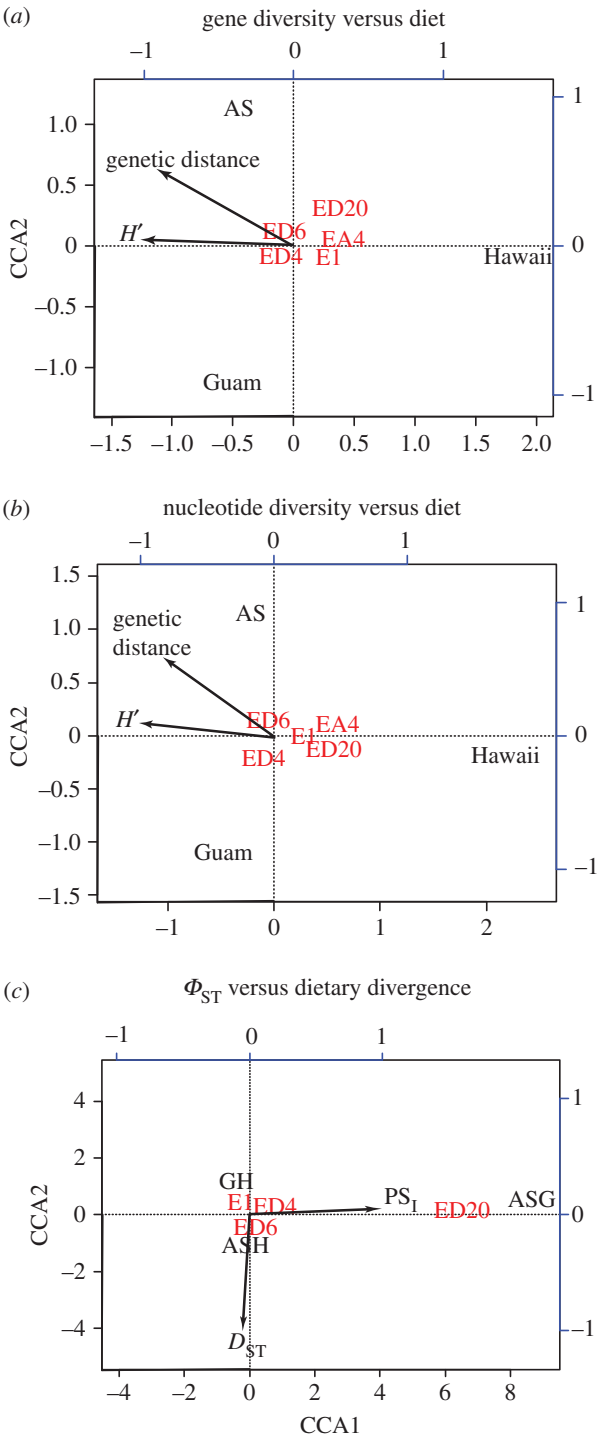

Figure 2. Ordination/biplots of canonical correspondence analyses of diversities and geographical divergence of conotoxin genes with heterogeneities of prey items. Arrows/vectors represent independent dietary variables. The dependent variables (conotoxin genes at three locations) are labelled with their names and positions indicating their relationships. Dashed lines are horizontal and vertical lines crossing the centroid. The bottom and left axes represent the first and second dimensions; the top and right axes show the loadings/weights of independent variables. AS, American Samoa. (*a*) Gene diversity and (*b*) nucleotide diversity of five conotoxin genes with diversities of prey items (H' and genetic distance) at the three locations. (*c*) Pairwise Φ_{ST} values of conotoxin genes with PS_I and D_{ST} values of prey items among locations. ASG, comparison of American Samoa and Guam; GH, comparison of Guam and Hawaii; and ASH, comparison of American Samoa and Hawaii.

at this gene—this gene exhibits extremely low diversity and does not show any significant differentiation of allelic composition among locations.

The patterns of geographical variation exhibited by the three highly polymorphic conotoxin loci, however, contrast strongly with the lack of differentiation presented at COI (table 1). This implies that patterns of variation at these loci may have been achieved by selection, despite high levels of gene flow among locations. Patterns of variation at all conotoxin genes examined are likely to have been affected by selection rather than drift and demography. Multiple tests of neutrality (electronic supplementary material, table S7) and the infinite K_a/K_s values (electronic supplementary material, figure S3) supported this notion. Moreover, different conotoxin genes appear to be subject to different modes of selection. Loci ED20 and EA4 may be subject to purifying selection based on their negative values of Tajima's *D* (electronic supplementary material, table S7). Locus E1 appears to be subject to diversifying selection, whereas loci ED6 and especially ED4 appear to be subject to contrasting modes of selection among locations based on the contrasting values of Tajima's *D* for the three populations (electronic supplementary material, table S7).

(b) Geographical variation in diet

Coincident with patterns of geographical variation at most venom genes, populations of *C. ebraeus* exhibit substantial differences in diets and considerable geographical heterogeneity in their interactions with prey (tables 1 and 2). Individuals at Hawaii possess the most distinct diet, with the lowest diversity and most uneven composition of prey species. At Guam and American Samoa, diets are broader and exhibit lower levels of divergence relative to Hawaii, but are still significantly different (tables 1 and 2). Because the methods we used to quantify and characterize diets measure the representation of particular species in diets (H' and PS_I) and the extent of the genetic diversity of prey items (D_{ST} and mean genetic distances), the differentiation of diets of these populations is consistently evident from both taxonomic and genetic perspectives. Although estimation of Shannon's index (H') requires that the sample sizes among locations are comparable [57], rarefaction of samples from Hawaii and Guam to the lowest sample size (19) revealed results consistent with those revealed from estimates of Shannon's indices: the American Samoa population has the highest degree of dietary diversity, whereas Hawaii exhibits the lowest (electronic supplementary material, figure S5).

Differences in prey utilization among populations may have several explanations. These differences may stem from a heterogeneous distribution of prey species that determines the local availability of prey and so influences access to particular prey species on a spatial scale. Schulze [56] and Schulze & Timm [58] investigated the diversity of *Palola* species in the western Pacific (Guam, Pohnpei Kosrae and Palau), South Pacific (American Samoa and Vanuatu; *P. viridis* only), eastern Pacific (Panama) and western Atlantic (Belize). These works demonstrate that the genus *Palola* is composed of a number of distinct clades that probably represent distinct species; some of these species may be geographically restricted while others may be relatively widespread. For example, *Palola* A1 occurs at sites that were sampled in both eastern and western Pacific, including Guam. *Palola* A2 and A6 were exclusively found at Guam. Nonetheless, none of the *C. ebraeus* individuals from Guam that were examined here consumed *Palola* A1, A2 or A6, but *Palola* A1 represents the primary prey item at Hawaii (table 2). Together, these observations suggest that some prey of *C. ebraeus* have the potential to possess broad distributions, but may be selectively targeted at different locations. However, temporal and seasonal variation in the availability or abundance of prey items in particular locations may also have contributed to geographical differences in diet. Our samples of *C. ebraeus* were mostly collected in June, July and August; the prey items identified may simply reflect the availability of polychaete species at this time of year. Temporal changes in prey communities that may be affected by specific periods of reproduction and settlement may also contribute to the geographical differences in prey utilization that were observed here. Alternatively, interspecific competition may limit access to prey, and such competition is heterogeneous among locations.

(c) Conotoxin gene evolution appears to be facilitated by differences in diet

The correspondence between patterns of variation of conotoxin genes and diets that we observed is manifested in two aspects: (i) levels of diversity of conotoxin genes are positively associated with levels of dietary diversity at each location; (ii) patterns of differentiation at conotoxin genes are concordant with patterns of heterogeneity in diet (figure 2; electronic supplementary material, tables S8 and S9). Because we are limited by the number of locations for which we have data, we cannot evaluate the significance of such an association, but canonical correspondence analyses permit an evaluation of the intercorrelated relationships of conotoxin genes and diets.

Correspondence between diversities of conotoxin genes and diet is not exclusive to *C. ebraeus*; such an association has also been observed in another worm-eating species, *C. miliaris*. This species is a widespread member of the Conidae and underwent ecological release at Easter Island, where it consumes a greater diversity of prey than elsewhere in the Indo-West Pacific [59]. Coincidently, two conotoxin genes (*MIL2* and *MIL3*) exhibit higher levels of diversity at Easter Island than at Guam and American Samoa [55], despite the fact that they show similar levels of diversity at the mitochondrial COI locus (as calculated from data of Duda & Lee [55]; electronic supplementary material, table S10). This phenomenon is also probably demonstrated by other venomous taxa. For example, snakes employ different envenomation strategies towards different prey [60], and prey species differ in their responses to venoms of different snakes [61,62] and spiders [63]. The positive relationship may be widely represented at other predation phenotypes, and especially those that are rapidly evolving.

Although Daltry *et al.* [11] detected a relationship between geographical divergence of venom and prey among populations of a Malayan pitviper species, debates arose over the universal applicability of this pattern and the existence of confounding factors [64]. For *C. ebraeus*, the association between differentiation at some conotoxin genes and divergence in prey utilization (figure 2*c*; electronic supplementary material, table S9) is not observed in all cases, but instead is only evident for comparisons involving Hawaii. At American Samoa and Guam, *C. ebraeus* shows considerable differences in prey

utilization but exhibits no differentiation at the highly polymorphic loci (tables 1 and 2, and figure 1). Selection regimes may be more intense at Hawaii than at these other locations, possibly as a consequence of episodic limited availabilities of resources at this locality, a phenomenon that accounts for selection on beak morphologies of Galapagos finches [65]. Similar to the peripheral speciation mechanism presented by Mayr [66], local selection pressures may generate more prominent effects at the range edges of species, because gene flow involving these locations is lower than in the centre of their distributions, a pattern evident in several *Conus* species [25]. Results from analyses of patterns of variation of *C. miliaris*, in which the most isolated and peripheral population at Easter Island exhibits the highest levels of differentiation at conotoxin genes and COI [55], support this scenario. In addition, even though it is a very minor degree of overlap, populations of *C. ebraeus* from Guam and American Samoa show more overlap in prey utilization than does either population with Hawaii. Perhaps this overlap prevents differentiation at the conotoxin loci we examined. Clearly, more studies of prey utilization and venom composition are needed to clarify the processes associated with the differentiation of venoms as impacted by diets.

The evolution of conotoxin genes exhibits high resemblance with that of major histocompatibility complex (MHC) genes in host–pathogen interactions. MHC gene polymorphism is maintained by heterozygote advantage, negative frequency-dependent selection or varying selection in response to temporal changes in associations with pathogens [67–70]. Similarly, patterns of diversity of the three 'highly polymorphic' conotoxin genes may be affected by negative frequency-dependent selection, in which rare alleles confer advantage in capturing prey. Temporal shifts of selection, affected by temporal changes in prey availability or prey preference, may also have led to patterns of variation that we detected. In some taxa, patterns of variation at MHC genes are substantially different from those of neutral loci [71,72]. For example, differentiation of MHC loci among populations of grey seals is associated with habitat differences [71]. Similarly, the significant geographical structure exhibited by conotoxin genes, in contrast to the lack of structure at a putative neutral locus (i.e. COI), may reflect an adaptive response of the venoms of populations to distinct sets of interactions with prey. Because cone snails are able to paralyse and consume organisms in captivity that they have never encountered before (e.g. earthworms; personal observations and communications with Alan Kohn, University of Washington, 2009, 2012), conotoxin gene evolution presumably depends more on the efficiency and speed of prey capture as opposed to simply the ability to capture prey.

The contrasting patterns of variation illustrated by different conotoxin genes, a phenomenon also detected in venoms of pitviper species [11,15], imply that conotoxin genes, including members of the same gene families, do not evolve in a concerted or universal manner, and functional roles of these genes' products differ. Some venom genes may track divergent targets and undergo adaptive divergence, whereas others track conserved targets and do not. The conotoxin gene families examined here target different ion channels and neuronal receptors: A-superfamily α-conotoxins (locus EA1) bind to nicotinic acetylcholine receptors, O-superfamily δ-conotoxins (loci ED4, ED6 and ED20) block voltage-gated sodium channels, and O-superfamily ω-conotoxins target calcium channels [18]. Differences in the evolution of genes from different gene families may be driven by heterogeneous evolutionary rates or trajectories of ion channels and neuronal receptors in prey.

5. Conclusion

Populations of *C. ebraeus* exhibit substantial differences in diet in terms of the particular species that are preyed upon and dietary breadth. Conotoxin loci show substantial variation in their patterns of diversity with among-locus differences in allelic richness, levels of differentiation among populations and modes of selection operating on loci. The impact of geographical mosaics of species interactions on the differentiation of genes associated with phenotypes that operate at the interface of these interactions is not entirely clear. Impact appears to be most pronounced among populations that have differences in the number and diversity of interacting species, but differences in the composition of prey species alone does not appear sufficient to drive differentiation at all venom genes examined. Nonetheless, we surveyed only a subset of genes that are related to prey capture and analyses of additional genes may reveal loci that do show patterns of differentiation that match differences in prey utilization. What is clear is that genes that contribute to the predatory phenotype of *C. ebraeus* are likely to exhibit distinct functions and are differentially affected by selection.

Data accessibility. DNA sequences used in this study have been uploaded to GenBank (accession numbers JX177103–JX177352, FJ804537–FJ804572 and FJ907334–FJ907342).

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References

1. Van Valen L. 1973 A new evolutionary law. *Evol. Theor.* **1**, 1–30.
2. Paterson S *et al.* 2010 Antagonistic coevolution accelerates molecular evolution. *Nature* **464**, 275–278. (doi:10.1038/nature08798)
3. Thompson JN. 2005 *The geographic mosaic of coevolution*. Chicago, IL: University of Chicago Press.
4. Kraaijeveld AR, Godfray HCJ. 1999 Geographic patterns in the evolution of resistance and virulence in *Drosophila* and its parasitoids. *Am. Nat.* **153**, S61–S74. (doi:10.1086/303212)
5. Brodie ED, Ridenhour BJ, Brodie ED. 2002 The evolutionary response of predators to dangerous prey: hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. *Evolution* **56**, 2067–2082. (doi:10.1111/j.0014-3820.2002.tb00132.x)

6. Schluter D, Grant PR. 1984 Ecological correlates of morphological evolution in a Darwin's finch, *Geospiza difficilis*. *Evolution* **38**, 856–869. (doi:10.2307/2408396)
7. Palkovacs EP, Post DM. 2008 Eco-evolutionary interactions between predators and prey: can predator-induced changes to prey communities feed back to shape predator foraging traits? *Evol. Ecol. Res.* **10**, 699–720.
8. Gross HP, Anderson JM. 1984 Geographic variation in the gillrakers and diet of European threespine sticklebacks, *Gasterosteus aculeatus*. *Copeia* **1984**, 87–97. (doi:10.2307/1445038)
9. Andrade SCS, Solferini VN. 2006 The influence of size on the radula of *Littoraria angulifera* (Gastropoda: Littorinidae). *Malacologia* **49**, 1–5. (doi:10.4002/1543-8120-49.1.1)
10. Sanford E, Worth DJ. 2010 Local adaptation along a continuous coastline: prey recruitment drives differentiation in a predatory snail. *Ecology* **91**, 891–901. (doi:10.1890/09-0536.1)
11. Daltry JC, Wuster W, Thorpe RS. 1996 Diet and snake venom evolution. *Nature* **379**, 537–540. (doi:10.1038/379537a0)
12. Gibbs HL, Sanz L, Chiucchi JE, Farrell TM, Calvete JJ. 2011 Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult dusky pigmy rattlesnakes (*Sistrurus miliarius barbouri*). *J. Proteomics* **74**, 2169–2179. (doi:10.1016/j.jprot.2011.06.013)
13. Mackessy SP, Sixberry NA, Heyborne WH, Fritts T. 2006 Venom of the brown treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. *Toxicon* **47**, 537–548. (doi:10.1016/j.toxicon.2006.01.007)
14. Smith TB. 1993 Disruptive selection and the genetic basis of bill size polymorphism in the African finch *Pyrenestes*. *Nature* **363**, 618–620. (doi:10.1038/363618a0)
15. Creer S, Malhotra A, Thorpe RS, Stocklin RS, Favreau PS, Hao Chou WS. 2003 Genetic and ecological correlates of intraspecific variation in pitviper venom composition detected using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and isoelectric focusing. *J. Mol. Evol.* **56**, 317–329. (doi:10.1007/s00239-002-2403-4)
16. Alape-Girón A, Sanz L, Escolano J, Flores-DiÃÅaz M, Madrigal M, Sasa M, Calvete JJ. 2008 Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *J. Proteome Res.* **7**, 3556–3571. (doi:10.1021/pr800332p)
17. Currier RB, Harrison RA, Rowley PD, Laing GD, Wagstaff SC. 2010 Intra-specific variation in venom of the African puff adder (*Bitis arietans*): differential expression and activity of snake venom metalloproteinases (SVMPs). *Toxicon* **55**, 864–873. (doi:10.1016/j.toxicon.2009.12.009)
18. Kaas Q, Westermann J-C, Craik DJ. 2010 Conopeptide characterization and classifications: an analysis using ConoServer. *Toxicon* **55**, 1491–1509. (doi:10.1016/j.toxicon.2010.03.002)
19. Chang D, Duda TF. 2012 Extensive and continuous duplication facilitates rapid evolution and diversification of gene families. *Mol. Biol. Evol.* **29**, 2019–2029. (doi:10.1093/molbev/mss068)
20. Duda TF, Palumbi SR. 1999 Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. *Proc. Natl Acad. Sci. USA* **96**, 6820–6823. (doi:10.1073/pnas.96.12.6820)
21. Abdel-Rahman MA, Abdel-Nabi IM, El-Naggar MS, Abbas OA, Strong PN. 2011 Intraspecific variation in the venom of the vermivorous cone snail *Conus vexillum*. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* **154**, 318–325. (doi:10.1016/j.cbpc.2011.06.019)
22. Rivera-Ortiz JA, Cano H, Marí F. 2011 Intraspecies variability and conopeptide profiling of the injected venom of *Conus ermineus*. *Peptides* **32**, 306–316. (doi:10.1016/j.peptides.2010.11.014)
23. Dutertre S, Biass D, Stöcklin R, Favreau P. 2010 Dramatic intraspecimen variations within the injected venom of *Conus* consors: an unsuspected contribution to venom diversity. *Toxicon* **55**, 1453–1462. (doi:10.1016/j.toxicon.2010.02.025)
24. Duda TF, Lessios HA. 2009 Connectivity of populations within and between major biogeographic regions of the tropical Pacific in *Conus ebraeus*, a widespread marine gastropod. *Coral Reefs* **28**, 651–659. (doi:10.1007/s00338-009-0485-9)
25. Duda TF, Terbio M, Chen G, Phillips S, Olenzek AM, Chang D, Morris DW. 2012 Patterns of population structure and historical demography of *Conus* species in the tropical Pacific. *Am. Malacol. Bull.* **30**, 175–187. (doi:10.4003/006.030.0116)
26. Duda TF, Chang D, Lewis BD, Lee TW. 2009 Geographic variation in venom allelic composition and diets of the widespread predatory marine gastropod *Conus ebraeus*. *PLoS ONE* **4**, e6245. (doi:10.1371/journal.pone.0006245)
27. Rambaut A. 2002 *Se-Al sequence alignment editor,* v. 2.0.a11. Oxford, UK: University of Oxford.
28. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739. (doi:10.1093/molbev/msr121)
29. Posada D. 2008 jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.* **25**, 1253–1256. (doi:10.1093/molbev/msn083)
30. Clement M, Posada D, Crandall KA. 2000 TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* **9**, 1657–1659. (doi:10.1046/j.1365-294x.2000.01020.x)
31. Excoffier L, Laval G, Schneider S. 2005 Arlequin (v. 3.0): an integrated software package for population genetics data analysis. *Evol. Bioinform.* **1**, 47–50.
32. Tamura K, Nei M. 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**, 512–526.
33. Lessios HA. 1992 Testing electrophoretic data for agreement with Hardy–Weinberg expectations. *Mar. Biol.* **112**, 517–523. (doi:10.1007/BF00356299)
34. Excoffier L, Smouse PE, Quattro JM. 1992 Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial-DNA restriction data. *Genetics* **131**, 479–491.
35. Tajima F. 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.
36. Fu YX. 1997 Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915–925.
37. Fu YX, Li WH. 1993 Statistical tests of neutrality of mutations. *Genetics* **133**, 693–709.
38. Librado P, Rozas J. 2009 DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452. (doi:10.1093/bioinformatics/btp187)
39. Nei M, Gojobori T. 1986 Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* **3**, 418–426.
40. Rousset F. 2008 GENEPOP'007: a complete reimplementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour.* **8**, 103–106. (doi:10.1111/j.1471-8286.2007.01931.x)
41. Raymond M, Rousset F. 1995 GENEPOP (v. 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249.
42. Hubisz MJ, Falush D, Stephens M, Pritchard JK. 2009 Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* **9**, 1322–1332. (doi:10.1111/j.1755-0998.2009.02591.x)
43. Huelsenbeck JP, Ronquist F. 2001 MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755. (doi:10.1093/bioinformatics/17.8.754)
44. Shannon CE. 1948 A mathematical theory of communication. *Bell Syst. Tech. J.* **27**, 623–656. (doi:10.1002/j.1538-7305.1948.tb00917.x)
45. Hurlbert SH. 1971 The nonconcept of species diversity: a critique and alternative parameters. *Ecology* **52**, 577–586. (doi:10.2307/1934145)
46. Oksanen J *et al*. 2012 Vegan: community ecology package. R package v. 2.0–3. See http://www.CRAN.R-project.org/package=vegan.
47. R Development Core Team. 2012 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
48. Whittaker RH. 1952 A study of summer foliage insect communities in the Great Smoky Mountains. *Ecol. Monogr.* **22**, 1–44. (doi:10.2307/1948527)
49. Pianka ER. 1974 Niche overlap and diffuse competition. *Proc. Natl Acad. Sci. USA* **71**, 2141–2145. (doi:10.1073/pnas.71.5.2141)
50. Kimura M. 1980 A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120. (doi:10.1007/BF01731581)
51. Ter Braak CJF. 1986 Canonical correspondence analysis: a new eigenvector technique for

multivariate direct gradient analysis. *Ecology* **67**, 1167–1179. (doi:10.2307/1938672)
52. Rodgers JL, Nicewander WA. 1988 Thirteen ways to look at the correlation coefficient. *Am. Stat.* **42**, 59–66. (doi:10.2307/2685263)
53. Spearman C. 1910 Correlation calculated from faulty data. *Brit. J. Psychol.* 1904–1920 **3**, 271–295.
54. Kendall MG. 1948 *Rank correlation methods*. Oxford, UK: Charles Griffin & Co.
55. Duda TF, Lee T. 2009 Ecological release and venom evolution of a predatory marine snail at Easter Island. *PLoS ONE* **4**, e5558. (doi:10.1371/journal.pone.0005558)
56. Schulze A. 2006 Phylogeny and genetic diversity of Palolo worms (*Palola*, Eunicidae) from the tropical North Pacific and the Caribbean. *Biol. Bull.* **210**, 25–37. (doi:10.2307/4134534)
57. Gotelli NJ, Ellison AM. 2013 EcoSimR 1.00. See http://www.uvm.edu/~ngotelli/EcoSim/EcoSim.html.
58. Schulze A, Timm L. 2012 Palolo and un: distinct clades in the genus *Palola* (Eunicidae, Polychaeta). *Mar. Biodivers.* **42**, 161–171. (doi:10.1007/s12526-011-0100-5)
59. Kohn AJ. 1978 Ecological shift and release in an isolated population: *Conus miliaris* at Easter Island. *Ecol. Monogr.* **48**, 323–336. (doi:10.2307/2937234)
60. Hayes W, Herbert S, Reeling G, Gennaro J. 2002 Factors that influence venom expenditure by vipers and other snakes during predatory and defensive contexts. In *Biology of the vipers* (eds GW Schuett, M Höggren, HW Greene), pp. 207–234. Traverse City, MI: Biological Sciences Press.
61. Barlow A, Pook CE, Harrison RA, Wüster W. 2009 Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proc. R. Soc. B* **276**, 2443–2449. (doi:10.1098/rspb.2009.0048)
62. Gibbs HL, Mackessy SP. 2009 Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* **53**, 672–679. (doi:10.1016/j.toxicon.2009.01.034)
63. Binford GJ. 2001 Differences in venom composition between orb-weaving and wandering Hawaiian Tetragnatha (Araneae). *Biol. J. Linn. Soc.* **74**, 581–595. (doi:10.1111/j.1095-8312.2001.tb01415.x)
64. Sasa M. 1999 Diet and snake venom evolution: can local selection alone explain intraspecific venom variation? *Toxicon* **37**, 249–252. (doi:10.1016/S0041-0101(98)00121-4)
65. Grant PR, Grant BR. 2002 Unpredictable evolution in a 30-year study of Darwin's finches. *Science* **296**, 707–711. (doi:10.1126/science.1070315)
66. Mayr E. 1963 *Animal species and evolution*. Cambridge, MA: The Belknap Press of Harvard University Press.
67. Borghans JAM, Beltman J, Boer R. 2004 MHC polymorphism under host-pathogen coevolution. *Immunogenetics* **55**, 732–739. (doi:10.1007/s00251-003-0630-5)
68. Hedrick PW. 2002 Pathogen resistance and genetic variation at MHC loci. *Evolution* **56**, 1902–1908. (doi:10.1111/j.0014-3820.2002.tb00116.x)
69. Slade RW, McCallum HI. 1992 Overdominant versus frequency-dependent selection at MHC loci. *Genetics* **132**, 861–864.
70. Spurgin LG, Richardson DS. 2010 How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proc. R. Soc. B* **277**, 979–988. (doi:10.1098/rspb.2009.2084)
71. Cammen K, Hoffman JI, Knapp LA, Harwood J, Amos W. 2011 Geographic variation of the major histocompatibility complex in Eastern Atlantic grey seals (*Halichoerus grypus*). *Mol. Ecol.* **20**, 740–752. (doi:10.1111/j.1365-294X.2010.04975.x)
72. Alcaide M, Edwards SV, Negro JJ, Serrano D, Tella JL. 2008 Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the lesser kestrel (*Falco naumanni*). *Mol. Ecol.* **17**, 2652–2665. (doi:10.1111/j.1365-294X.2008.03791.x)