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Ecological specialization is associated with genetic structure in the ant-associated butterfly family Lycaenidae

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The role of specialization in diversification can be explored along two geological axes in the butterfly family Lycaenidae. In addition to variation in host-plant specialization normally exhibited by butterflies, the caterpillars of most Lycaenidae have symbioses with ants ranging from no interactions through to obligate and specific associations, increasing niche dimensionality in ant-associated taxa. Based on mitochondrial sequences from 8282 specimens from 967 species and 249 genera, we show that the degree of ecological specialization of lycaenid species is positively correlated with genetic divergence, haplotype diversity and an increase in isolation by distance. Nucleotide substitution rate is higher in carnivorous than phytophagous lycaenids. The effects documented here for both micro- and macroevolutionary processes could result from increased spatial segregation as a consequence of reduced connectivity in specialists, niche-based divergence or a combination of both. They could also provide an explanation for the extraordinary diversity of the Lycaenidae and, more generally, for diversity in groups of organisms with similar multi-dimensional ecological specialization.

1. Background

‘What drives the diversification of life?’ is a fundamental question in biology. Although the role of long-term geographical isolation in giving rise to the divergence between allopatric populations is well known [1], the idea that speciation may also result from ecologically based divergent selection driving the evolution of reproductive isolation has seen a recent revival (e.g. ‘speciation-with-gene-flow’) [2]. For example, ecological specialization of conspecific herbivorous insects feeding on different host-plants (host races) [3] may enhance diversity within species [4] and promote the evolution of barriers to gene flow via host-associated differentiation [5]. The formation of host races appears to be associated with subsequent speciation and radiation in some cases [6] (but see [7,8]).

Rates of molecular evolution have been shown to accelerate in specialized parasitic [9,10] as well as mutualistic symbiont lineages [11,12] compared with generalists. However, theoretical predictions disagree as to how coevolution should affect rates of molecular evolution in those two cases. According to the ‘red queen hypothesis’, coevolving organisms must continuously adapt and

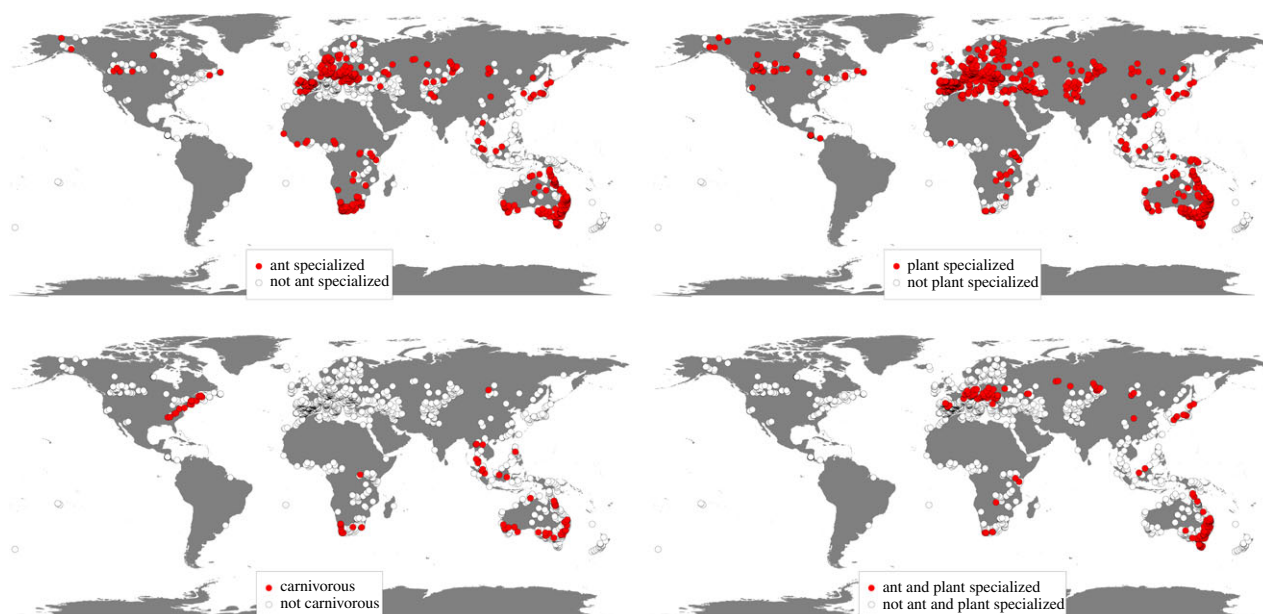


Figure 1. Distribution map of the 8282 samples of lycaenid butterflies used in this study grouped by their life-history strategy in regard to specialization. (Online version in colour.)

evolve in order to survive in an environment of ever-evolving antagonistic organisms if their interaction is locked [13,14]. Thus, accelerated rates of molecular evolution can be expected in antagonistic (parasitic) relationships. On the other hand, this is not always true in ‘red king’ scenarios involving mutualists where the symbiont evolving mutualistic traits more quickly could end up providing more help than it receives in return [15].

The butterfly family Lycaenidae is globally distributed and unusually diverse, particularly considering that it is among the youngest families of Lepidoptera (approx. 90 Myr old) [16]. The juveniles of many lycaenid species engage in symbiotic associations with ants (myrmecophily). Their caterpillars typically possess multiple, specialized ant-associated organs that form the basis for this association by appeasing ants that might otherwise be predators [17]. In return, the ants protect the caterpillars from natural enemies [18]. Degree of myrmecophily in the Lycaenidae ranges from no interactions, to obligate ones [19]. Most described lycaenid–ant interactions are facultative and appear to be mutualistic. These loose, facultative symbioses are not specific, and the caterpillars of a single lycaenid species can be found associating with up to 11 different species of ants [20].

About 30% of the Lycaenidae are obligately ant-associated in the sense that juveniles are never found without ants and often only associate with a single or closely related group of ant species [19]. Obligate lycaenid–ant symbioses can be mutualistic or parasitic. Parasitic caterpillars typically enter the ant nest where they either eat the brood directly or solicit the workers for food regurgitations (kleptoparasitism). Myrmecophilous and non-myrmecophilous species occur in most lycaenid subfamilies, although a few, such as the Lycaeninae and Lipteninae, are rarely ant associated [19]. The often specific interactions between lycaenids and ants add another dimension to the lycaenids’ ecological specialization beyond the host-plant associations of most Lepidoptera, providing a potential explanation for their disproportionately high diversity [21,22].

The hypothesis of ‘ecological islands’ [23,24] (suitable areas where host plants and ant associates intersect)

affecting genetic structure has been tested with data from 34 species of Lycaenidae collected in Romania [25]. Effective population sizes were found to be smaller in mutualistic species with a ‘high degree of myrmecophily’ compared with species with a ‘low degree of myrmecophily’ [25]: ant specialization may drive diversification in the Lycaenidae by decreasing effective population sizes. In addition, several studies have investigated the possibility of genetic differentiation between conspecific lycaenid populations associated with different ants, without finding any evidence [26–28].

Here we re-evaluate the possible association between genetic structure and myrmecophily, and more generally ecological specialization in the Lycaenidae, using a substantially larger and more representative dataset than has been assembled before. We explore the hypothesis that host-ant and host-plant specialization, as well as dual specialization on ants and plants simultaneously and carnivory, are associated with both micro- and macroevolutionary patterns.

2. Methods

A total of 8282 samples representing eight subfamilies, 249 genera and 967 species of Lycaenidae from all over the world were analysed (figure 1; electronic supplementary material, table S1). The degree of specialization on host ants and host plants for each species is shown in electronic supplementary material, table S2. Phylogenetic relationships between species based on the within-species consensus of the mitochondrial gene cytochrome oxidase subunit I (COI) are shown in electronic supplementary material, figure S1. Publicly available COI sequences for the family Lycaenidae were downloaded from the Barcoding Of Life Data Systems (BOLD) or GenBank. Additionally, 707 samples were sequenced to complete the dataset for underrepresented species. DNA extraction, PCR reactions and sequencing were similar to the conditions described in [29] using the primer pair ‘LCO1490’ (forward) [30] and ‘Nancy’ (reverse) [31]. GENEIOUS v. 6.1.2 (created by Biomatters; <http://www.geneious.com/>) was used for assembly of chromatograms and to edit alignments. A maximum-likelihood phylogenetic tree for the investigated species (electronic supplementary material, figure S1) was obtained based on the majority-rule consensus DNA barcodes for each species using

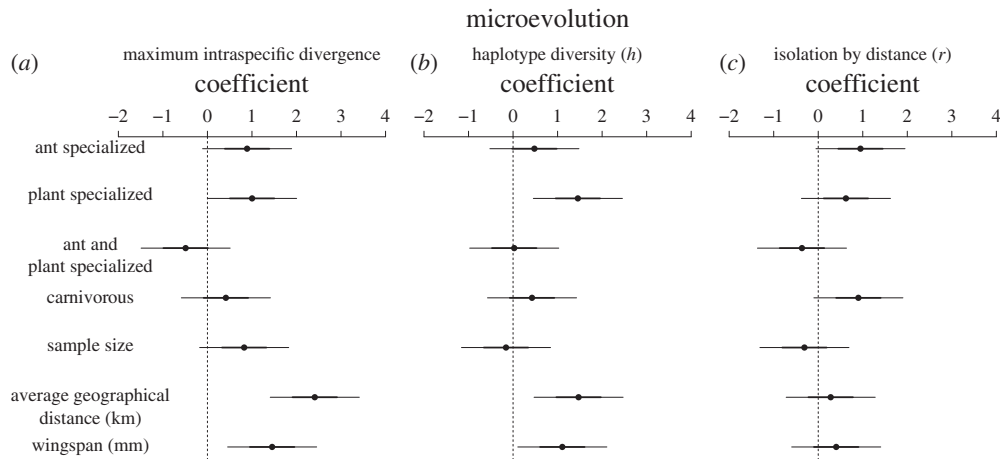


Figure 2. Coefficient plots from the three generalized linear mixed models investigating microevolution using the full dataset. The response variables are maxima of intraspecific genetic divergence (a), haplotype diversity, h (b) and IBD regression coefficients, r (c). Coefficients adjusted for comparison by division by two s.d. are shown for explanatory variables included in the models (mean: dots, standard deviations, s.d.: bold lines, 2s.d.: thin lines).

the program RAxML [32] through the raxmlGUI v. 1.5b1 interface [33] using the GTR + G model of sequence evolution with 100 bootstraps. Monophyly of subfamilies and their relationships were constrained according to [34]. Species for which at least five sequenced specimens with published sampling location existed were selected for microevolutionary analysis of intraspecific lineage accumulation. Location descriptions lacking coordinates were geo-referenced (Google Maps). Degree of ant specialization was quantified using a binary categorical predictor with the levels 'ant specialized' (obligate association with ants) and 'not ant specialized' (not obligatorily associated with ants), based on literature records and expert opinions (electronic supplementary material, table S1). Plant specialization was similarly used as a binary categorical variable with the levels 'plant specialized' (feeding on only one plant genus) and 'not plant specialized' (feeding on more than one plant genus). To investigate a third type of specialization, 'carnivorous' (defined here as strictly carnivorous during all larval stages) versus 'not carnivorous', diet was included as a binary categorical predictor, based on literature records (electronic supplementary material, table S1), the Lepidoptera host plant database of the Natural History Museum (<http://www.nhm.ac.uk/our-science/data/hostplants/>) and expert opinions (electronic supplementary material, table S2; Acknowledgements). As a possible confounder, body size (measured as wingspan, based on data published in field guides) was included (electronic supplementary material, table S2). Associations between levels of specialization and multiple genetic parameters, related to both micro- and macroevolution processes, were analysed.

(a) Microevolution

Matrices of divergence (K80 model of DNA evolution [35]) between all combinations of individual sequences and haplotype diversity [36] were calculated for species using the R packages 'ape' [37], 'adephylo' [38] and 'Biostrings' [39] in R v. 3.2.2 [40]. Average geographical distances between samples were calculated per species using the 'sp' package [41]. Associations between measures of intraspecific lineage accumulation (maximum divergence and haplotype diversity, h), and specialization categories were analysed using generalized linear mixed models in the R package 'MCMCglmm' [42]. Maximum divergence (square root transformed) and h were included as independent, Gaussian variables. Ant and plant specialization categories, double specialization (on ants and plants), carnivory, sample size and average geographical distance between samples of the same species were

included as fixed effects. Phylogeny was included as a random effect (as a phylogenetic covariance matrix [43]). Possible correlations between significant predictors were assessed using Pearson's product-moment correlation. There was a significantly positive correlation between body size and specialization (Pearson's product-moment correlation: $t_{129} = 2.9$, $p = 0.004$), but not between body size and average geographical distance (Pearson's product-moment correlation: $t_{129} = 1.24$, $p = 0.217$). Isolation by distance (IBD) was investigated for each species using Mantel tests comparing matrices of pairwise genetic distances (K80 model of DNA evolution [35]) between individuals (R package 'Biostrings' and 'ape') with their geographical distances (R package 'sp') using the R package 'adeget' [38]. The association between the resulting correlation coefficients (r) and specialization was investigated in 'MCMCglmm', with Gaussian residual distribution. The dependent variable r was predicted by the fixed effects wingspan, average geographical distance, sample size, carnivory, ant and plant specialization, and their interaction. Phylogeny was included as a random effect.

(b) Macroevolution

Molecular substitution rate was analysed as the branch length of each species (tip) to the root, extracted from the ML phylogeny described above. Branch lengths to root were log-transformed for the analysis in order to approach a normal distribution. Ant- and plant specialization, their interaction, carnivory and average geographical distance between samples were included as fixed predictors, and a phylogeny without branch lengths was included as a random effect.

All MCMCglmm models were run for 1.3×10^6 iterations with a burn-in period of 3×10^5 iterations (thinning interval: 500 iterations). In order to investigate a possible bias introduced by unequal sample sizes, models for analysis of microevolutionary processes were repeated with average values of each parameter obtained from 10^3 iterations of a random selection of five sequences per species. In addition, all models were repeated comparing the subset of mutualistic obligate ant symbionts to parasitic ones. The R scripts used are available in electronic supplementary material, appendix S1.

3. Results

Species whose larvae specialize on plants had higher maxima of intraspecific genetic divergence than their

Table 1. Results from generalized linear mixed models using the 'MCMCglmm' R package [42] with 1.3×10^6 iterations, and a burn-in period of 3×10^5 iterations. Phylogeny (electronic supplementary material, figure S1) was included as a random effect in all models. Significant *p*-values are printed in italics.

response variable	fixed effects	posterior mean	lower 95% confidence limit	upper 95% confidence limit	effective sample size	MCMC <i>p</i> -value	
microevolution							
maximum divergence, all samples	ant specialization	0.02982	0.00118	0.06437	2000	0.063	
	plant specialization	0.01937	0.00142	0.03820	2000	<i>0.043</i>	
	ant specialization and plant specialization	-0.02669	-0.07753	0.02466	2151	0.318	
	carnivory	0.02118	-0.02427	0.07353	2000	0.424	
	average geographical distance between samples	0.00002	0.00001	0.00003	2510	< <i>0.001</i>	
	sample size	0.00009	-0.00002	0.00020	1836	0.103	
	body size (wingspan mm)	0.00164	0.00059	0.00273	2000	<i>0.004</i>	
	obligate ant parasites versus obligate ant mutualists	0.00179	-0.08052	0.07748	2000	0.955	
	average geographical distance between samples	0.00003	-0.00001	0.00007	2000	0.064	
	sample size	0.00006	-0.00041	0.00056	2000	0.827	
	body size (wingspan mm)	0.00229	-0.00095	0.00572	2000	0.164	
	haplotype diversity (<i>h</i>), all samples	ant specialization	0.07692	-0.08681	0.22820	1741	0.326
		plant specialization	0.13150	0.03575	0.22000	2000	<i>0.008</i>
		ant specialization and plant specialization	0.01256	-0.23530	0.27780	1904	0.920
carnivory		0.09692	-0.12020	0.32950	2000	0.394	
average geographical distance between samples		0.00006	0.00002	0.00010	2000	<i>0.003</i>	
sample size		-0.00010	-0.00063	0.00045	2000	0.732	
body size (wingspan mm)		0.00617	0.00079	0.01159	2215	<i>0.025</i>	
obligate ant parasites versus obligate ant mutualists		-0.04421	-0.32710	0.24460	2548	0.724	
average geographical distance between samples		0.00005	-0.00007	0.00019	2000	0.448	
sample size		-0.00066	-0.00241	0.00119	2085	0.456	
body size (wingspan mm)		0.00249	-0.00893	0.01324	2000	0.624	
IBD coefficient (<i>r</i>)		ant specialization	0.19730	-0.01266	0.39530	2000	0.063
		plant specialization	0.08445	-0.02880	0.22410	2000	0.191
		ant specialization and plant specialization	-0.13190	-0.47370	0.26230	2333	0.476
	carnivory	0.25710	-0.02395	0.52020	1763	0.072	
	average geographical distance between samples	0.00002	-0.00004	0.00007	1827	0.595	
	sample size	-0.00024	-0.00101	0.00054	2000	0.540	
	body size (wingspan mm)	0.00347	-0.00400	0.01189	2367	0.381	
	obligate ant parasites versus obligate ant mutualists	-0.10310	-0.42890	0.24340	2191	0.521	
	average geographical distance between samples	0.00005	-0.00010	0.00021	2000	0.504	
	sample size	-0.00103	-0.00302	0.00100	1768	0.312	
	body size (wingspan mm)	0.00403	-0.00899	0.01730	2295	0.518	
	macroevolution						
	molecular substitution rate (branch length to root)	ant specialization	-0.01577	-0.05338	0.02656	2000	0.460
		plant specialization	0.01510	-0.00853	0.03656	2000	0.210
ant specialization and plant specialization		0.05319	-0.00406	0.10790	1521	0.067	
carnivory		0.09501	0.02730	0.16563	2000	<i>0.012</i>	

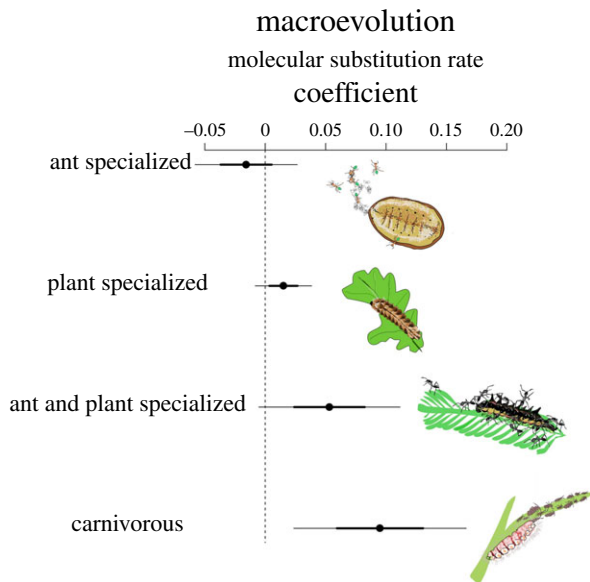


Figure 3. Coefficient plot from the generalized linear mixed model investigating macroevolution using the full dataset. The response variable is molecular substitution rate (branch length). Coefficients are shown for explanatory variables included in the model (mean: dots; standard deviations, s.d.: bold lines, 2 s.d.: thin lines). (Online version in colour.)

generalist counterparts, and a similar trend was found for ant specialized and carnivorous species (figure 2*a* and table 1; electronic supplementary material, appendix S2, table S3). Maximum divergence also increased with average geographical distance and body size (table 1 and figure 2*a*). The species mean for maximum divergence was 0.015 ± 0.014 (s.d.), ranging between 0 and 0.086. With the mean of 1000 iterations of five randomly chosen specimens per species, all results were consistent with results obtained from the full dataset (electronic supplementary material, appendix S2, table S3). Haplotype diversity was higher for species with a high degree of plant specialization than for generalist plant feeders (table 1). Haplotype diversity was also correlated with average geographical distance and body size (table 1). Average h was 0.592 ± 0.274 , ranging between 0 and 0.969. Results for h were equivalent to those obtained for the full dataset when five randomly selected sequences per species were used (mean of 1000 iterations; electronic supplementary material, appendix S2, table S3). There was a trend of regression coefficients obtained in Mantel tests for IBD being higher in carnivorous than in non-carnivorous species, and in ant-specialized compared with not ant-specialized species (table 1 and figure 2*c*). Average geographical distance between conspecific individuals was also not correlated with the strength of IBD of that species (table 1). The average for r was 0.398 ± 0.351 ranging between -0.328 and 1. When five specimens were randomly selected per species (1000 iterations), the results were similar to those obtained in the analysis of all specimens (electronic supplementary material, appendix S2, table S3). None of the investigated microevolutionary response variables differed significantly between species whose larvae are either obligate mutualists or parasites of ants (table 1). Molecular substitution rate (branch length) was significantly higher in carnivorous compared with non-carnivorous species (table 1 and figure 3).

4. Discussion

We found a positive association between genetic structure and ecological specialization. Species' maximum genetic divergence and h are higher in lycaenids that are specialists on host-plants compared to generalists (figure 2*a*). Alternatively, specialization may be promoted in species with increased genetic structure (due to dispersal limitation, fragmentation, etc.). These results could reflect reduced gene flow among populations due to decreased dispersal, stronger fragmentation and/or disruptive selection in specialists. Decreased dispersal in specialists may result from selection due to coevolution with local ant/plant associates. That is, selection may favour symbionts specializing on local partners because of increased fitness benefits of co-adaptation. Disruptive effects of distance and/or biogeographical barriers may be magnified in specialists because of fewer 'stepping stones' (suitable habitat patches), limiting dispersal. Disruptive selection in species specializing on different host plants in sympatry is known for phytophagous insects [44]. Such host races could exist within butterfly species living in close association with different ant species. Here we found that h was indeed higher in plant specialists than generalists (figure 2*b* and table 1). However, patterns consistent with IBD increased in carnivores, suggesting stronger spatial segregation in these species, and there was a general trend of stronger IBD patterns with increased specialization (figure 3). This suggests that diversification is not driven purely by ant specialization in sympatry, but that distance or biogeographic barriers play a role. Certain highly specialized lycaenid species have been shown to use ants themselves as cues in egg-laying, a mechanism that could potentially produce an amplification of their host-plant range [45,46]. This could in turn lead to greater opportunities for diversification if different host-plants occupy different ecological niches.

Molecular substitution rate (branch length from tip to root in the species tree) increased significantly in strictly carnivorous species, but not in moderately specialized species (on only ants or plants; figure 3). Thus, the effects at the macroevolutionary scale (rate of molecular evolution) were only visible in extreme specialists (carnivorous). Nevertheless, at least in the most extreme cases, the microevolutionary mechanisms documented here seem to be translated to macroevolutionary scales. Accelerated rates of molecular evolution have previously been documented in parasites, and this phenomenon has been explained by their typically small population sizes [9,10].

Specialists could be more susceptible to fragmentation and reduced population sizes than generalists during broad-scale environmental changes such as climatic oscillations. It is frequently argued that specialist species are more likely to go extinct due to narrower and more complex habitat requirements (specialization as an evolutionary dead end; but see [47,48]). However, interactions involving that are simultaneously specialized lycaenids with respect to both attendant ants and host plants simultaneously were not significantly correlated with divergence, h and r (table 1). Perhaps these dual specialists have reached a point of ecological refinement where they are simply constrained from further population subdivision.

In summary, increased genetic structuring of specialists could be a result of smaller population sizes, reduced dispersal, ant-induced host-plant shifts, divergent selection on

different ecological niches, relaxed selection or any combination of these factors. The assessment of ecological niche width and its translation to total range and connectivity of habitats in geographical space and time (the factors potentially linked to genetic population structure) are difficult. Moreover, the dimensions analysed here for life-history specialization do not cover those species that specialize in additional dimensions. Future comparative studies, including detailed host use data (for both ants and plants) at a population level for a large number of species with different degrees of specialization, may help to identify mechanisms leading to the observed increase in genetic divergence in specialists. Comparative analyses at an intraspecific level shed light on microevolutionary processes that may give rise to macroevolutionary patterns of diversification. Our results support the importance of ecological specialization in general, and of species interactions in particular, in generating biodiversity.

Ethics. This study has been conducted using published DNA sequences or collection material that belongs to the Museum of Comparative Zoology, and procedures have been subject to relevant institutional oversight.

Data accessibility. All primary data are accessible via the electronic supplementary material, GenBank or BOLD (see electronic

supplementary material, table S1 for individual GenBank Accession numbers and BOLD access codes).

Authors' contributions. N.E.P., R.V., R.E., D.R.N. and S.S. conceived of the study; N.E.P., R.V., R.E., S.S., A.K. and M.E. contributed with DNA material; S.S., K.G.A., A.K., J.H.B. and M.E. performed laboratory work; S.S., G.T. and D.R.N. analysed data; all authors contributed to writing the manuscript.

Competing interests. We declare we have no competing interests.

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