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Integrating biogeographic and genetic approaches to investigate the history of bioluminescent colour alleles in the Jamaican click beetle, *Pyrophorus plagiophthalamus*

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Abstract

Bioluminescent colour in the Jamaican click beetle, *Pyrophorus plagiophthalamus*, is an ideal system for studies moving from gene to landscape to gain a holistic understanding of the molecular, ecological, and historical bases for adaptation. Previous studies have established the genetics of bioluminescent colour variation in the beetle to the level of the nucleotide base pair in the target gene *luciferase*. Three different *luciferase* colour alleles affecting ventral light organ colour [yellow-green (vYG), yellow (vYE), and orange (vOR)] were found segregating in *P. plagiophthalamus* populations. These alleles differ from each other in a number of replacement mutations (14 total), the majority of which (11) have a measurable effect on colour. Phylogenetic analysis revealed a long-term adaptive trend on Jamaica towards longer wavelength bioluminescence, culminating in the most recently derived vOR allele. Here, we further investigate the historical and geographic context of adaptive colour evolution by testing a vicariance model for the origins of the extant ventral light organ polymorphism: that the vOR allele arose and differentiated in an isolated deme on the east side of Jamaica before spreading westward. Comparisons of colour phenotypes, *luciferase* coding sequences, the third intron of the gene, mtDNA, and microsatellite data provided evidence for past population subdivision on Jamaica and ongoing gene flow, as has been found for other island endemics. However, the pattern of differentiation supported the allopatric divergence of vYG and vYE alleles. The vOR gene appears to have arisen relatively recently from a vYE precursor and postdates the period of major biogeographic isolation. We discuss the implications of the results for discerning ecological causation in the adaptive sequence from nucleotide to landscape to population change for bioluminescent colour.

Keywords: biogeography, Caribbean click beetle, gene flow, microsatellite, population structure, *Pyrophorus plagiophthalamus*

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Introduction

Recent advances in linking gene products with their phenotypic function have opened new vistas for the study of biological cause and effect (Sinclair *et al*. 2003; Tittiger 2004). In particular, these advances are helping address a central problem of molecular ecologists: the identification and characterization of the nucleotide substitutions that matter in nature (Hoffmann *et al*. 1995; Lewontin 2002; Via 2002).

Much work has relied on comparative population and phylogenetic approaches to discern the molecular 'footprint' of selection (Kreitman & Hudson 1991; Kreitman & Akashi 1995; Graur & Li 2000). Statistical analysis of patterns of genetic variation through time and space have been used to identify nucleotide sites deviating from neutral expectations, implying the action of natural selection (Graur & Li 2000). When these data were coupled with information on protein structure (e.g. the locations of amino acids comprising active, binding and/or recognition sites), the case for adaptive significance was strengthened

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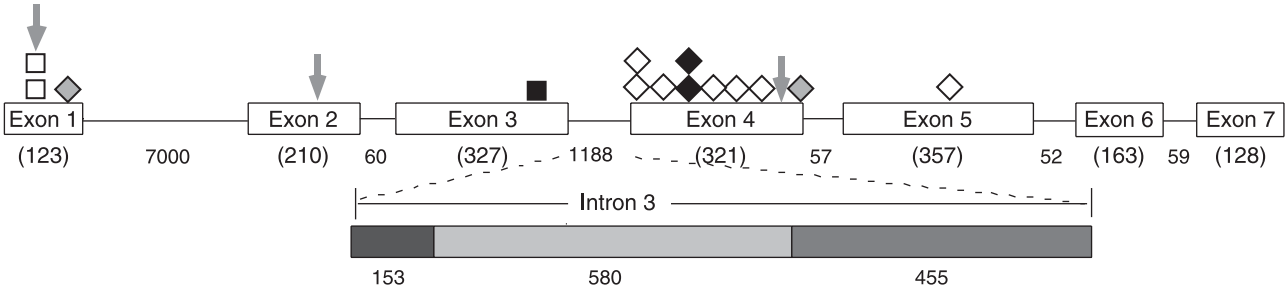

Fig. 1 Structure of *Pyrophorus plagiophthalamus* ventral *luciferase* locus. Shown are the seven exons and six introns comprising the gene along with their sizes (in base pair; exon lengths are given in parentheses). The third intron is enlarged to highlight flanking regions shared by dorsal and ventral loci (darker shaded region), as well as central insert unique to *P. plagiophthalamus* (grey shaded region). Sequence analysis was based on the second shared intron region (455 bp indicated by the second dark shaded box). Arrows designate synonymous substitutions, and boxes and diamonds nonsynonymous substitutions, in the ventral locus of the Jamaican click beetle. Diamonds represent mutations having a measurable effect on bioluminescent colour, as determined by spectrophotometer analysis. The shading of the symbol indicates whether the mutation is the derived state in the yellow-green (grey), yellow (white), or orange (black) clade of *P. plagiophthalamus* colour alleles. Note that 10 of the 11 derived mutations affecting colour resulted in a shift towards longer-wavelength light (only exception was the change at base pair position 121 for vYG), supporting an evolutionary trend towards more orange-coloured bioluminescence on Jamaica.

(Hughes & Nei 1988, 1992). However, it is still rare to find instances where specific mutations' phenotypic and natural-population fitness effects have been precisely determined or quantified (Watt 1992; Feder 1999; Kingsolver *et al*. 1999; Watt *et al*. 2003). The main body of research has instead focused on resolving population genetic issues of the relative importance (frequency of action) and intensity of natural selection at the molecular level (Kreitman *et al*. 1991, 1995). The biological basis for selection was presumed from the nature of the genetic sequences or codons being analysed, but usually not directly tested. Consequently, our understanding of the successive steps in natural selection (the 'adaptive recursion') moving from nucleotide, to gene, to phenotype, to ecology, to differential survival (fitness), to evolutionary change in populations is generally incomplete for any given system.

From an ecological perspective, the problem has been the reverse: a difficulty in moving to the level of the gene. Tests of selection in the wild have established fitness differences for several biologically relevant traits (e.g. body size, colour, beak shape) (Endler 1986; Feder 1999; Watt *et al*. 2003). While these studies have greatly added to our understanding of natural history and the link of ecology in the adaptive chain of events, most of the traits are polygenic and influenced by multiple loci, making detailed gene mapping from phenotype via development daunting (Keller *et al*. 2001; Grant & Grant 2002).

Advances in molecular genetic and genomic techniques are making the entire adaptive recursion more accessible to study. This is due, in part, to an increased ability to reverse the investigatory sequence of the problem and to the delivery of the power of the molecular genetic tool kit into the hands of the practicing field ecologist. Researchers have traditionally chosen characters for study based on their relevance to natural history and worked outward to investigate ecological causation. Now the increased ease to study gene function provides the opportunity to initially identify interesting suites of traits that are both genetically and developmentally tractable to verify the action of natural selection at the molecular level before proceeding to ecological hypothesis testing. This strategy alleviates a major criticism of what was called the 'adaptationist program' by Gould & Lewontin (1979): just-so natural selection story telling.

Bioluminescent colour in the Jamaican click beetle, *Pyrophorus plagiophthalamus* (Coleoptera: Elateridae), provides an opportunity to study the entire adaptive sequence from gene to landscape. The genetics of bioluminescence in these beetles are known to the level of the individual nucleotide (Wood *et al*. 1989; Wood 1990, 1995; Viviani & Bechara 1997; Viviani *et al*. 1999; Stolz *et al*. 2003). Molecular analysis has also provided strong evidence for selection acting on *luciferase* (Fig. 1), the gene controlling bioluminescent colour in *P. plagiophthalamus*. In particular, sequence data imply that a series of selective substitutions has occurred in the *luciferase* gene affecting ventral light organ colour, producing a long-term adaptive trend on Jamaica favouring longer wavelength (more orange-coloured) bioluminescence (Stolz *et al*. 2003). However, the ecological and demographic bases for colour selection are unresolved. Questions also remain concerning the biogeographic context in which the adaptive colour shift towards orange has occurred.

Three different ventral colour alleles segregate in *P. plagiophthalamus* (yellow-green, yellow, and orange) distinguishable from one another by a number of fixed replacement differences. The pattern of extensive molecular differentiation, unbroken by recombination, suggests

that the *luciferase* colour alleles may have originally evolved in allopatry. Under this biogeographic scenario, selection favouring longer-wavelength emission initially occurred in a reproductively isolated subdeme in Jamaica leading to the most recently derived orange allele. Following subsequent contact, the orange allele began spreading across the island, generating the current colour polymorphism in *P. plagiophthalamus*. Support for this vicariance hypothesis comes from the observation that the orange allele (as well as the yellow-green) is most prevalent on the east side of the island, and rare in the west.

Here, we test the biogeographic hypothesis for colour polymorphism in *P. plagiophthalamus* through a comparative analysis of different classes of nuclear and mitochondrial genetic markers. Our goal is to equate ventral *luciferase* gene levels of differentiation with other potential molecular indicators of population divergence, in an attempt to gain insight into the timeframe and geographic context of bioluminescent colour evolution. Such an approach represents an important component of a general research strategy linking genes to landscape in the wild, leading to the ultimate objective of discerning the ecology and demography responsible for orange-colour selection.

Materials and methods

The study system

Pyrophorus plagiophthalamus is a member of a Caribbean, Central, and South American distributed genus containing 26 described species (Costa 1976). *Pyrophorus* beetles bioluminesce at night, using their light as a species recognition cue in mating much like fireflies. However, unlike fireflies, male *P. plagiophthalamus* beetles remain continuously lit for several seconds while in flight, bioluminescing from a ventral light organ located in the abdominal cleft and do not flash (Fig. 2A; Costa 1976; Colepicolo-Neto *et al*. 1986; Bechara 1988). Our field observations suggest that receptive *P. plagiophthalamus* females respond to males from a pair of dorsal light organs in the posterior pronotum (Fig. 2B). There is no sexual dimorphism in *P. plagiophthalamus*. Both sexes have functional dorsal and ventral organs. However, the various *Pyrophorus* species do differ from one another in their ventral organ light colour, ranging from green to orange. The male mating signal (ventral light colour) has therefore evolved repeatedly in the genus, despite an apparent lack of intraspecific variation. Dorsal organ colour (the female mating and general startle response signal for beetles) is predominantly green. Like the ventral organ, there is little, if any, intraspecific variation for dorsal light colour. The exception is *P. plagiophthalamus*, which displays striking colour polymorphisms for both its dorsal and ventral organs. Individuals differ in their dorsal light colour from green to yellow green and in ventral light colour from yellow-green, to yellow, to orange. As such, *P. plagiophthalamus* represents the only known example of colour polymorphism for a bioluminescent organism.

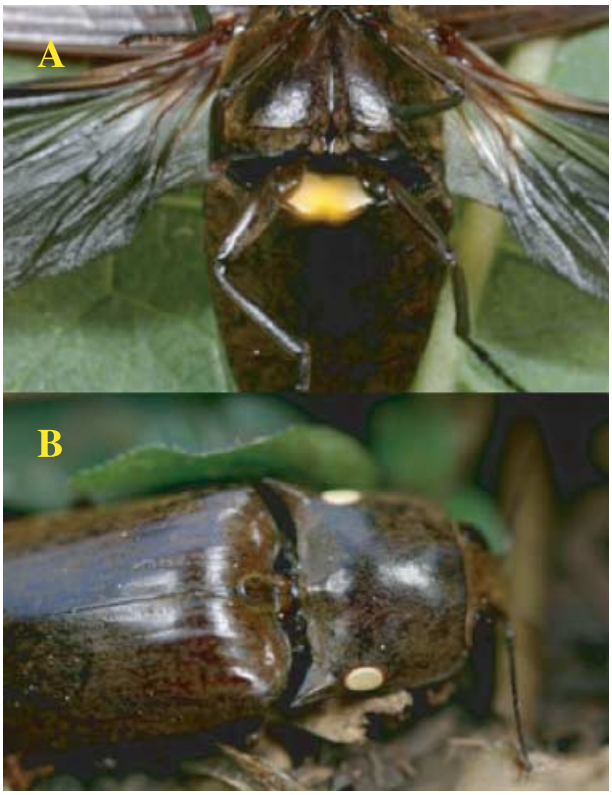

Fig. 2 (A) Ventral bioluminescent light organ. This organ is only seen while the beetle is in flight (i.e. elytra open). Photo was taken by manually exposing the organ and agitating the specimen. (B) Paired dorsal light organs.

The genetic basis for colour polymorphism in *P. plagiophthalamus* has been established and involves specific amino acid substitutions in the *luciferase* gene (Wood *et al*. 1989; Stolz *et al*. 2003). The ability to clone, sequence, and spectrophotometrically measure the phenotypic expression of cloned beetle *luciferase* genes in bacteria has resulted in (i) precise characterization of the effects that specific nucleotide substitutions (both natural variants and laboratory-induced mutations) have on bioluminescent colour, (ii) establishment of separate *luciferase* loci controlling dorsal and ventral colour, and (iii) identification of different colour classes of dorsal and ventral *luciferase* alleles segregating in beetle populations [dorsal locus: green (dGR) and yellow-green (dYG) alleles; ventral locus: yellow-green (vYG), yellow (vYE), and orange (vOR) alleles]. Phylogenetic analysis has shown that the ventral locus on Jamaica has accumulated a series of 14 replacement mutations compared to three synonymous mutations (Fig. 1). The pattern of *luciferase* molecular evolution implied that a long-term adaptive trend has occurred in *P. plagiophthalamus*

towards longer wavelength (more orange-coloured) ventral light (Stolz *et al*. 2003). Moreover, these data suggested that the vOR allele is most recently derived and deterministically increased in frequency. The derived orange allele may therefore constitute the latest chapter in the evolution of longer wavelength bioluminescence on Jamaica, with the extant polymorphism reflecting an adaptive colour shift in progress.

Genetic markers

Four different categories of genetic markers were used to assess the population structure of *P. plagiophthalamus* and test the biogeographic hypothesis for bioluminescent colour evolution. These markers included (i) a 1165-bp sequence of the coding region of the ventral *luciferase* gene (exons 1–4 and the first half of exon 5; Fig. 1), (ii) a 948-bp portion of the third intron of the ventral *luciferase* gene (Fig. 1), (iii) nine GT-dinucleotide repeat microsatellite loci, and (iv) a 494-bp region of the mitochondrial encoded cytochrome oxidase I (COI) gene. The coding regions of the *luciferase* gene provided information concerning the genealogical relationship of colour alleles and their degree of differentiation. The intron was analysed to gain a clearer understanding of colour allele differentiation and age for a putatively noncoding, neutral, and genetically and genealogically linked region embedded within the coding portions of the *luciferase* gene. Microsatellites were scored to provide a general assessment of the degree of nuclear genome differentiation on the island, while mtDNA yielded information from a haploid and maternally inherited gene that might give insight into possible differences in male vs. female migration patterns.

Beetle collection and spectrophotometry

Beetles were collected at night at three different locations on Jamaica in the spring of 1996, 1997, and 2004 (see Fig. 3A for map of Ecclesdown, Kingston, and Windsor Caves study sites and Supplementary material tables for sample sizes). *In vivo* spectral emission was quantified for dorsal and ventral light organs using a portable spectrophotometer (Spectra-Match GT, CVI Laser). The tip of the fibre optic input cable was held flush to a beetle's organ, and the individual was gently agitated to induce bioluminescence. Spectral emission curves were recorded for a minimum of three trials per beetle on a computer using the manufacturer's software. A smoothing function was applied to each curve to determine peak emission wavelength. Previous studies have shown a 1:1 relationship between colour phenotype and genotype (as determined by cloning of expressed *luciferase* mRNA) for the ventral light organ (Stolz *et al*. 2003). Estimates of ventral *luciferase* allele frequencies in populations were therefore derived from ventral light organ phenotypes assuming beetles bioluminescing in the range of 558–560 nm to be vYG/vYG homozygotes, 563–570 nm to be vYG/vYE heterozygotes, 573–582 nm to be vYE/vYE homozygotes, 584–588 nm to be vYE/vOR heterozygotes, and 591–596 nm to be vOR/vOR homozygotes. Following spectral recording, beetles were marked, placed in separate plastic vials, and frozen in dry ice for later genetic analysis.

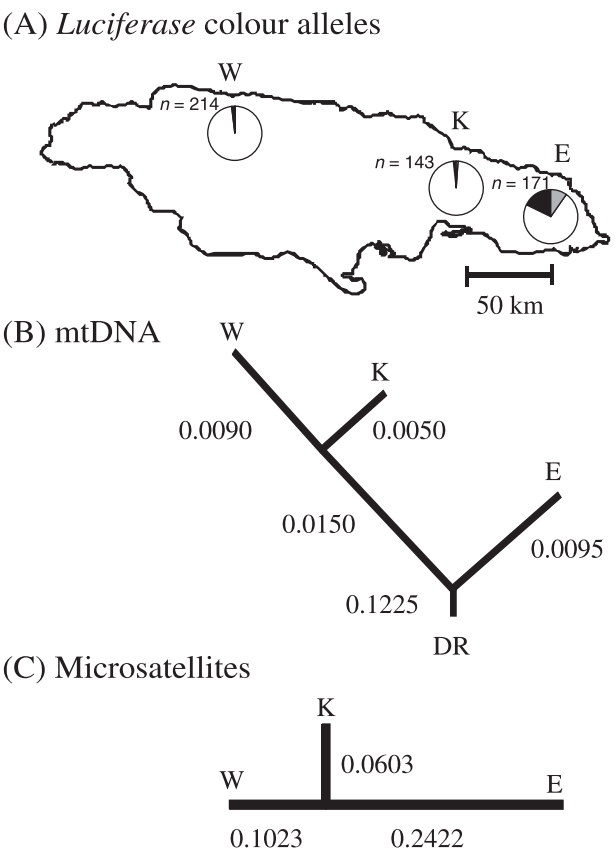

Fig. 3 (A) Frequencies of *luciferase* colour alleles [yellow-green (grey shading), yellow (white), and orange (black) coloured proportions of pie diagrams] estimated for Jamaican click beetle populations at Ecclesdown (E), Kingston (K), and Windsor Caves (W) collecting sites. Frequencies represent pooled totals for 1996, 1997, and 2004 samples. See Table S1-A for breakdown of allele frequencies at sites for each year. (B) Neighbour-joining mtDNA network (COI gene) for Jamaican beetle populations constructed from pairwise Tamura–Nei genetic distance estimates. Network is rooted using the beetle *Pyrophorus mellifluus* from the Dominican Republic (DR) as an outgroup. Gene tree for mtDNA is given in Fig. 4B and haplotype frequencies for sites are provided in Table S1-B. (C) Unrooted neighbour-joining network for Jamaican populations derived from nine microsatellite loci based on pairwise Nei genetic distances estimated across loci. See Table S2 for microsatellite allele frequencies.

Luciferase *gene cloning and sequencing*

Details of the cloning methodology used to generate sequence data for the coding and intron regions of the ventral *luciferase* gene are described in Stolz *et al*. (2003). *Luciferase* clones

were generated for individual *P. plagiophthalamus* beetles from the 1997 field sample in two different ways (i) Reverse transcription–polymerase chain reaction (RT–PCR) of expressed mRNA isolated from each beetle's dorsal and ventral organs, and (ii) PCR of genomic DNA isolated from the heads of the same specimens. Comparisons of cDNA and genomic sequences from the same individual were initially used in Stolz *et al*. (2003) to deduce the genotypes of beetles and to establish the existence of separate dorsal and ventral *luciferase* locus. Here, mRNA-generated sequences from the ventral light organ were used to assess ventral *luciferase* coding variation, while genomic clones formed the basis for analysis of the third intron. Genomic intron clones included a 107-bp segment of the fourth exon of the *luciferase* gene, which was used to distinguish dorsal from ventral and colour allele associations for intron sequences. During the course of analysis, it was found that the ventral third intron sequence of *P. plagiophthalamus* contained a unique 580-bp insert not found in the dorsal *luciferase* gene or ventral introns of other *Pyrophorus* species. The presence of the insert provided an additional basis for distinguishing ventral genomic *P. plagiophthalamus* intron clones from dorsal sequences. However, due to the unique nature and uncertain origin of the insert, we limited phylogenetic analysis of the third intron to a 455-bp shared region flanked by the insert and fourth exon (Fig. 1).

cDNA clones (10 per organ) and genomic DNA clones (10–12 per individual) were sequenced in both the 5′ and 3′ directions on an ABI 3700 automated DNA analyser (Applied Biosystems) using the BigDye Terminator version 3.1 kit. Bases were automatically called by SEQUENCING ANALYSIS version 3.7 software (Applied Biosystems). Only mutations that were present in more than one clone per individual and in both 5′ and 3′ sequencing directions were included for statistical and phylogenetic analyses. GenBank Accession nos for the *luciferase* coding sequences are AF543372, AF543378, AF543399, AF543402, AF543413, AF545853, and AF545854 and for the intron sequences are DQ092643–DQ092701.

Mitochondrial DNA gene cloning and sequencing

A 495-bp fragment of the mtDNA COI gene was cloned and sequenced from total genomic DNA isolated from 15 beetles each from Ecclesdown, Kingston and Windsor Caves (1997 sample) using PUREGENE DNA Purification Kits (Gentra Systems). Mitochondrial DNA sequences were PCR amplified using 5′-CTCCCAGGATTTGGAATGATC-3′ forward and 5′-TCTTAGAGGAATGCCACGAC-3′ reverse primers, and HotMaster *Taq* polymerase (Brinkmann Instruments), following the manufacturer's instructions and with supplied buffers. Thermocycler conditions were 3 min at 94 °C, 1 min 56 °C, and 1 min 68 °C for 1 cycle; 1 min 94 °C, 1 min 56 °C, 1 min 68 °C for 35 cycles; 10 min 68 °C final extension cycle. PCR products were run on a 0.8% agarose gel, cut, and extracted from the gel using the Montage DNA Gel Extraction Kit (Millipore Corp.). Fragments were then cloned using the TOPO TA Cloning Kit with TOP10 *Escherichia coli* competent cells and the pCR 2.1-TOPO cloning vector (Invitrogen Corp.). Colonies were picked and grown overnight on LB+ ampicillin media at 37 °C and plasmids isolated with the Eppendorf Perfectprep Plasmid 96 Vac Direct Bind kit (Brinkmann Instruments). Five cloned inserts were sequenced for each beetle in both directions using standard M13 specific forward (5′-GTAAAACGACGGCCAGTG) and reverse (5′-GGAAACAGCTATGACCATG) primers on an ABI 3700 automated DNA analyser (Applied Biosystems) with the BigDye Terminator v3.1 kit. All mtDNA sequences were deposited in GenBank (Accession nos AY747259–AY747305).

Microsatellite loci

Details concerning the isolation, characterization, and internal repeat structure of the *P. plagiophthalamus* microsatellite loci used in the study can be found in Velez & Feder (2005). Nine variable GT dinucleotide repeat microsatellites designated usat # 1, 2, 3, 6, 11, 12, 21, 29, and 36 were chosen for population-level analysis due to their scoring reliability and consistency in comparisons of cloned vs. PCR-generated microsatellite alleles (Velez & Feder 2005). PCR was performed on total genomic beetle DNA isolated from 1997 collected field specimens using locus-specific fluorescence-labelled 5′ microsatellite primers and HotMaster *Taq* kits (Brinkmann Instruments), with final reagent concentrations as follows: Mg^{2+} at 2.5 mM, dNTP mix at 0.25 mM, primers at 0.5 μM, and 50 ng of template DNA. Reactions were run on a RoboCycler 96 thermal cycler (Stratagene) for 3 min at 94 °C, 1 min 56 °C, and 1 min at 68 °C (1 cycle); 1 min at 94 °C, 1 min at 56 °C, 1 min at 68 °C (35 cycles); and 10 min at 68 °C (final extension cycle). Allele sizes were determined from PCR amplifications using a CEQ 8000 Genetic Analysis System (Beckman-Coulter Inc.), following the manufacturer's protocols. Negative controls with no DNA were included in all experiments.

Phylogenetic and sequence analysis

Sequences were visualized and manually aligned in SE-AL software (Rambaut 1996). Sequences containing an abundance of ambiguous base calls were eliminated from further analysis. In addition, Stolz *et al*. (2003) have previously identified a portion of the coding region of the *P. plagiophthalamus luciferase* locus that underwent a dorsal to ventral intergenic recombination event. Converted base pairs at coding positions # 58, 83, 670, 672, 806, 974, 1010, 1026, 1045, 1101, 1111, 1112, 1119, 1120, 1129, 1134, and 1150 were therefore also excluded from gene tree construction and genetic distance estimates.

Maximum-parsimony (MP) and maximum-likelihood (ML) gene trees were constructed using PAUP*b10 (Swofford 2003). Dorsal and ventral *luciferase* alleles for *P. plagiophthalamus* were compared with those for *Pyrophorus mellifluus*, the sister taxon to *P. plagiophthalamus* (Costa 1976) from the Dominican Republic (dorsal organ peak bioluminescence = 549 nm, ventral organ = 554 nm), as well as *Pyrophorus noctilucus* (Trinidad; d = 548 nm, v = 584 nm), *Pyrophorus luscus* (Belize; d = 547 nm, v = 578 nm) and *Pyrearinus termitilluminans* (Viviani *et al*. 1999) to root trees. mtDNA gene trees were rooted using *P. mellifluus* as an outgroup. MP and ML analyses yielded very similar results, and so only MP gene trees are presented here. MP gene trees were constructed using the heuristic search option of PAUP*b10, with tree-bisection–reconnection (TBR) branch swapping and 1000 taxon addition replicates. Branch support was assessed by non-parametric bootstrapping (Felsenstein 1985), with 10 000 pseudo-replicates performed using the heuristic search and TBR branch swapping options in PAUP*b10.

Neighbour-joining mtDNA and microsatellite genetic distance networks for Jamaican beetle populations were constructed using PHYLIP (Felsenstein 1989). Pairwise Tamura–Nei genetic distances (mtDNA) and Nei distances (microsatellite loci) (Nei *et al*. 2000) were estimated using MEGA3 (Kumar *et al*. 2004). Analysis of molecular variation (AMOVA) was performed using ARLEQUIN (Schneider *et al*. 2000). The molecular clock was tested for *luciferase* coding and intron sequences, as well as mtDNA, for *P. plagiophthalamus* and *P. mellifluus* by comparing log-likelihood scores enforcing versus relaxing the clock hypothesis for the best supported DNA substitution model identified by MODELTEST (Posada & Crandall 1998). Intragenic recombination was tested by using the method of Hudson & Kaplan (1985), as implemented in DNASP (Rozas & Rozas 1999). Estimates of nucleotide diversity and Tajima's *D*-tests (Tajima 1989) were also performed using DNASP, while heterozygosity values for microsatellites were derived by MSA (Dieringer & Schlotterer 2003). Relative node depths were calculated by dividing mean Tamura–Nei distances separating *P. plagiophthalamus luciferase* colour alleles or major mtDNA haplotypes by the corresponding distances between *P. plagiophthalamus* and *P. mellifluus*. Under the assumption of a molecular clock and modest effective population sizes, relative node depths provide a gauge of the coalescence times for intraspecific variants in *P. plagiophthalamus* (demarking possible vicariance events on Jamaica) compared to the baseline divergence of *P. plagiophthalamus* and *P. mellifluous*.

Results

Luciferase *colour alleles and coding sequence*

For each year in which samples were collected (1996, 1997, and 2004), the frequency of ventral *luciferase* alleles differed significantly across the three Jamaican locales (1996: G-heterogeneity test = 83.8, $P < 0.0001$, 4 d.f.; 1997: G = 45.0, $P < 0.0001$, 4 d.f.; 2004: G = 31.6, $P < 0.0001$, 2 d.f.; see Fig. 3A and Table S1-A, Supplementary material, for allele frequencies). Differentiation was also indicated by a high and significant F_{ST} value of 0.132 for colour alleles among sites ($P < 0.0001$, as determined by 10 000 random permutations; Table 1). The Ecclesdown population in the John Crow Mountains on the east side of Jamaica possessed much higher frequencies of the vYG (*c.* 10%) and vOR (*c.* 19%) alleles then either the Kingston or Windsor Caves sites to the west (Fig. 3A). At Kingston and Windsor Caves, the vYE allele predominated, being found at frequencies > 0.97. All wavelength measurements fell well inside the predefined allele classes.

Table 1 Results of AMOVA quantifying variation among the three Jamaican beetle populations (Ecclesdown, Kingston and Windsor Caves) or among the three colour classes of ventral *luciferase* alleles (orange, yellow, and yellow-green) for indicated genetic data (Locus). Given are fixation indices (F_{ST} or Φ_{ST} values) for analysis based on either population gene frequency estimates (*luciferase* ventral colour alleles, microsatellite loci) or genetic distances derived from DNA sequence data (mtDNA, *luciferase* coding region and intron). Microsatellite result is the composite value derived from locus by locus analysis for the five variable loci scored in the study

Locus	Level of variation	F_{ST}/Φ_{ST} value
Microsatellites	Among populations	0.0159 (F_{ST})
Luciferase colour alleles	Among populations	0.1322**** (F_{ST})
Luciferase intron	Among populations	0.2856*** (Φ_{ST})
mtDNA	Among populations	0.7223**** (Φ_{ST})
Luciferase intron	Among colour alleles	0.3865**** (Φ_{ST})
Luciferase coding region	Among colour alleles	0.8985**** (Φ_{ST})

***$P < 0.001$, ****$P < 0.0001$, as determined by 10 000 random permutations of the data.

The ventral *luciferase* coding sequence did not deviate significantly from a molecular clock ($\chi^2 = 15.73$, $P = 0.999$, 37 d.f.; TrN + I best-fit substitution model based on AIC). A minimum of two exchange events were inferred for the *luciferase* coding region by the method of Hudson & Kaplan (1985).

The gene tree for the ventral *luciferase* gene strongly supported the hypothesis that vOR is the most recently derived colour allele, evolving from a vYE sequence (see Fig. 4A for one of six best MP trees representative of the general gene genealogy). As previously reported by Stolz *et al*. (2003), substantial numbers of fixed nucleotide substitutions separated vYG, vYE, and vOR colour alleles (14 between vYG and vYE, 17 between vYG and vOR, and 3 between vYE and vOR). The extensive interclass variation was reflected by the high Φ_{ST} value (0.8985, $P < 0.0001$) estimated among the three classes of colour alleles by AMOVA (Table 1). The majority of the nucleotide differences

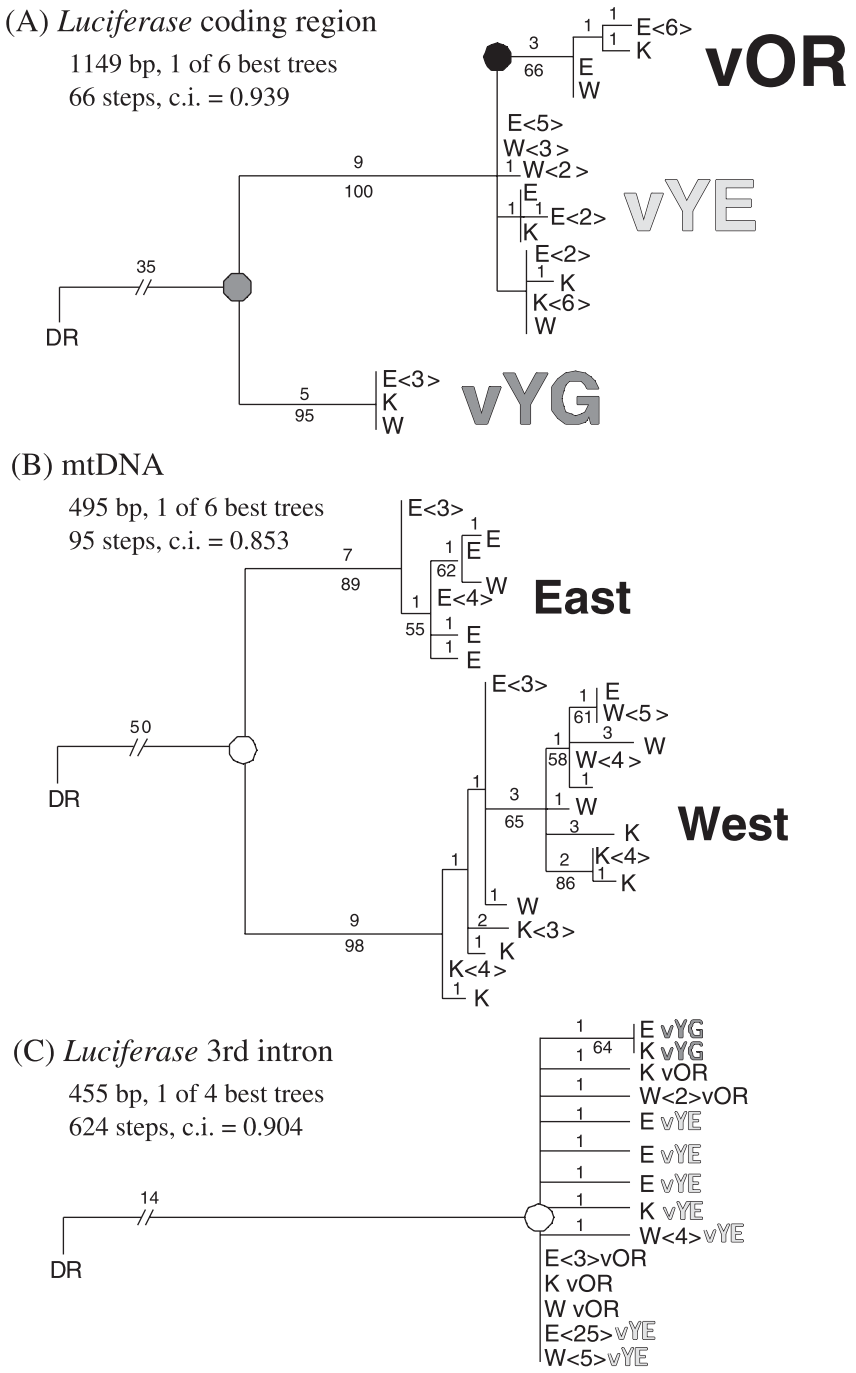

Fig. 4 Representative maximum-parsimony (MP) gene trees for (A) the ventral *luciferase* locus (exons 1–4, and the first half of exon 5), (B) the mtDNA COI gene, and (C) the latter portion of the third intron of the ventral *luciferase* gene. Trees are scaled so that they are proportionally the same length from the outgroup *Pyrophorus mellifluous* [Dominican Republic (DR)] to the tip of the longest branch in *Pyrophorus Plagiophthalamus*. Note the similar relative depth between the basal node connecting Jamaican yellow-green (vYG) and yellow (vY)/orange (vOR) clades of ventral colour alleles (grey-coloured circle in Fig. 4A) and the node connecting the two major clades of Jamaican east and west mtDNA haplotypes (black circle in Fig. 4B). E, Ecclesdown study site; K, Kingston; W, Windsor Caves. Length of the sequenced region for each locus (in base pair), the total number of evolutionary steps, and the consistency indices for the representative MP gene trees are given, along with branch lengths (numbers above branches) and bootstrap support (10 000 replicates) for key nodes (numbers below branches). Numbers in arrow brackets following site designations indicate the number of identical sequences of the same colour class for *luciferase* or haplotype class for mtDNA sequenced from different individuals at the site.

distinguishing the three colour alleles were replacement substitutions (14/17 = 82.3%), 11 of which had a measurable phenotypic effect on light colour (Fig. 1; Stolz *et al*. 2003). The three inferred synonymous mutations were all unique to the vYG clade (Figs 1 and 4A). The majority of mutations assigned to the branch connecting the basal vYG/vYE-vOR node of *Pyrophorus plagiophthalamus* genes (yellow-green coloured circle in Fig. 4A) to the outgroup *Pyrophorus mellifluous* were also nonsynonymous substitutions (23.5/35 = 67.1%), suggesting a history of positive selection on the ventral *luciferase* gene. The relative depth for the vYG/vYE-vOR node was 0.2897, while it was 0.0857 for the vYE/vYE node (orange coloured circle in Fig. 4A).

In contrast to the extensive differentiation seen among vYG, vYE, and vOR alleles, relatively little sequence variation was observed within colour classes among populations (Fig. 4A). Hierarchical AMOVA quantified the pattern: 87.79% of the molecular variation was described by differences

among colour classes, 2.65% by differences within colour classes among populations, and 9.56% by differences within colour classes within populations.

Luciferase *intron sequence*

The distal most 455-bp region of the third intron of the ventral *luciferase* gene did not deviate significantly from a molecular clock ($\chi^2 = 1.89$, $P > 0.999$, 57 d.f.; HKY best-fit substitution model based on AIC). No exchange event was inferred for this segment of the intron. However, the low level of variation observed for the region (see below) made it difficult to detect recombination, as no pair of sites displayed the four possible gametic types. Analysis of the unique *P. plagiophthalamus* ventral intron sequence immediately proximal to the 455-bp segment did provide evidence for recombination. A minimum of two exchange events was inferred and seven pairs of sites displayed all four possible gametic types.

The 455-bp segment of the third intron showed reduced levels of polymorphism in *P. plagiophthalamus* ($\pi = 0.00098 \pm 0.00023$ SD, $n = 57$) compared to the *luciferase* coding region ($\pi = 0.00454 \pm 0.00086$, $n = 37$). Despite a moderately high and significant Φ_{ST} value of 0.2856 among sites ($P < 0.0001$, Table 1), the MP gene tree for the intron showed little evidence for population differentiation (Fig. 4C). Intron variation among the three colour classes was also limited. Only one nucleotide substitution distinguished vYG-associated introns from vYE and vOR-related sequences (Fig. 4C). No diagnostic mutation in the intron differentiated vOR and vYE colour alleles (Fig. 4C). The lack of intron differentiation could not be explained by genetic constraint, as *P. plagiophthalamus* from Jamaica and *P. mellifluous* from the Dominican Republic differed by an average of 14.23 substitutions from each other. Interspecific sequence divergence was therefore not dramatically different between the intron [0.03425 ± 0.00387 SD substitutions per site (Juke–Cantor corrected)] and *luciferase* coding region (0.0429 ± 0.0065). The proportion of segregating sites was also similar for the intron vs. coding region (8 variable intron sites = 1.76% of 455 bp vs. 20 variable coding sites = 1.72% of 1165 bp). Instead, the major difference was that the segregating sites in the intron were all low frequency polymorphisms. As a result, the intron displayed a significant negative Tajima's *D* value (–1.978, $P < 0.05$), while the coding region had a positive value (0.346, $P > 0.10$). Individually, however, both the vYE and vOR colour class coding regions had negative, nonsignificant, Tajima's *D* values (–0.936 and –0.297, respectively, $P > 0.10$).

Mitochondrial DNA

Similar to the *luciferase* coding region, and in contrast to the third intron, the mtDNA COI gene displayed pronounced geographic differentiation among beetle populations on Jamaica ($\Phi_{ST} = 0.7223$, $P < 0.0001$; Table 1), as well as polymorphism within populations (Ecclesdown $\pi = 0.0176 \pm 0.00415$ SD, $n = 15$; Kingston $\pi = 0.0097 \pm 0.00091$, $n = 15$; Windsor Caves $\pi = 0.0096 \pm 0.00454$, $n = 15$). Two major mtDNA haplotypes were found in *P. plagiophthalamus* (Fig. 4B), one predominating in the eastern side of the Island at Ecclesdown (designate the East haplotype) and the other at high frequency at the Kingston and Windsor Caves sites (West haplotype). The two haplotypes showed substantial sequence divergence [0.0402 ± 0.0030 SD substitutions per site (Jukes–Cantor corrected)], with a relative node depth of 0.2995 to the outgroup *P. mellifluous* (see dark circle in Fig. 4B), the latter mirroring the value seen between vYG and vYE/vOR *luciferase* colour alleles (0.2897). The genetic break observed in mtDNA haplotype frequencies between Ecclesdown and Kingston (see Fig. 3B for mtDNA neighbour-joining network of populations) also corresponded to the major transition on the Island in *luciferase* colour allele frequencies. No cytonuclear disequilibrium was observed between mtDNA haplotypes and colour alleles, however, within the Ecclesdown population ($P > 0.72$ genotypic cytonuclear disequilibrium, $P > 0.22$ allelic cytonuclear disequilibrium, Markov chain Monte Carlo analysis, 100 000 repetitions, as determined by CNDm; Basten & Amussen 1997). The mtDNA did not deviate significantly from a molecular clock ($\chi^2 = 32.9$, $P = 0.891$, 44 d.f.). Based on insect mtDNA COI clock of 1.15×10^{-8} substitutions/bp/year (Brower 1994), the estimated time to coalescence of the East and West haplotypes would be *c.* 1.75 million years ago (Ma).

Microsatellite Loci

The nine microsatellite loci displayed high levels of variation within *P. plagiophthalamus* (mean number of alleles per locus = 10.7 ± 5.39 SD, range 3–21; mean observed heterozygosity = 0.665 ± 0.739 SD, range 0.433–0.739; see Table S2, Supplementary material, for microsatellite allele frequencies at sites). The microsatellite loci showed little differentiation among populations, as measured by F_{ST} (0.0159, $P = 0.226$). However, an unrooted neighbour-joining genetic distance network for the microsatellites implied that Kingston and Windsor Cave beetle populations are more genetically similar than either is to Ecclesdown (Fig. 3C).

Discussion

Previous studies have mapped the specific nucleotide substitutions in the target *luciferase* gene to quantifiable phenotypic effects on colour (Wood *et al*. 1989; Stolz *et al*. 2003). In addition, molecular analysis has statistically confirmed the action of natural selection on the gene and

its specific nucleotides, implying a long-term adaptive trend towards more orange-coloured ventral organ bioluminescence (Stolz *et al*. 2003). These data justify repeated hypotheses testing concerning the ecological and demographic bases for selection.

The goal of the current study was to connect the next link in the adaptive sequence from gene to population by placing *luciferase* colour variation within the context of the genetic landscape and phylogeography of *Pyrophorus plagiophthalamus*. In particular, we focused on examining a biogeographic hypothesis for the origin and spread of ventral *luciferase* colour variation on Jamaica. Our rationale was that distinct clades of colour alleles persisting in the ventral *luciferase* gene, despite evidence for recombination, could reflect periods of past geographic subdivision in which directional selection differentially fixed diverged suites of colour-affecting replacement mutations in isolated subdemes. Following contact, gene flow resulted in the current colour polymorphism in *P. plagiophthalamus*, an apparent rarity in other *Pyrophorus* species and for bioluminescent organisms, in general. We were especially interested in the vOR as a potential demonstration of a vicariance and gene flow phenomenon in progress, with the vOR allele extending the long-term evolutionary progression on Jamaica favouring longer-wavelength bioluminescence and sweeping the other colour variants from the population.

Our results support a role for biogeography in the evolution of bioluminescent colour, but the details differ from the model formulated for the vOR allele. MtDNA displayed evidence for a past vicariance event on Jamaica involving *P. plagiophthalamus*. The major genetic subdivision for mtDNA corresponded to the major shift in colour allele frequencies for *P. plagiophthalamus*, as well as the break zone for other Jamaican endemics between the eastern and central/western regions of the Island (Gill *et al*. 1973; Schubart *et al*. 1998). The estimated time of coalescence for the beetles (*c*. 1.75 Ma) is at the upper end of other estimates for land crabs, lizards, and frogs, which range from 7 to 1.9 Ma (Schubart *et al*. 1998). However, such interpretations must be viewed cautiously due to potential vagaries in the molecular clock within and between species. Nevertheless, the relative depth for the basal node connecting *P. plagiophthalamus* mtDNA haplotypes (0.2995) coincided with that for the divergence of vYG and vYE/vOR colour classes (0.2897), not the derivation of the vOR allele from vYE (0.0857). The implication is that a major biogeographic event played a role in the evolution of the vYE and vYG alleles from a shorter-wavelength precursor [the outgroup *P. mellifluous* bioluminesces green (553 nm) and all seven derived mutations leading to vYE result in longer wavelength emission]. Moreover, the vYE allele most likely diverged on the western side of the island, given the current distribution of vYE and vYG alleles (Fig. 3A). Secondary contact and gene flow followed, with the vYE allele selectively pushing vYG lower in frequency and eastward on Jamaica. The derived vOR allele appears to have originated more recently than the period of major isolation and contact, however. It is possible that the vOR allele arose in a small, as yet unidentified, isolate in the East that has subsequently fused with the main body of the *P. plagiophthalamus* population. Our current data are not sufficient to resolve this hypothesis, however. Clarification of the biogeography for the vOR allele will require additional sampling of beetles across Jamaica, especially from the East side of the Island.

Six issues must still be addressed, however, concerning the congruence of mtDNA and vYG/vYe-vOR relative node depths and the inferred origins of the *luciferase* colour alleles and their evolutionary fate. First, the dorsal to ventral intergenic recombination event inferred in *P. plagiophthalamus* by Stolz *et al*. (2003) required elimination of converted base pair positions from the calculations of node depth. To the extent that the gene conversion event postdated the split of *P. plagiophthalamus* and *P. mellifluous*, it could shorten the relative node depth estimate for vYG/vYe-vOR divergence.

Second, the ventral *P. plagiophthalamus luciferase* has the molecular footprint of selection, so substitution rates may have been accelerated in the *P. plagiophthalamus* lineage, leading to a deeper estimate for the vYG/vYe-vOR node. We did not, however, observe a significant departure from a molecular clock for the *luciferase* coding region. Also, although selection appears intense in the vYE clade (all nine diagnostic mutations are derived replacement changes), it also seems strong in the branch connecting the basal vYG/vYe-vOR node to *P. mellifluous* (23.5 nonsynonymous/35 total substitutions = 67.1%, Dn/Ds ratio = 0.628). Only the vYG does not display an elevated Dn/Ds ratio (0.199). Thus, major differences in selection intensity are unlikely to have overly biased the result.

Third, the ventral *luciferase* intron displays little differentiation among colour alleles. But if the vYG/vYE-vOR split represents a past biogeographic event that occurred on the order of 1.75 Ma, as suggested by mtDNA, then we might expect to see similar levels of sequence divergence for the third intron and flanking coding regions of the ventral *luciferase* between vYG vs. vYE-vOR colour classes. This does not appear to be the case. Only one fixed difference separated vYG from vYE and vOR third intron sequences. Assuming limited recombination among colour alleles and that the third intron is neutral (no open reading frame was found in the intron nor homology to other sequences in GenBank), we would have expected about 4.26 fixed substitutions distinguishing vYG from vYE/vOR sequences. Despite this, the difference between observed (1) vs. expected (4.26) substitutions for the third intron is actually not statistically significant, based on a time homogeneous-Poisson mutation process ($P < 0.087$). Consequently, the

limited divergence seen for the third intron does not discount the biogeographic hypothesis for vYG/vYE-vOR.

Fourth, Tajima's *D* values differed in sign across the ventral *luciferase* gene; the third intron displayed a significant, negative value (–1.978), while flanking *luciferase* coding regions had a positive value (0.346). The difference cannot be explained by demographic factors such as expanding population size, as demography should affect all sequences (intron and coding) equally. More detailed analysis, however, indicates that the positive Tajima's *D* value for the coding region is due to pooling sequences across colour allele classes. When the vYE and vOR classes are considered separately, both have negative Tajima's *D* values (–0.936 and –0.297, respectively). Moreover, there is almost no polymorphism in coding sequences from base pair 362 in exon 3 to base pair 1165 in exon 5 within vOR ($\pi = 0.00000$, $n = 9$), vYE ($\pi = 0.00021 \pm 0.00012$ SD, $n = 23$) or vYG ($\pi = 0.00000$, $n = 5$) colour allele classes, concordant with the limited variation present in the third intron ($\pi = 0.00098 \pm 0.00023$ SD, $n = 57$). All of the variation for the coding region represents fixed substitutions distinguishing the colour alleles. Thus, the data are consistent with repeated selective sweeps occurring in isolated demes generating the diverged colour alleles followed by gene flow creating population polymorphism.

Fifth, microsatellites indicate only low-level population subdivision, albeit greater differentiation between Ecclesdown and Kingston than elsewhere on Jamaica. One explanation for the result is that a rapid rate of microsatellite mutation resulted in significant homoplasy of repeat variants among sites. Alternatively, the reduced microsatellite divergence could reflect elevated nuclear vs. mtDNA gene flow, potentially male driven. This migration scenario would be consistent with the natural history of *P. plagiophthalamus*, as we have observed that males fly extensive distances at night, while females tend to remain stationary. Nuclear gene flow could also contribute to the general similarity of colour allele sequences across populations.

Finally, we had presumed that the evidence for a rapid, deterministic increase of the vOR allele on the east side of Jamaica portended its eventual fixation in *P. plagiophthalamus*. However, the temporal distribution of ventral phenotypes from 1996 to 2004 questions our interpretation of imminent change, as there was no obvious trend for an increase in vOR frequency through time. Indeed, the orange allele decreased in frequency at the Ecclesdown site in 2004 compared to the other years (Table S1, Supplementary material), although not significantly so ($P > 0.092$, one-tailed Fisher exact test, 1 d.f.). This could be because the current status of the colour polymorphism is in a balanced state near equilibrium or that our sample size and/or time span between collections was not sufficient to detect only strong, natural selection. It is also conceivable that gene flow is disproportionate from the West to East on the Island. If so, then any selective advantage of the vOR allele may be partially or fully negated by an influx of vYE alleles from the West. Support for this hypothesis comes from the relatively high frequency of mtDNA-West haplotypes at the Ecclesdown site compared to East haplotypes at Kingston or Windsor Caves. Detailed population sampling and more extensive genetic surveying of the nuclear genome will help resolve the issue of whether beetle densities and gene flow are greater from the West.

In conclusion, our findings provide insight into the nature of the bioluminescent colour polymorphism on Jamaica and its history. Biogeography appears to have played an important role in the generation of distinct colour variants in *P. plagiophthalamus* and differential gene flow may be complicating a simple selective sweep story. Many other organisms such as land crabs, frogs, birds, and lizards also show geographic breaks in morphology and genetics between the volcanic origin Blue Mountains and limestone-dominated John Crow Mountains on the eastern side of Jamaica vs. the limestone-based geology of the West (Gill *et al*. 1973; Hedges 1989; Hedges & Burnell 1990; Hass & Hedges 1991; Schubart *et al*. 1998). The breaks in biogeography have been traced to major fluctuations in sea level in the Caribbean basin (Haq *et al*. 1987) that coincide with speciation events in the Jamaican fauna (Schubart *et al*. 1998). The pattern of colour variation in *P. plagiophthalamus* is thus consistent with the known biogeography of other Jamaican endemics. Our results also support an origin for the vYE in the western side of the island and vOR and vYG in the East. It is tempting to speculate that sexual selection in small, isolated demes led to a runaway process, perhaps initiated by drift, that produced rapid colour shifts. However, this is not the only possible explanation for the polymorphism, as differential predation by bats, for example, or reproductive character displacement in light colour due to interspecific spectral competition from fireflies or a related, but now extinct, *Pyrophorus* beetle could also be involved. We are now in position to connect the final ecological link in the causal chain in this colourful example of adaptive evolution from nucleotide to landscape, as well as explore related *Pyrophorus* for evidence that they represent current end states of similar phenomena that are unfolding in *P. plagiophthalamus*.

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Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2793/MEC2793sm.htm

Table S1 Gene frequencies for (A) *luciferase* colour alleles (YG, yellow green; YE, yellow; OR, orange) and (B) mtDNA haplotypes (Hap E, east haplotype; Hap W, west haplotype) for three Jamaican beetle populations surveyed in the study (Ecclesdown, Kingston, and Windsor Caves) in indicated years. Estimates of *luciferase* allele frequencies were derived from spectrophotometer determined colour phenotypes of beetles as described in text. *n*, sample size (number of beetles scored)

Table S2 Allele frequencies for nine microsatellite loci (ustat # 3, 1, 11, 12, 6, 21, 2, 29, and 36) for three Jamaican beetle populations surveyed in the study (Ecclesdown, E; Kingston, K; and Windsor Caves, W) in 1997. Number designations for alleles represent the total length (in base pair) of the PCR-amplified gene fragment, not the internal number of dinucleotide repeats. *n*, sample size (number of beetles scored)

References

Basten CJ, Asmussen MA (1997) The exact test for cytonuclear disequilibria. *Genetics*, **146**, 1165–1111.

Bechara EJH (1988) Luminescent elaterid beetles: biochemical, biological, and ecological aspects. *Advances in Oxygenated Processes*, **1**, 123–178.

Brower AVZ (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceedings of the National Academy of Sciences, USA*, **91**, 6491–6495.

Colepicolo-Neto P, Costa C, Bechara EJH (1986) Brazilian species of luminescent Elateridae. *Insect Biochemistry*, **5**, 803–810.

Costa C (1976) Speciation and geographical patterns in *Pyrophorus* Bilberg, 1820 (Coleoptera, Elateridae, Pyrophorini). *Papeis Avulsos de Zoologia*, **29**, 141–154.

Dieringer D, Schlotterer C (2003) MICROSATELLITE ANALYSER (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, **3**, 167–169.

Endler JA (1986) *Natural Selection in the Wild*. Princeton University Press, Princeton, New Jersey.

Feder ME (1999) Engineering candidate genes in studies of adaptation: The heat-shock protein Hsp70 in *Drosophila melanogaster*. *American Naturalist*, **154**, S55–S66.

Felsenstein J (1985) Confidence-limits on phylogenies — an approach using the bootstrap. *Evolution*, **39**, 783–791.

Felsenstein J (1989) PHYLIP (version 3.2) — Phylogeny Interface Package. *Cladistics*, **5**, 164–166.

Gill FB, Stokes FJ, Stokes C (1973) Contact zones and hybridization in the Jamaican Hummingbird, *Trochilus polytmus* (L.). *Condor*, **75**, 170–176.

Gould SJ, Lewontin RC (1979) Spandrels of San-Marco and the Panglossian paradigm — a critique of the adaptationist program. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **205**, 581–598.

Grant PR, Grant BR (2002) Unpredictable evolution in a 30-year study of Darwin's finches. *Science*, **296**, 707–711.

Graur D, Li W-H (2000) *Fundamentals of Molecular Evolution*, 2nd edn. Sinauer Associates, Sunderland, Massachusetts.

Haq B, Hardenbol J, Vail P (1987) Chronology of fluctuating sea levels since the Triassic. *Science*, **235**, 1156–1167.

Hass C, Hedges SB (1991) Albumin evolution in West Indian frogs of the genus *Eleutherodactylus* (Leptodactylidae): Caribbean biogeography and a calibration of the albumin immunological clock. *Journal of Zoology*, **225**, 413–426.

Hedges SB (1989) An island radiation: allozyme evolution in Jamaican frogs of the genus *Eleutherodactylus* (Leptodactylidae). *Caribbean Journal of Science*, **25**, 123–147.

Hedges SB, Burnell K (1990) The Jamaican radiation of *Anolis* (Sauria: Iguanidae): an analysis of relationships and biogeography using sequential electrophoresis. *Caribbean Journal of Science*, **26**, 31–44.

Hoffmann A, Sgro C, Lawler S (1995) Ecological population genetics: the interface between genes and the environment. *Annual Review of Genetics*, **29**, 349–370.

Hudson RR, Kaplan NL (1985) Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics*, **111**, 147–164.

Hughes AL, Nei M (1988) Pattern of nucleotide substitution at major histocompatibility complex class-I loci reveals overdominant selection. *Nature*, **335**, 167–170.

Hughes AL, Nei M (1992) Maintenance of Mhc polymorphism. *Nature*, **355**, 402–403.

Keller LF, Grant PR, Grant BR, Petren K (2001) Heritability of morphological traits in Darwin's finches: misidentified paternity and maternal effects. *Heredity*, **87**, 325–336.

Kingsolver JG, Hoekstra H, Hoekstra J *et al.* (1999) The strength of phenotypic selection in natural populations: a review. *American Zoologist*, **39**, 9A–10A.

Kreitman M, Akashi H (1995) Molecular evidence for natural selection. *Annual Review of Ecology and Systematics*, **26**, 403–422.

Kreitman M, Hudson RR (1991) Inferring the evolutionary histories of the Adh and Adh-dup loci in *Drosophila melanogaster* from patterns of polymorphism and divergence. *Genetics*, **127**, 565–582.

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

Lewontin R (2002) Directions in evolutionary biology. *Annual Review of Genetics*, **36**, 1–18.

Nei M, Rogozin IB, Piontkivska H (2000) Purifying selection and birth-and-death evolution in the ubiquitin gene family. *Proceedings of the National Academy of Sciences, USA*, **97**, 10866–10871.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Rambaut A (1996) SE-AL. *Sequence Alignment Program*. version 1.0 Alpha 1. Department of Zoology, University of Oxford, Oxford, UK.

Rozas J, Rozas R (1999) DNASP (version 3): an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: A software for population genetics data analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland.

Schubart C, Diesel R, Hedges S (1998) Rapid evolution to terrestrial life in Jamaican crabs. *Nature*, **393**, 363–365.

Sinclair BJ, Vernon P, Klok CJ, Chown SL (2003) Insects at low temperatures: an ecological perspective. *Trends in Ecology & Evolution*, **18**, 257–262.

Stolz U, Velez S, Wood K, Wood M, Feder J (2003) Darwinian natural selection for orange bioluminescent color in a Jamaican click beetle. *Proceedings of the National Academy of Sciences, USA*, **100**, 14955–14959.

Swofford DL (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima F (1989) Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.

Tittiger C (2004) Functional genomics and insect chemical ecology. *Journal of Chemical Ecology*, **30**, 2335–2358.

Velez S, Feder JL (2005) Isolation and characterization of novel dinucleotide repeat microsatellites in the Jamaican click beetle *Pyrophorus Plagiophthalamus* (Coleptera: Elateridae). *Molecular Ecology Notes*, in press. doi: 10.1111/j.1471-8286.2005.01106.x

Via S (2002) The ecological genetics of speciation. *American Naturalist*, **159**, S1–S7.

Viviani VR, Bechara EJH (1997) Bioluminescence and biological aspects of Brazilian railroad-worms (Coleoptera: Phengodidae). *Annals of the Entomological Society of America*, **90**, 389–398.

Viviani VR, Bechara EJH, Ohmiya Y (1999) Cloning, sequence analysis, and expression of active Phrixothrix railroad-worms *luciferases*: relationship between bioluminescence spectra and primary structures. *Biochemistry*, **38**, 8273–8279.

Watt WB (1992) Eggs, enzymes, and evolution — natural genetic-variants change Insect fecundity. *Proceedings of the National Academy of Sciences, USA*, **89**, 10608–10612.

Watt WB, Wheat CW, Meyer EH, Martin JF (2003) Adaptation at specific loci. VII. Natural selection, dispersal and the diversity of molecular-functional variation patterns among butterfly species complexes (Colias: Lepidoptera, Pieridae). *Molecular Ecology*, **12**, 1265–1275.

Wood KV (1990) Luc genes — introduction of color into bioluminescence assays. *Journal of Bioluminescence and Chemiluminescence*, **5**, 107–114.

Wood KV (1995) The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochemistry and Photobiology*, **62**, 662–673.

Wood KV, Lam YA, Seliger HH, McElroy WD (1989) Complementary-DNA coding click beetle *luciferases* can elicit bioluminescence of different colors. *Science*, **244**, 700–702.

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