Rapid evolution to terrestrial life in Jamaican crabs

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Crabs of the family Grapsidae are abundant organisms in most intertidal communities. However, relatively few species live in complete independence of the sea[1]. Of those species that do, Jamaica's nine endemic species of land crabs are unique in their exceptional adaptations to terrestrial life, which include the only active brood-care for larvae and juveniles known in crabs[2–6]. These adaptations, and the morphological similarity to a group of southeast Asian land-dwelling crabs, have raised the question of the number and age of land invasions of the Jamaican species. Here we present molecular evidence that Jamaican land crabs represent a single adaptive radiation from a marine ancestor that invaded terrestrial habitats only 4 million years (Myr) ago. A Late-Tertiary origin has also been found for lizards and frogs of Jamaica[7–9] and probably reflects the Mid-Tertiary inundation of that island[10].

To allow survival in the freshwater environment, all Jamaican crabs of the endemic genera *Sesarma* and *Metopaulias* undergo an abbreviated non-feeding larval development phase[2,6,11]. Offspring survival is further enhanced by highly complex brood-care strategies in locally confined breeding habitats[2–6]. For example, the bromeliad crab, *M. depressus*, raises its young in water-filled bromeliad leaf axils. The mother crab manipulates water quality by removing detritus, circulating the water to oxygenate it, and carrying empty snail shells into leaf axils as both a calcium source and a pH buffer[2–4]. She also protects the leaf axil against potential predators, including damselfly nymphs and spiders[2,5]. The snail-shell crab, *S. jarvisi*, breeds in empty shells of the snail *Pleurodonte*; the crab either turns the snail shells upside down to collect rainwater or carries water into the shell[6]. Both species feed their offspring, which remain in the nursery habitat for several months[2,6]. In the bromeliad crab, successive broods may coexist on the same host plant, resulting in the formation of family groups[2].

Species of the endemic Jamaican land crabs occupy various terrestrial and freshwater habitats and show different degrees of dependence on water. Morphological differences between these species are often pronounced and can be related to their habitats[11,12]. For example, the flattened body of the bromoliad crab allows it to squeeze into the leaf axils of bromeliads. Such major changes in the body plan have made it difficult to study the phylogenetic history of these crabs by looking at morphological characters. Initially it was suggested that the radiation of land crabs arose from the only marine *Sesarma* species of Jamaica, *S. curacaoense*, or from a related stock[11,13]. However, other morphological studies[1,14–16] have suggested that at least some of the Jamaican land crabs are more closely related to freshwater species (genus *Sesarmoides*) from southeast Asia.

We obtained sequences from two mitochondrial genes, the large subunit ribosomal RNA (16S rRNA) and cytochrome oxidase I (COI), from 22 crab species of the family Grapsidae, including *Metopaulias* and all the American representatives of the genus *Sesarma*. We constructed phylogenetic trees using parsimony[17] and distance methods[18] (Fig. 1); these trees indicate that the Jamaican endemic terrestrial crabs are monophyletic, the result of a single colonization event. The closest relatives of the Jamaican terrestrial crabs seem to be marine intertidal American representatives of the genus *Sesarma* and not southeast Asian Sesarmoides. These results indicate that the bromeliad crab *M. depressus* should be placed in the same genus as the other Jamaican endemic crabs, of the genus *Sesarma*.

The well constrained dating of the geological closure of the Panama landbridge (3.1 Myr ago)[19,20] allowed us to calibrate a molecular clock for the evolution of *Sesarma* using genetic distances between recognized trans-isthmian sister species groups (see Methods). We used this molecular clock to estimate a separation of the Jamaican endemic *Sesarma* and *Metopaulias* from their

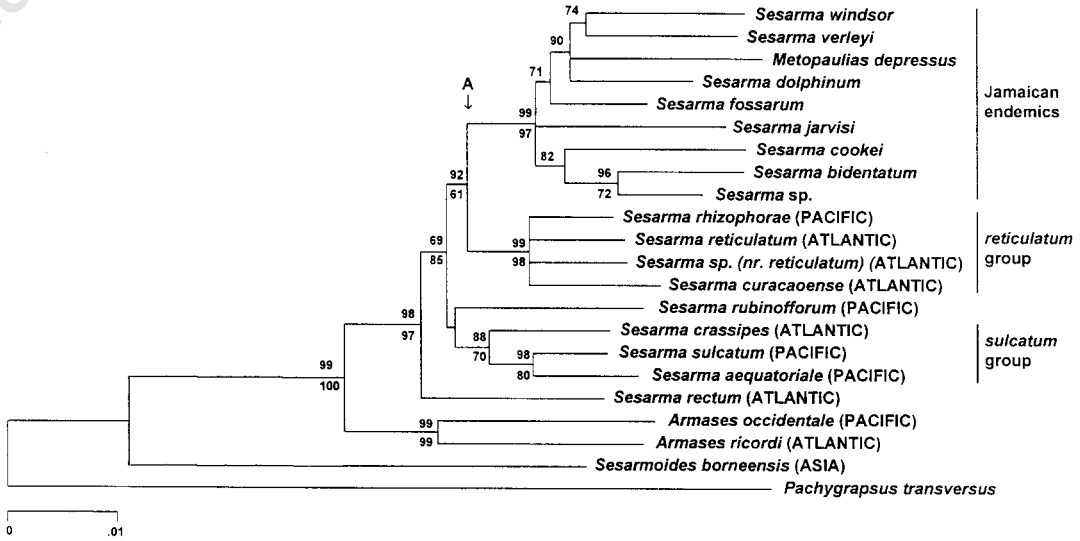

Figure 1 Molecular phylogeny of grapsid crabs, subfamily Sesarminae, inferred from an NJ analysis of DNA sequences of two mitochondrial genes, the large subunit (16S) rRNA and the cytochrome oxidase I genes. *Pachygrapsus transversus* (subfamily Grapsinae) was used to root the tree. Numbers are confidence values from an NJ analysis using the interior-branch method[18] (above lines) and an MP analysis using the bootstrap method (below lines). Interior branches of $d < 0.001$ and confidence values of <50% not shown. The *reticulatum* and *sulcatum* groups contain the trans-isthmian species used in calibration of the molecular clock; node A represents the origin of the Jamaican lineage (see Methods). Scale bar, genetic distance.

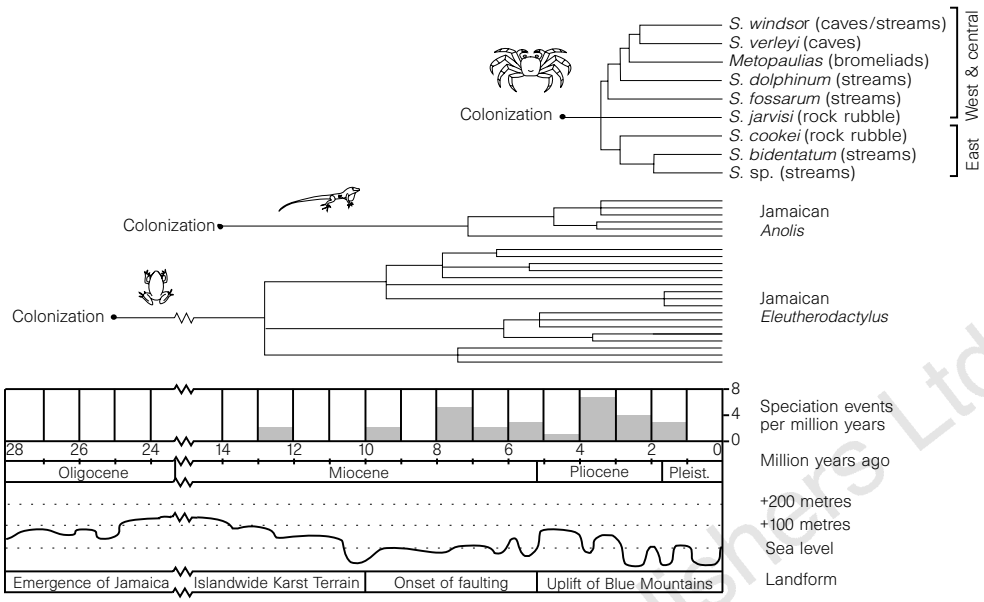

Figure 2 Phylogeny and adaptive radiation of the nine terrestrial crabs on Jamaica, with estimated times of divergence based on molecular clock calibrations. Phylogenetic trees of Jamaican lizards (*Anolis*)[7] and frogs (*Eleutherodactylus*)[8] are added for comparison of divergence times. The crab phylogeny is from Fig. 1. The histogram indicates the total number of speciation events per million years in all three groups. Sea-level changes are shown[26]. Pleist., Pleistocene.

marine ancestor at 4.5 ± 0.42 Myr ago. Independent rate calibrations from two other studies of crustaceans[21,22] also support a recent origin for the Jamaican lineage (see Methods). Invasion of freshwater and terrestrial habitats probably occurred between that time and the time of the earliest speciation events within Jamaica, at ~3.4 Myr ago (Fig. 2); subsequent speciation events occurred in the Late Pliocene epoch (3.4–1.9 Myr ago). Adaptive radiations of Jamaican lizards of the genus *Anolis*[7] and frogs of the genus *Eleutherodactylus*[8] also took place during the Late Miocene and Pliocene epochs (Fig. 2). These now are the best-documented terrestrial adaptive radiations of Jamaica and all three are relatively recent in origin. They occurred during the end of the Tertiary period, when terrestrial habitats became available for colonization after a Mid-Tertiary inundation of that island by the Caribbean Sea[10].

The rapid radiation of Jamaican land crabs is characterized by an initial split into two geographic groups (Fig. 2), the position of *S. jarvisi* being unresolved. Both of these lineages include mountain-stream species and morphologically more derived and ecologically more specialized species. This suggests that two or more radiations took place simultaneously in distinct geographic regions of Jamaica, both independently giving rise to species with similar ecologies and morphologies. A similar geographic pattern is seen in the frogs of Jamaica[8].

The colonization of terrestrial habitats by different animal groups is generally believed to be a long-term process involving many morphological, physiological and behavioural adaptations[23,24]. Here it is shown that the complex adaptations of Jamaican terrestrial crabs[2–6] evolved during a relatively short time span only a few million years ago. In contrast, marine species in the same genus separated for about the same amount of time (by the Isthmus of Panama) are ecologically and morphologically very similar[25]. The Late-Tertiary emergence of Jamaica[10] provided unoccupied terrestrial and freshwater habitats for exploitation by marine intertidal crabs and other organisms. The timing of speciation events in the subsequent radiations of Jamaican crabs, lizards and frogs coincides with unusually pronounced cycles of fluctuating sea levels[26], which may have isolated different populations (Fig. 2). Thus, the evolution of terrestrial habits in Jamaican crabs is another example of how mechanisms of evolution can be better understood by the study of island life[27]. □

Methods

Amplification and sequencing. All crabs were collected by C.D.S. and R.D. in Jamaica, and North and Central America, and were preserved in 75% ethanol. Specimens of *Sesarma sulcatum* (Mexico), *Sesarma* sp. (nr. *reticulatum*) (Texas) and *Sesarmoides borneensis* (Borneo) were gifts. Genomic DNA was isolated from the muscle tissue of one of the crab's walking legs using a phenol/chloroform extraction. Selective amplification was carried out for portions of the two mitochondrial genes by polymerase chain reaction (PCR) (33–40 cycles: with 94° C/15 s, 50–55° C/15 s and 72° C/45 s denaturing, annealing and extension temperatures), using the primers 16sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16sbr (5′-ACGTGATCTGAGTTCAGACCGG-3′) (ref. 28), in addition to internal primers 5′-TGACCGTGCAAAGGTAGCATAA-3′ and 5′-TTATCRCCCCAATAAAATA-3′ (16L12 and 16H16, S.B.H. lab), for the 16S gene. For the COI gene, we used primers COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′), COIf (5′-CCTGCAGGAGGAGGAGAYCC-3′ (ref. 28) and the internal primers 5′-ATAATYTCYCAYATYATTAAYCAAGA-3′ and 5′-TTTGDGWTCCRTGRARRGTWCTWARTCA-3′) (COIL2 and COIH2, S.B.H. lab). Double-stranded PCR products were purified and used for asymmetric PCR (40–45 cycles: with 94° C/15 s, 55–60° C/15 s and 72° C/45 s). The resulting single-stranded DNA was filtered and sequenced by dideoxy chain termination with S35 radioactive labelling. The sequences have been deposited in the EMBL database (AJ225849–AJ225982).

Phylogenetic analysis. Alignments were done with ESEE[29]. Of 1,073 total aligned sites, 345 were variable and 218 were informative for maximum parsimony (MP)[17]. Transversions were weighted 3 × transitions to correct for different substitution rates. Kimura 2-parameter distances were analysed by the neighbour-joining (NJ) method[18]. Statistical significance of resultant groups in the constructed trees was evaluated by the bootstrap method with 2,000 iterations (MP) or with an interior branch test (NJ)[18].

Time estimation. Rate constancy[30] was rejected if all taxa were included, because of a faster rate of substitution in the Jamaican lineage, but was not rejected if that lineage was excluded. For this reason, divergence times were estimated using a lineage-specific method[30]. In this method, the substitution rate was determined for non-Jamaican species and then used to estimate a date for the origin of the Jamaican lineage (node A in Fig. 1 and 'colonization' point in Fig. 2). This date, in turn, was used to estimate the substitution rate and

divergence times for the Jamaican lineage. To determine the non-Jamaican rate, we used the mean genetic distance ($d = 0.036$) of two *Sesarma* species group comparisons. In each comparison, the genetic distance was determined for species on either side of the Isthmus of Panama. For the *reticulatum* group, this was *S. rhizophorae* versus *S. reticulatum*, *S. curacaoense*, and *S.* sp. (nr. *reticulatum*), $d = 0.031$ (16S rRNA, 0.020; COI, 0.041). For the *sulcatum* group, this was *S. crassipes* versus *S. sulcatum* and *S. aequatoriale*; $d = 0.042$ (16S rRNA, 0.021; COI, 0.062). These trans-isthmian species of intertidal and supratidal crabs presumably have not been isolated for more than 3.1 Myr (refs 19, 20). The resulting average rate of pairwise sequence divergence (which equals the non-Jamaican rate) was 1.17% per Myr (0.65% for 16S rRNA and 1.66% for COI, if analysed separately).

The mean genetic distance between the Jamaican lineage and the *reticulatum* group is 0.063 ± 0.005 (s.e.m.). A relative rate test[28] revealed a distance of 0.037 on the Jamaican lineage and 0.026 on the *reticulatum* group lineage. The time of divergence between the two lineages (4.54 ± 0.42 Myr) was estimated on the *reticulatum* group lineage by applying the non-Jamaican rate; the standard error of the divergence times is the standard error of the mean genetic distance divided by this rate. That time estimate (4.54 Myr), in turn, was used to calibrate the rate of pairwise sequence divergence within the Jamaican lineage (1.63% per Myr; 0.88% for 16S rRNA and 2.33% for COI) and estimate divergence times among species (Fig. 2) from the length of internal branches in Fig. 1. A nearly identical time estimate (4.56 Myr) for the origin of the Jamaican lineage was obtained if a gamma correction[18] ($\alpha = 1.5$) was used to account for rate variation among sites. In two other decapod crustacean studies, estimated rates of pairwise sequence divergence for these two genes were higher: 2.2% per Myr for 16S rRNA in hermit crabs[21] and 2.2–2.6% per Myr for COI in snapping shrimps[22]. Although the rate calibrated within *Sesarma* is preferred, application of those independent rates would result in an even more recent time estimate (2.4–2.9 Myr ago) for the origin of the Jamaican lineage.

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The biosynthetic pathway of vitamin C in higher plants

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Vitamin C (L-ascorbic acid) has important antioxidant and metabolic functions in both plants and animals, but humans, and a few other animal species, have lost the capacity to synthesize it[1]. Plant-derived ascorbate is thus the major source of vitamin C in the human diet. Although the biosynthetic pathway of L-ascorbic acid in animals is well understood[2], the plant pathway has remained unknown[3]—one of the few primary plant metabolic pathways for which this is the case. L-ascorbate is abundant in plants (found at concentrations of 1–5 mM in leaves and 25 mM in chloroplasts[3,4]) and may have roles in photosynthesis and transmembrane electron transport[3–5]. We found that D-mannose and L-galactose are efficient precursors for ascorbate synthesis and are interconverted by GDP-D-mannose-3,5-epimerase. We have identified an enzyme in pea and *Arabidopsis thaliana*, L-galactose dehydrogenase, that catalyses oxidation of L-galactose to L-galactono-1,4-lactone. We propose an ascorbate biosynthesis pathway involving GDP-D-mannose, GDP-L-galactose, L-galactose and L-galactono-1,4-lactone, and have synthesized ascorbate from GDP-D-mannose by way of these intermediates *in vitro*. The definition of this biosynthetic pathway should allow engineering of plants for increased ascorbate production, thus increasing their nutritional value and stress tolerance.

Evidence for the ascorbate-biosynthesis pathways so far proposed for plants is inconclusive. The most effective exogenous precursor of L-ascorbic acid is L-galactono-1,4-lactone, which is converted directly to ascorbate by a mitochondrial enzyme, L-galactono-1,4-lactone dehydrogenase[6,7]. However, the role of L-galactono-1,4-lactone as a physiological precursor has been disputed[8]. L-galactono-1,4-lactone has not been detected in plants and, more significantly, its proposed production by D-galacturonic acid[9] requires inversion of the hexose carbon skeleton, which extensive radiolabel tracer studies have shown does not occur during ascorbate synthesis from glucose[10]. An alternative pathway, involving the unusual intermediates D-glucosone and L-sorbosone, has been suggested[11]; this pathway involves no inversion of the carbon chain. However, the evidence that the physiological precursors are osones is also not conclusive.

If plants could produce L-galactono-1,4-lactone without inversion of the carbon chain, evidence for its involvement in ascorbate biosynthesis would be more powerful. To investigate the possible source of L-galactono-1,4-lactone in plants, we supplied L-galactose to barley leaf slices. This resulted in a rapid and substantial increase in the foliar ascorbate concentration, similar to that induced by L-