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LETTERS

Evolutionary origin and development of snake fangs

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Many advanced snakes use fangs—specialized teeth associated with a venom gland[1,2]—to introduce venom into prey or attacker. Various front- and rear-fanged groups are recognized, according to whether their fangs are positioned anterior (for example cobras and vipers) or posterior (for example grass snakes) in the upper jaw[3–5]. A fundamental controversy in snake evolution is whether or not front and rear fangs share the same evolutionary and developmental origin[3–9]. Resolving this controversy could identify a major evolutionary transition underlying the massive radiation of advanced snakes, and the associated developmental events. Here we examine this issue by visualizing the tooth-forming epithelium in the upper jaw of 96 snake embryos, covering eight species. We use the sonic hedgehog gene as a marker[10–13], and three-dimensionally reconstruct the development in 41 of the embryos. We show that front fangs develop from the posterior end of the upper jaw, and are strikingly similar in morphogenesis to rear fangs. This is consistent with their being homologous. In front-fanged snakes, the anterior part of the upper jaw lacks sonic hedgehog expression, and ontogenetic allometry displaces the fang from its posterior developmental origin to its adult front position—consistent with an ancestral posterior position of the front fang. In rear-fanged snakes, the fangs develop from an independent posterior dental lamina and retain their posterior position. In light of our findings, we put forward a new model for the evolution of snake fangs: a posterior subregion of the tooth-forming epithelium became developmentally uncoupled from the remaining dentition, which allowed the posterior teeth to evolve independently and in close association with the venom gland, becoming highly modified in different lineages. This developmental event could have facilitated the massive radiation of advanced snakes in the Cenozoic era, resulting in the spectacular diversity of snakes seen today[6,14,15].

Many advanced snakes (Caenophidia, in the sense of ref. 16) use venom, with or without constriction, to subdue their prey[6,15]. Their venom-delivery system includes a post-orbital venom gland associated with specialized venom-conducting fangs[2]. Fangs can occupy various positions on the upper jaw, but are always located on the maxilla and never on any other tooth-bearing bone[17] (Fig. 1c).

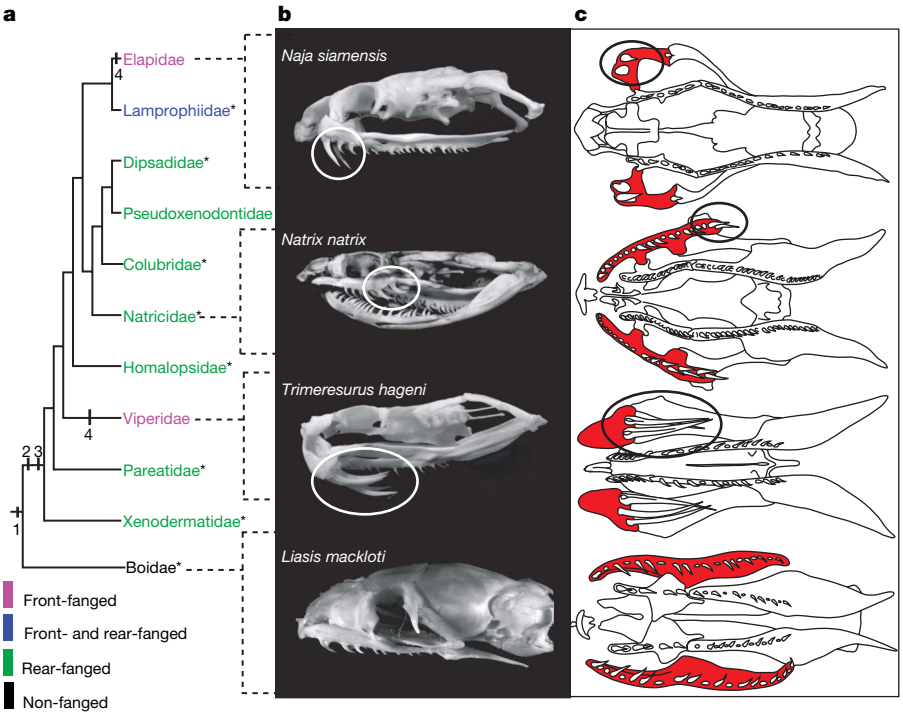

Figure 1 | Adult maxillary dentition mapped onto a molecular snake phylogeny to show relative positions of the various fang types. **a**, Phylogeny from ref. 16. **b**, **c**, Adult skulls (Supplementary Table 4): lateral views (**b**); palate, schematic ventral views (**c**; maxilla coloured, fangs circled). Asterisks indicate species studied by electron microscopy (Supplementary Fig. 5, Supplementary Table 3). The evolutionary changes leading from an unmodified maxillary dentition to the different fang types in advanced snakes are indicated at the nodes: (1) continuous maxillary dental lamina, no specialized subregions—ancestral condition for advanced snakes; (2) evolution of posterior maxillary dental lamina—developmental uncoupling of posterior from anterior teeth; (3) starting differentiation of the posterior teeth with the venom gland; (4) loss of anterior dental lamina and development of front fangs.

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Viperidae (vipers and pit vipers), *Atractaspis* (Lamprophiidae, in the sense of ref. 16) and Elapidae (cobras and their relatives) have tubular front fangs (Fig. 1b, c). The remaining lineages do not have front fangs, being either 'non-fanged' (no distinguishable enlarged posterior tooth) or rear-fanged[6,17] (Fig. 1b, c). Rear fangs can be solid or slightly or deeply grooved, but are never tubular[17].

There has been active debate concerning the evolutionary origin of these different fang types, and their relationships to the simple, unmodified teeth of non-fanged[18] basal snakes[3–9,18] such as pythons and boas (Boidae). Proposed hypotheses include the following: (1) front-fanged snakes form a monophyletic group and their fangs are derived from rear fangs[8,19,20]; (2) elapid fangs are derived from front teeth and viperid fangs from rear fangs[21,22]; (3) elapid and viperid fangs are both independently derived from rear fangs[6,7]. Establishing the origin and evolutionary transformation series between these dentition types requires a robust phylogeny to map the characters onto. Because recent molecular phylogenies of advanced snakes place the front-fanged Viperidae as relatively basal and the front-fanged Elapidae as more recently derived[6,16] (Fig. 1a), the current evidence seems to support an 'independent-origin' hypothesis[6].

So far, this issue has not been examined using a molecular developmental approach, not least because of the difficulty of obtaining snake embryos of all the different species. The development of fangs and venom glands has been studied before in viperids[23,24], *Natrix*[25,26] (Natricidae, in the sense of ref. 16), *Spalerosophis*, *Thamnophis* and *Telescopus*[1,2] (Colubridae). Those morphological studies identified a common primordium of the venom gland and fangs[1,23,25], but did not visualize the odontogenic band (tooth-forming epithelium: a band of epithelial tissue that invaginates and forms a dental lamina). Therefore, no conclusions could be drawn about the origin and evolutionary transformation series of the fangs.

Here we have carried out *in situ* hybridization of the sonic hedgehog (*shh*) gene in 96 snake embryos of multiple stages, and three-dimensionally reconstructed the development of the maxillary dentition in 41 of these through serial sections. We use nine advanced snake species, comprising two front- and two non-front-fanged lineages. As outgroup we included the non-fanged water python, *Liasis mackloti* (Boidae), which is basal to advanced snakes (Fig. 1a). *Shh* is expressed in the odontogenic band in different vertebrate species[10,13]. By visualizing this band, we aim to find evidence for the ancestral condition of the maxillary dentition. A list of all material studied can be found in Supplementary Tables 1–4. We map our characters onto the recently published, robust molecular phylogeny of advanced snakes obtained in ref. 16 (Fig. 1a).

In the water python, *shh* expression reveals one continuous maxillary odontogenic band (Fig. 2a). As confirmed by serial sections of embryos ranging from young to old, this band invaginates to form one dental lamina—a single continuous, invaginating epithelium that will develop a row of teeth (Fig. 3a, Fig. 4j–l). This is consistent with a recent morphological study of *Python sebae*[27]. The odontogenic band, with its associated lamina, appears along the entire rostral–caudal extent of the upper jaw—from the premaxilla to the mandibular articulation (Fig. 2a). This suggests that the ancestral condition for the maxillary dentition of advanced snakes is one dental lamina that appears along the entire rostral–caudal extent of the upper jaw, lacking specialized subregions.

The early odontogenic bands in the non-front-fanged grass snake, *Natrix natrix* (Natricidae), and the rat snake *Elaphe obsoleta* (Colubridae) are similar to that of the water python (Fig. 2c, Supplementary Fig. 2f, i). However, we show that there are two dental laminae which invaginate separately (Figs 3b, e and 4a–c, Supplementary Fig. 3e–g) and fuse during development (Fig. 4c, Supplementary Fig. 3h–i). The anterior lamina bears only teeth (Fig. 4c, Supplementary Fig. 3f) and is similar in development to that in the water python (Fig. 4j–l). The posterior lamina, however, bears teeth and forms the common primordium with a post-orbital gland (Figs 3b and 4b, c; Supplementary Fig. 3i). These develop into the rear fangs and venom gland in the grass snake, and probably represent the first differentiation of the posterior teeth with a venom gland in the rat snake. The latter observation is consistent with a recent magnetic resonance imaging and histology study[15] showing the presence of a small gland in rat snakes. To verify that the anterior and posterior dental laminae are truly developmentally independent, we ablated the primordium of the anterior lamina in isolated developing upper jaws of the dice snake, *Natrix tessellata*[28] (Supplementary Fig. 3m–o)[29]. We found that, after cultivation under the yolk sac membrane, the posterior lamina, with its venom gland and fangs, developed normally in the absence of the anterior lamina (Supplementary Fig. 4), showing that they are developmentally independent.

In the five front-fanged species examined (Viperidae and Elapidae), the maxillary odontogenic band is found in the posterior part of the upper jaw (Fig. 2b, d; Supplementary Fig. 2a, d, g). There is no *shh* expression or dental lamina in the anterior region (verified by histology; data not shown). In contrast, in the water python, the grass snake and the rat snake, the odontogenic band and associated dental laminae appear along the entire rostral–caudal extent of the upper jaw. We find that, during development, the 'rear' fang is displaced to its adult 'front'

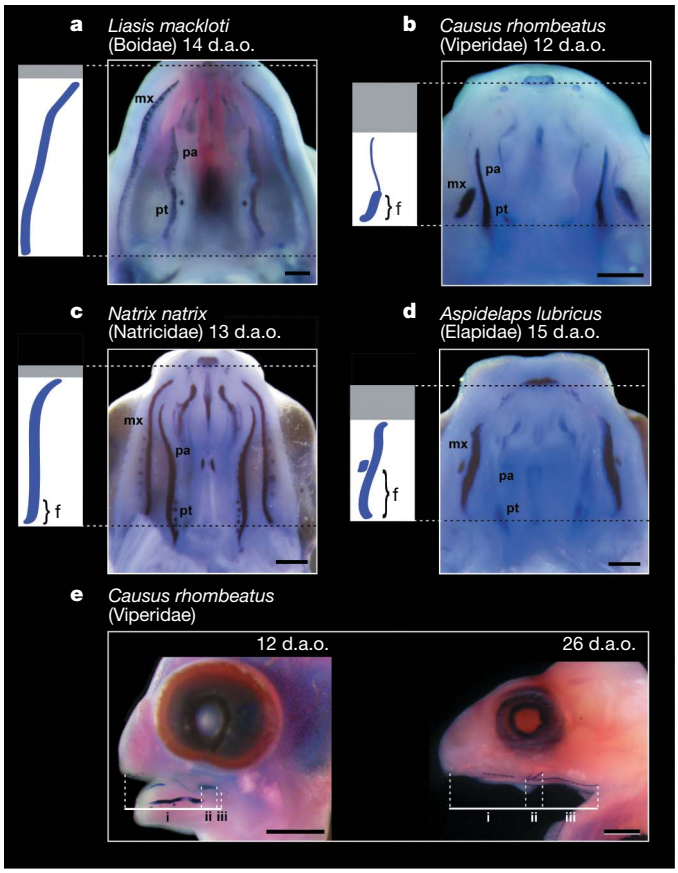

Figure 2 | *Shh* expression in the embryonic snake palate, showing the posterior developmental origins of front fangs. **a–d**, Palate, ventral view: top, anterior; scale bar, 0.5 mm; dotted lines, upper jaw (posterior margin of premaxilla to attachment of the mandible); boxes, schemes of maxillary odontogenic band (purple, *shh* expression; grey, no *shh* expression). Positions of fangs in **b–d** were identified histologically (Fig. 3, Supplementary Fig. 3). The odontogenic band in the front-fanged species is located posterior in the upper jaw (**b**, **d**). In the non-fanged outgroup (**a**) and the rear-fanged *Natrix* (**c**), the odontogenic band extends along the entire upper jaw. f, fang; mx, maxillary odontogenic band; pa, palatine odontogenic band; pt, pterygoid odontogenic band. **e**, Ontogenetic allometry in the fang in the front-fanged *Causus* displaces the fang along the upper jaw (Supplementary Figs 5–9, Supplementary Tables 5–9). Scale bars, 1 mm. We note the change in relative size of the upper jaw subregions: i, anterior; ii, fang; iii, posterior. d.a.o., days after oviposition.

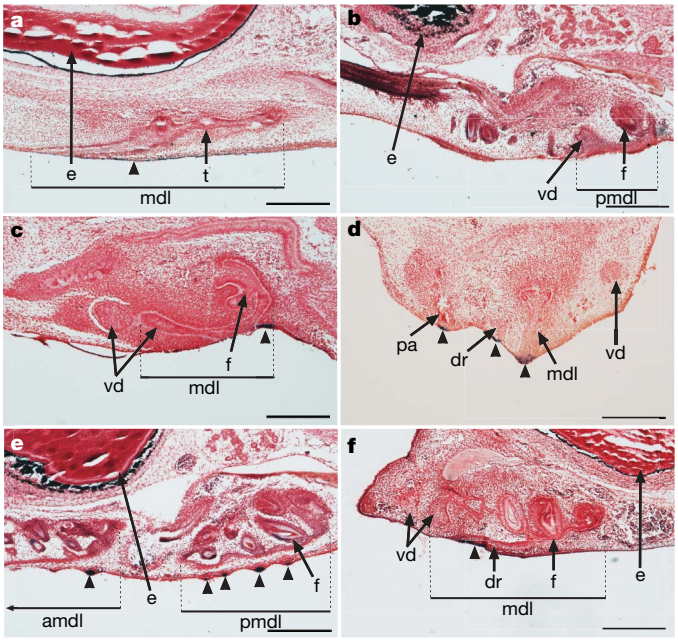

Figure 3 | Sections of the *shh in situ* hybridizations of the embryonic upper jaw in five snake species, showing the posterior and anterior dental laminae. **a–c**, **e–f**, Sagittal sections, anterior to the left, of *L. mackloti* (Boidae) 22 d.a.o. (**a**), *N. natrix* (Natricidae) 22 d.a.o. (**b**), *Calloselasma rhodostoma* (Viperidae) 8 d.a.o. (**c**), *N. natrix* 22 d.a.o. (**e**), *Naja siamensis* (Elapidae) 23 d.a.o. (**f**). **d**, Transverse section, medial to the left, of *Trimeresurus hageni* (Viperidae) 8 d.a.o. The posterior maxillary dental laminae in **b** and **e** are similar in morphogenesis to the maxillary dental laminae in all front-fanged species examined (**c**, **d**, **f**; see also Fig. 4). Arrowheads, *shh* expression; amdl, anterior maxillary dental lamina; dr, dental ridge; e, eye; f, fang; mdl, maxillary dental lamina; pa, palatine dental lamina; pmdl, posterior maxillary dental lamina; t, tooth bud; vd, primordium of venom gland; scale bars, 300 μm.

position by ontogenetic allometry (Supplementary Figs 6, 7; Supplementary Table 6 for statistical analyses), suggesting a posterior evolutionary origin for the front fangs. Histology shows that although the odontogenic band invaginates normally and forms one dental lamina (in contrast to the non-front-fanged snakes described above), in all front-fanged species the fangs develop from the posterior-most part of this lamina and there are no developing teeth in the anterior part (Fig. 3f, Fig. 4d–i). This apparently toothless part of the dental lamina has been described before only in *Vipera aspis* (Viperidae), and termed the 'dental ridge'[24,30]. We find it here in Elapidae (Fig. 3f, Supplementary Fig. 3j–l). The fact that viperids and elapids share the dental ridge and a posterior developmental origin for their front fangs is interesting, because they are phylogenetically not closely related (Fig. 1a).

Because Elapidae and Viperidae do not form a monophyletic group (Fig. 1a), the dental ridge, the posterior developmental origin of the fangs and the ontogenetic allometry in both lineages may reflect convergent evolution. However, our three-dimensional reconstructions show that there is a striking similarity in morphogenesis of all front and rear fangs examined (Fig. 4b–i), despite the large variation in adult morphology. The toothless dental ridge seen in elapids and viperids is similar to the part of the posterior dental lamina that fuses with the anterior dental lamina in the grass snake and the rat snake (Fig. 4). Although developmental similarity is not conclusive proof of structural homology, this is especially interesting in light of the posterior developmental origin of the front fangs in both elapids and viperids mentioned above. These results are difficult to reconcile with the independent-origin hypothesis, but are consistent with the hypothesis that elapid and viperid front fangs, and the posterior dental lamina in non-front-fanged snakes, represent homologous structures.

Our results suggest a new model for the evolution of snake fangs. A posterior subregion of the ancestral tooth-forming epithelium became developmentally uncoupled from the remaining dentition, resulting in posterior and anterior dental laminae that are developmentally independent (Supplementary Fig. 1). This condition is retained in the non-front-fanged snakes, such as the grass and rat snake. This model would imply that the front-fanged elapids and viperids have independently lost the anterior dental lamina (Fig. 1), which is supported by the lack of *shh* expression anterior in their upper jaws.

Because obtaining developmental data for each non-front-fanged advanced snake lineage is impracticable, convergence cannot be ruled out completely. We have, therefore, examined the adult maxillary tooth morphologies through scanning electron microscopy in the water python, the grass snake, the rat snake and a wide range of other non-front-fanged advanced snake species (Fig. 1a). We aimed to

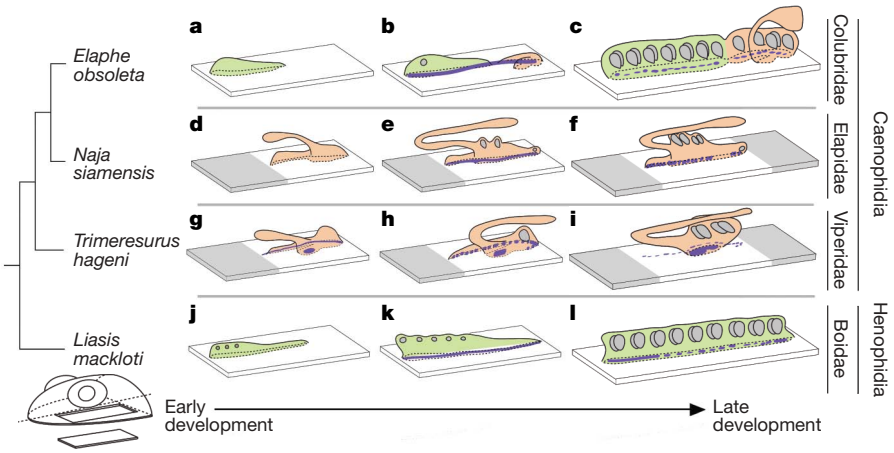

Figure 4 | Schematic three-dimensional reconstructions showing the similarity in morphogenesis between the rear and front fangs. Derived from serial sections (Fig. 3, Supplementary Fig. 3); materials analysed are listed in Supplementary Tables 1, 2, 4. Left-hand side of the upper jaw is depicted, and only epithelial components are shown. Purple, *shh* expression; grey, tooth buds; green, unspecialized maxillary dental lamina; orange, specialized maxillary dental lamina that bears fangs. The specialized dental lamina is dilated into a bifurcated epithelial sac, the lateral part giving rise to the venom duct and venom gland by growing rostrad (see also Fig. 3b–d, f), then turning caudad to reach the post-orbital region (as previously described for vipers[23,24], *Natrix*[25,26] and *Spalerosophis*[1,2]). In *Elaphe obsoleta* (**a–c**) and *Natrix natrix* (data not shown), fangs develop rostrally and caudally alongside the base of the venom duct; in *Naja siamensis* (**d–f**) and *Trimeresurus hageni* (**g–i**) the rostral part regresses, remaining visible only as the dental ridge, whereas in **b** and **c** this part bears fangs and fuses with the anterior dental lamina. The unspecialized dental lamina in *E. obsoleta* (**a–c**) and the outgroup *Liasis mackloti* (**j–l**) starts developing anterior and grows caudad.

find differences in the maxillary dentition, which might suggest the presence of two maxillary dental laminae in additional lineages. Our results show that there is indeed a consistent difference in anterior versus posterior tooth morphologies in other advanced snake lineages (Supplementary Fig. 5d–x, Supplementary Table 3). In contrast, the maxillary teeth of the examined boids do not show a morphological difference between anterior and posterior teeth (Supplementary Fig. 5f). These results suggest the possible presence of two dental laminae in other non-front-fanged advanced snake lineages, and provide additional support to our proposed model.

The developmental uncoupling of the posterior from the anterior tooth region could have allowed the posterior teeth to evolve independently and in close association with the venom gland. Subsequently, the posterior teeth and venom gland could have become modified and formed the fang-gland complex—an event that underlies the massive radiation of advanced snakes during the Cenozoic era[6,14,15].

METHODS SUMMARY

Snake embryos. Snake eggs and embryos were acquired in accordance with local and international regulations from European, Israeli and Australian breeders and zoos. Eggs were incubated at 30 °C and embryos fixed in 4% paraformaldehyde in PBS at 4 °C overnight. They were dehydrated through graded methanols and stored at −18 °C.

***In situ* hybridization.** The RNA probe was based on the partial PCR product of sonic hedgehog using the complementary DNA of a one-day-old rhombic night adder (*Causus rhombeatus*) embryo as template. Hybridization was performed according to standard protocols. In all species examined, the odontogenic band (tooth-forming epithelium) always expressed *shh* (Fig. 2, Supplementary Fig. 2). This shows that, as in other vertebrate groups[10–13], *shh* is also a marker for odontogenic epithelium in snakes. A list of embryos studied can be found in Supplementary Table 1.

Histology. Embryos were dehydrated using ethanol or methanol, cleared in HistoClear or tetrahydronaphthalene and embedded in paraffin. Sections were cut at 5–7 μm and stained with H&E or counterstained using neutral red. A list of embryos studied can be found in Supplementary Table 2.

Three-dimensional modelling. Schematic three-dimensional models were drawn from analyses of the serial sections of the embryos using a Nikon Eclipse E800 microscope. All models were drawn using Adobe Illustrator and Adobe Photoshop.

Scanning electron microscopy. The maxillary bone on one side was dissected out of the specimen, allowed to dry and mounted on a stub, using double-sided tape, with teeth pointing upwards. Specimens were sputter-coated with gold, and examined using a JEOL JSM-T300 scanning electron microscope, at an acceleration voltage of 15 kV. A list of specimens examined, with their museum numbers, can be found in Supplementary Table 3.

Ablation experiment. The ablation experiment was performed as previously described[29].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions F.J.V.: study concept and design; embryo and skull collection, acquisition and processing; probe synthesis; *in situ* hybridizations; histology; figures; paper. J.F.A.: three-dimensional reconstructions, figures. K.J.: scanning electron microscopy. R.R.: study concept, ablation experiment, histology. E.K.: study concept, embryological data. K.V., I.v.d.B., M.v.A.: *in situ* hybridizations, histology. M.A.G.d.B.: probe design, *in situ* hybridizations. A.B.: *Naja* embryo collection. P.J.M.: supply of *Naja* material. B.G.F.: study concept, supply of *Causus* material. A.W.: provision of laboratory space. E.B., F.W.: morphometric analyses. M.K.R. (project leader): study concept and design, provision of funding and laboratory space.

Author Information Sonic hedgehog complementary DNA clone sequence for the rhombic night adder, *Causus rhombeatus*, has been deposited in the Genbank database under accession number EU236145. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.K.R. (m.k.richardson@biology.leidenuniv.nl).