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Genetic changes associated with floral adaptation restrict future evolutionary potential

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A commonly accepted evolutionary principle is that adaptive change constrains the potential directions of future evolutionary change^{1–3}. One manifestation of this is Dollo's law, which states that character elimination is irreversible^{4,5}. Although the common occurrence of irreversibility has been documented by

phylogenetic analyses of phenotypic transitions, little is known about the underlying causes of this phenomenon⁴. One explanation for evolutionary irreversibility relies on the fact that many characteristics result from interactions between multiple gene products^{4,6}. Such characteristics may often be eliminated by inactivation of just one gene in the network. If they serve no other functions, other genes of the network are then free to accumulate mutations or evolve new functions. Evolutionary change after character loss results in the accumulation of redundant loss-of-function mutations. Such pathway degeneration makes it very unlikely that the characteristic will re-evolve, because multiple simultaneous mutations would be required⁴. Here we describe what appear to be the initial stages of such degeneration in the anthocyanin pigment pathway associated with an adaptive change from blue to red flowers in the morning glory *Ipomoea quamoclit*.

The ancestral floral colour in the genus *Ipomoea* is blue/purple^{7,8} (Fig. 1a, b). A number of independent transitions to other colours have occurred in this genus^{7–9}. One such transition is represented by a small, well-supported clade¹⁰ of red-flowered species in the section *Mina*, subgenus *Quamoclit*, including *I. quamoclit*⁷ (Fig. 1a, b), which are pollinated primarily by hummingbirds^{8,11,12}. Unlike typical blue-flowered *Ipomoea* species, which are pollinated primarily by bees and exhibit a set of characteristics associated with adaptation to bee pollination (for example, a broad floral tube, small quantities of nectar, inserted stigma and anthers and non-versatile anthers)^{8,13}, *Mina* species exhibit a suite of floral traits, including red pigmentation, typically associated with bird pollination (for example, narrow tube, copious nectar production, exerted stigma and anthers and versatile anthers)^{7,8,13}. The transition to red flowers in this group appears to represent an adaptive shift to association with a different type of pollinator.

Floral colour in *Ipomoea* is determined largely by the type of anthocyanin pigment produced. Pigments derived from cyanidin typically produce blue/purple flowers, whereas pigments derived from pelargonidin typically produce red flowers^{14–16}. Cyanidin differs from pelargonidin by possessing an extra hydroxyl group, which is added by the enzyme flavonoid 3'-hydroxylase (F3'H)¹⁶. This enzyme creates a branch in the biosynthetic pathway, with the enzymes downstream of F3'H (that is, dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and UDP glucose flavonoid 3-glucosyltransferase (UF3GT)) participating in both branches (Fig. 2). In *I. nil*, *I. purpurea* and *I. tricolor*, virtually all of the flux normally flows down the cyanidin branch of the pathway, producing blue flowers. However, mutations in these species that block the cyanidin branch produce red pigments and flowers (Fig. 1c), indicating that these downstream enzymes are substrate generalists capable of metabolizing both the hydroxylated and non-hydroxylated intermediates^{16,17}.

Thin-layer chromatography of anthocyanidins from several *Mina* species reveals that the red floral pigments of these species are derived from pelargonidin (Fig. 1b, c). The evolutionary transition to red flowers in this group was thus brought about by inactivation of the cyanidin branch, which can be accomplished in two ways, either by inactivation of F3'H or by the evolution of substrate specificity—loss of the ability to metabolize hydroxylated substrates—by one of the downstream enzymes, DFR or ANS. Substrate specificity of DFR has been demonstrated in several unrelated genera, including *Petunia*¹⁸ and *Arabidopsis*¹⁹. Here we show that both of these types of change have occurred in the lineage leading to *I. quamoclit*.

The single-copy *F3'h* gene from *I. quamoclit* exhibits a high sequence similarity to its homologue from *I. purpurea*. The two sequences exhibit no indels and are 94.7% similar at the nucleotide level and 93.5% similar at the amino-acid level. Although no obvious features (for example, premature stop codons or frame shifts) indicate that the gene is non-functional, our experiments

suggest there has been both regulatory and functional inactivation of F3'H in *I. quamoclit*.

Regulatory inactivation is revealed by slot-blot assays using labelled cDNA from floral bud tissue as a probe. These assays indicate that *F3'h* expression levels are dramatically reduced in *I. quamoclit* compared with the blue-flowered *I. purpurea*, whereas other genes of the anthocyanin pathway are expressed at comparable levels (Fig. 3d). Reductions of this magnitude in the expression of other anthocyanin genes block pigment production in a variety of *Ipomoea* species²⁰. Absence of cyanidin-based pigments in *I. quamoclit* can be explained entirely by a lack of F3'H in developing flowers and a consequent redirecting of flux down the pelargonidin branch of the pathway.

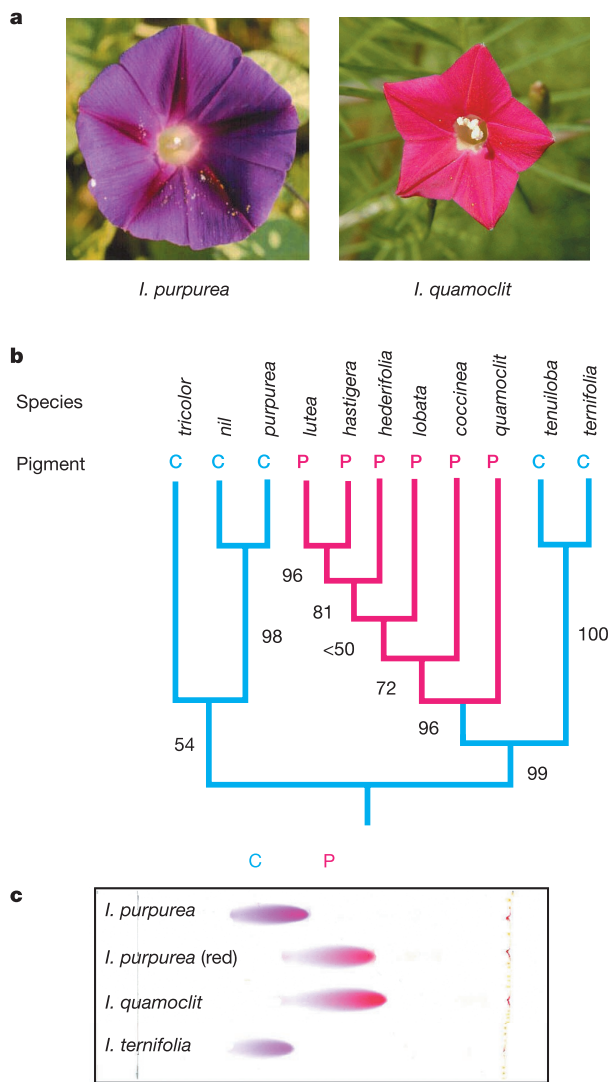


Figure 1 Flower colours and floral pigments of *Ipomoea* species. **a**, *I. purpurea* exhibits blue/purple flowers and inserted stigma and anthers, whereas *I. quamoclit* exhibits red flowers and exerted stigma and anthers. **b**, Phylogenetic relationships among closely related *Ipomoea* species, based on ribosomal internal transcribed sequence. Red branches: species in subgenus *Quamoclit*, section *Mina*, which have red flowers with bird-pollination syndrome. Blue branches: blue/purple flowers and bee-pollination syndrome. Pigment: floral anthocyanin pigments are derived either from cyanidin (C) or pelargonidin (P). Bootstrap support (out of 100 replicates) for the branches is indicated by the numbers next to the branches. **c**, Thin-layer chromatogram illustrating anthocyanin pigments from three species. *I. purpurea* corresponds to the normal blue/purple-flowered morph. *I. purpurea* (red) corresponds to a red-flowered mutant in which *F3'h* is inactivated^{16,17}.

Functional inactivation of *I. quamoclit* F3'H is suggested by complementation assays, in which we transformed the *F3'h*-coding region from *I. quamoclit* and *I. purpurea* (this copy serving as a control) linked to a constitutive promoter into an *Arabidopsis thaliana* mutant strain lacking a functional copy of *F3'h*¹⁹. Although the *I. purpurea* *F3'h* gene complemented the *A. thaliana* knockout in four independent transformations, and produced anthocyanins in the leaves of nutrient-stressed plants, no such complementation occurred in any of four transformations with *I. quamoclit* *F3'h* (Fig. 3c). Moreover, no anthocyanins were detectable, by extraction and thin-layer chromatography, in the leaves of any of the *I. quamoclit* *F3'h* transformants. Although we did not quantify transcript levels using quantitative polymerase chain reaction (PCR), standard reverse-transcribed PCR (RT-PCR) on leaf RNA from the four transformants of each species yielded bands of comparable intensity on agarose gels, indicating that failure to complement is probably not caused by markedly reduced transcript levels.

This failure to complement does not appear to be due to an inactivation of catalytic activity in the *I. quamoclit* F3'H. Qualitative enzyme assays using thin-layer chromatography indicate that the *I. quamoclit* gene is capable of metabolizing its natural substrate dihydrokaempferol (Fig. 3a). Quantitative assays suggest that the efficiency of the *I. quamoclit* F3'H on this substrate may be slightly lower than that of the *I. purpurea* F3'H, but this difference is not statistically significant (Fig. 3b). The catalytic activity of the *I. quamoclit* F3'H thus is not substantially impaired. This result suggests that some aspect of the *I. quamoclit* F3'H enzyme other than its catalytic activity (for example, cellular localization) is defective, preventing it from functioning properly *in vivo*. Although we cannot completely rule out the possibility that failure to complement is an experimental artefact of heterologous transformation (for example, due to the lack of a necessary co-factor present in *I. quamoclit* but absent in both *I. purpurea* and *A. thaliana*), we believe this possibility is unlikely because functional anthocyanin pathway genes from very distantly related species (for example, maize) typically complement *A. thaliana* mutants¹⁹.

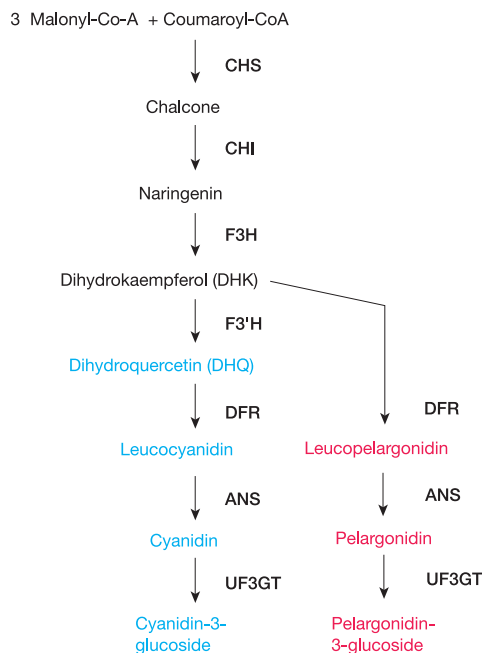


Figure 2 The core anthocyanin biosynthetic pathway. Names in lower case represent pathway intermediates and products. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase. Purple intermediates and products indicate the cyanidin branch of the pathway. Red intermediates and products indicate pelargonidin.

Several properties of DFR demonstrate that evolutionary changes in this enzyme in *I. quamoclit* are sufficient to block the production of cyanidin-based pigments. In *Ipomoea*, *Dfr* is represented by a small family of three paralogues, of which only *Dfr-B* is expressed in floral tissue²¹. The sequence of *Dfr-B* that we cloned from *I. quamoclit* is 95% similar in nucleotide composition and 93% similar in amino-acid composition to its *I. purpurea* orthologue in regions outside of indels. It also exhibits four small in-frame insertions totalling 27 nucleotides, as well as a large 98-nucleotide insertion at the extreme 3' end of the gene (Fig. 4a). This insertion, which is highly similar to the 98 base pairs (bp) just 3' of it, causes a frame shift, which converts a TAA sequence into a stop codon 59 bp upstream of the original stop codon. In addition, two amino-acid substitutions occur in a 13 amino-acid portion of the gene that has been implicated in determining substrate specificity (Fig. 4a)^{22,23}.

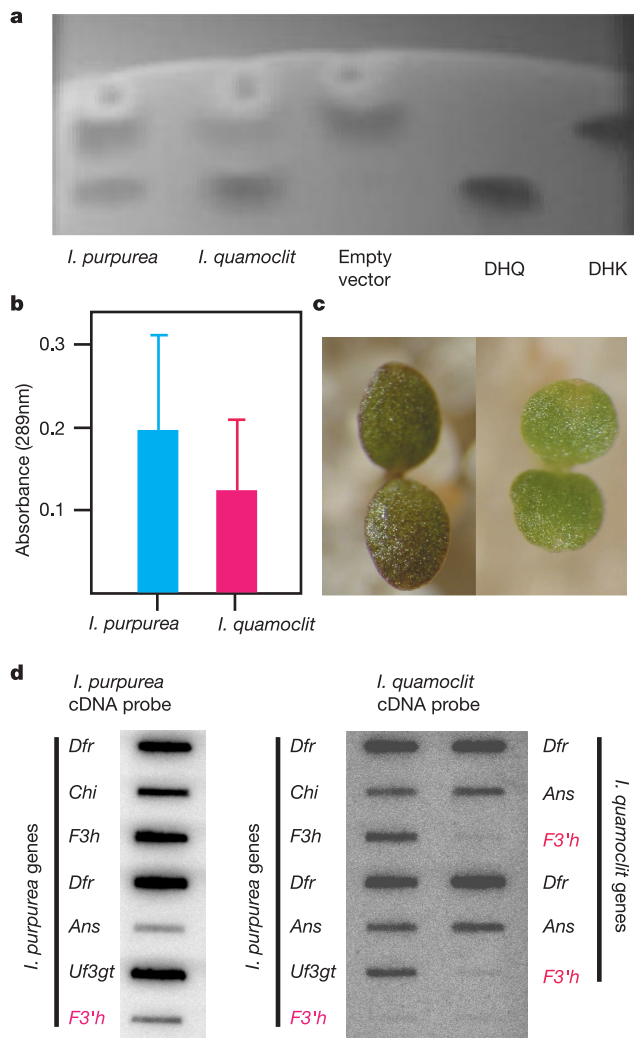


Figure 3 Functional and expression analysis of *F3'h*. **a**, Thin-layer chromatogram illustrating that *F3'h* from both *I. purpurea* and *I. quamoclit* metabolize dihydrokaempferol (DHK) to produce dihydroquercitin (DHQ) in *in vitro* assays. **b**, Mean production of DHQ in *in vitro* enzyme assays. Bars are one standard error. **c**, Accumulation of anthocyanins in *F3'h*-null *A. thaliana* seedlings carrying the transgene *F3'h* from (left) *I. purpurea* (substantial accumulation) and (right) *I. quamoclit* (no visible accumulation). **d**, Anthocyanin gene expression patterns. *F3'h* is highlighted with a red label. Left panel, spotted *I. purpurea* genes probed with *I. purpurea* cDNA. All genes, including *F3'h*, exhibit substantial expression. Right panel, left lane contains spotted genes from *I. purpurea*, right lane genes from *I. quamoclit*. Note that only *Dfr*, *Ans* and *F3'h* have been spotted (each gene twice), because the other anthocyanin genes have not been cloned from *I. quamoclit*. Both lanes of right panel were probed with *I. quamoclit* cDNA.

To determine whether any of these differences influence the ability of the *I. quamoclit* DFR-B to metabolize dihydroquercitin (DHQ) or dihydrokaempferol (DHK), precursors to cyanidin and pelargonidin, respectively, we performed complementation tests and *in vitro* enzyme assays. For complementation, *Dfr-B* from *I. quamoclit* and from *I. purpurea* as a control were transformed into a strain of *A. thaliana* carrying a knockout allele of the sole copy of *Dfr*. In four independent transformations, the *I. purpurea* *Dfr* complemented this knockout and produced pigmented leaves (Fig. 4b). By contrast, in four independent transformations the *I. quamoclit* *Dfr* failed to complement (Fig. 4b), despite substantial expression of the transgene in leaf tissue. Because *A. thaliana* produces only cyanidins (the pelargonidin branch of the pathway is inactive)¹⁹, this failure to complement indicates that the *I. quamoclit* DFR has lost the ability to metabolize DHQ. However, because pelargonidin is produced in the red flowers of *I. quamoclit*, this DFR-B clearly retains the ability to metabolize DHK. Results of *in vitro* enzyme assays support this conclusion. *I. purpurea* DFR metabolizes the two substrates with roughly equal efficiency, whereas the efficiency at which DHQ is metabolized by *I. quamoclit* DFR is markedly reduced, compared with DHK (Fig. 4c). *I. quamoclit* DFR has thus evolved to become a substrate specialist, and this specificity has introduced another block to the cyanidin branch of the anthocyanin pathway in *I. quamoclit*.

Phylogenetic analyses indicate that production of blue/purple,

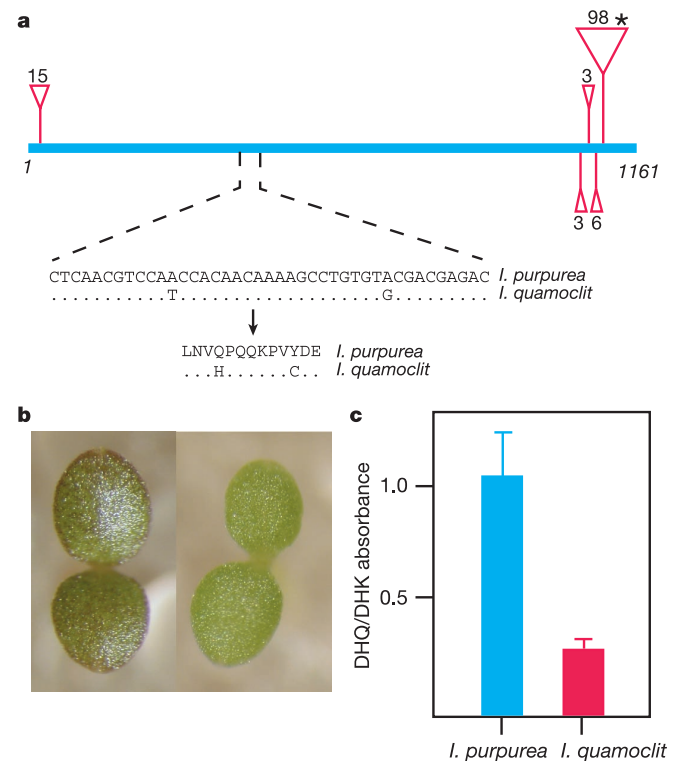


Figure 4 Functional analysis of *Dfr*. **a**, Structural organization. Blue bar, coding region of *I. purpurea* gene. Red open triangles, insertions in *I. quamoclit* (sizes (bp) indicated at bases of triangles). Italic numbers, initial and final bp number. Asterisk, novel stop codon in *I. quamoclit* sequence. Also shown are the nucleotide and amino-acid sequences of the purported substrate specificity-determining region^{22,23}. **b**, Accumulation of anthocyanins in *Dfr*-null *A. thaliana* seedlings carrying the transgene *Dfr* from (left) *I. purpurea* (substantial accumulation) and (right) *I. quamoclit* (no visible accumulation). **c**, Mean and standard error of the ratio of DHQ production to DHK production in *in vitro* assays. The *I. purpurea* enzyme metabolizes DHQ and DHK with equal efficiency, whereas the *I. quamoclit* enzyme metabolizes DHQ with only about quarter the efficiency it metabolizes DHK. The difference in ratio between species is highly significant ($P < 0.01$, *t*-test).

cyanidin-based pigments was the ancestral state in *Ipomoea*^{7,8}, and also in *I. tenuiloba* and *I. ternifolia*, members of the sister clade to the *Mina* section (Fig. 1). The change from blue to red flowers in the ancestral lineage of the *Mina* group, which is associated with a transition from bee to bird pollination, appears to have been brought about by inactivation of the cyanidin branch of the anthocyanin biosynthetic pathway and a redirecting of flux down the pelargonidin branch. We have shown that in *I. quamoclit* this inactivation involves at least two genetic changes: (1) reduction of *F3'h* expression to barely detectable levels; and (2) a marked reduction in the capacity of DFR to metabolize DHQ. In addition, our experiments also suggest that the functionality of the enzyme F3'H may be impaired *in vivo*. Because each of these changes is probably sufficient to block cyanidin production, there has evolved in the cyanidin branch at least one (and probably two) redundant block, that is, a block that presumably evolved after the flower colour had changed to red.

Relaxation of selection following the initial blockage of the cyanidin branch thus seems to have facilitated further degeneration in that pathway. This degeneration makes it very unlikely that *I. quamoclit* would be able to re-evolve cyanidin-based blue/purple pigments, since this would require at least two (restoration of *F3'h* expression and either restoration of the ability of DFR to metabolize DHQ or recruitment of expression of a paralogous copy of DFR in flowers), and possibly three (restoration of function to F3'H), simultaneous mutations to restore a functional cyanidin branch, and perhaps more if the enzymes ANS and UF3GT have evolved substrate specificity like that seen in DFR. The potential for evolutionary change in an important ecological characteristic seems to have been restricted in *I. quamoclit* by genetic changes that are a consequence of adaptation in that characteristic. □

Methods

Plant materials

Leaf and floral tissue from *I. quamoclit* and *I. purpurea* were collected from populations of these species near Durham, North Carolina.

Pigment identification

Anthocyanidins were extracted from flowers by boiling corollas in 2 M HCl, then extracting with iso-amyl alcohol. This extract was lyophilized, then resuspended in methanol with 1% HCl. Anthocyanidins were separated using thin-layer chromatography on cellulose-coated glass plates in forestal solvent²⁴.

Cloning of *F3'h* and *Dfr-B*

Full-length clones of the entire coding regions of *F3'h* and *Dfr-B* were obtained using RT-PCR and primers based on known sequences from *I. purpurea*. Template cDNA was obtained from floral buds. PCR products were cloned into a TOPO vector and subcloned into the transformation vector *pBI 1.4t* containing the constitutive CaMV 35S promoter and kanamycin and gentamicin resistance for complementation analysis. Sequences of the *I. quamoclit* genes have been deposited in GenBank (accession numbers AY463156 and AY463157).

Complementation analyses

To determine whether *F3'h* and *Dfr* are functional *in vivo*, we employed the complementation analysis using *A. thaliana* strains that were homozygous for a null mutant of the gene to be tested (either *tt7-1* for *F3'h* or *tt3-1* for *Dfr*)¹⁹. Genes in the vector *pBI 1.4t* were transformed first into the *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw protocol²⁵, then into the appropriate *A. thaliana* mutant strain using the standard dip protocol²⁶. Transformants were selected by growing on MS medium containing kanamycin. Anthocyanin phenotypes of T₂ plants were scored by germinating seeds in sand under high light. DNA was extracted from mature T₂ plants and scored for the presence of the transgene by PCR cloning, followed by sequencing. Expression of the transgene was assayed by RT-PCR using cDNA from seedlings grown in sand under high light.

Enzyme assays

Clones of *Dfr* and *F3'h* from both *I. purpurea* and *I. quamoclit* in the *pBI 1.4t* vector were expressed in *Escherichia coli*. Proteins were extracted from cell culture by disruption in 100–200 µl HEPES buffer with 10 µl of 100 mg ml⁻¹ lysozyme for 30 min on ice. F3'H activity was measured using a previously described protocol^{18,27}. Three reactions of each type were run. DFR activity was also assayed using a previously described method²⁸. Seven reactions of each type were run.

F3'h expression assays

To determine the expression levels of *F3'h* and *Dfr*, relative to other anthocyanin structural

genes, DNA slot-blots were constructed using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad) following the manufacturer's instructions. Anthocyanin genes obtained from *I. purpurea* and *I. quamoclit* were spotted onto a Zeta-Probe membrane (Bio-Rad) and fixed by exposure to ultraviolet light. ³²P-labelled cDNA probes were constructed from total RNA extracted from bud tips (the distal half of the bud) of *I. purpurea* and *I. quamoclit* that were 1 day before opening. Membranes were probed overnight at 65 °C and washed according to the manufacturer's instructions. The probe signal was captured and visualized using a phosphorimager (Molecular Dynamics).

Phylogenetic analysis

A phylogeny of 13 *Ipomoea* species (including those species shown in Fig. 1 plus *I. cairica* and *I. sepiaria* as outgroups) was determined based on the sequence of the internal transcribed spacers of nuclear ribosomal DNA (ITS). ITS sequences were obtained from references 9, 10 and 28. A maximum likelihood analysis based on the HKY85+G model was performed in PAUP* version 4.0b10²⁹. Branch support was estimated with 100 bootstrap replicates.

A detailed description of the methods is provided in Supplementary Information.

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