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Regulatory Genes Control a Key Morphological and Ecological Trait Transferred Between SpeciesMinsung Kim, *et al.**Science* **322**, 1116 (2008);

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Typically, early in leaf development, *SPCHpro::SPCH-YFP* is expressed in many small cells, but fluorescence diminishes as cells become morphologically distinct meristemoids (2) (Fig. 2K). Relative to equivalently staged *SPCHpro::SPCH-YFP* plants, *SPCH* variants with strong overproliferation phenotypes displayed increased numbers of YFP-positive cells early (Fig. 2L) and a trend toward increased protein persistence into meristemoid, GMC, and guard cell stages later (Fig. 2L and fig. S8). When expressed in a *CA-YODA* background (in which *SPCH* was predicted to be phosphorylated), full-length *SPCH-GFP* was not visible, nor could it promote stomatal development (figs. S2 and S9C). However, GFP-*SPCHΔ49*, which is missing phosphorylatable residues, was detectable and was able to drive asymmetric divisions (fig. S9D).

SPCH is closely related to two other bHLH transcription factors that control stomatal development. We have shown, however, that a novel domain of *SPCH* renders it uniquely subject to phospho-regulation by a group of kinases that have been demonstrated to transduce signals downstream of both cell-cell and plant-environment interactions (fig. S10). In general, the domain mediates repression of *SPCH* and does so in a quantitative manner; the more potential MAPK sites eliminated, the stronger the effect of the *SPCH* variant on stomatal development. However, one specific residue phosphorylated by MPK6, Ser¹⁹³, is required positively for activity, which suggests that the MPKTD is the integration site for complex regulatory inputs. The MPKTD is of unknown origin; it is not present in *Arabidopsis* proteins other than *SPCH* but is found in *SPCH*

homologs from a variety of plant species (fig. S11) (25), hence MAPK regulation of a stomatal bHLH is likely to be a widespread regulatory strategy.

SPCH solves a problem intrinsic to MAPK signaling—how is a set of generally used MAPKs recruited to a specific biological event?—by providing the important effector in a spatially and temporally restricted domain. From the perspective of stomatal control, *SPCH* guards the entry into the stomatal lineage, including the production of self-renewing cells that contribute to later flexibility in epidermal development. This important decision point is likely the target of developmental, physiological, and environmental regulation (26, 27). Coupling the MPK3/6 signaling module to the activity of *SPCH* provides a unified, yet tunable, output for the complex set of inputs from these sources. Understanding the elements of the MAPK/*SPCH* regulatory system that coordinate stomatal production with the prevailing climate may allow the production of food or bioenergy crops with the ability to respond and adapt to changes in that climate.

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Materials and Methods
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Regulatory Genes Control a Key Morphological and Ecological Trait Transferred Between Species

Minsung Kim,^{1,3*} Min-Long Cui,^{1*} Pilar Cubas,^{1,4*} Amanda Gillies,² Karen Lee,¹ Mark A. Chapman,^{2,5} Richard J. Abbott,² Enrico Coen^{1†}

Hybridization between species can lead to introgression of genes from one species to another, providing a potential mechanism for preserving and recombining key traits during evolution. To determine the molecular basis of such transfers, we analyzed a natural polymorphism for flower-head development in *Senecio*. We show that the polymorphism arose by introgression of a cluster of regulatory genes, the *RAY* locus, from the diploid species *S. squalidus* into the tetraploid *S. vulgaris*. The *RAY* genes are expressed in the peripheral regions of the inflorescence meristem, where they promote flower asymmetry and lead to an increase in the rate of outcrossing. Our results highlight how key morphological and ecological traits controlled by regulatory genes may be gained, lost, and regained during evolution.

Changes in regulatory genes have been implicated in a range of evolutionary transitions, operating from the micro- to macro-evolutionary scales (1–3). These changes have largely been considered as occurring independently within different species. However, it is

also possible that interspecific hybridization plays an important role in evolution (4). One consequence of such exchanges is that they may allow traits that are lost because of short-term selective pressures to be regained at a later stage. For example, members of the sunflower family (Asteraceae)

share a composite flower head, with each head comprising numerous small flowers (florets). In radiate species, the outer florets (ray florets) have large attractive petals, whereas the inner florets (disc florets) tend to be less conspicuous. Loss of the radiate condition has occurred multiple times within the Asteraceae, yielding nonradiate species with only disc florets (5). These events often correlate with shifts to higher levels of self-pollination (6), which should be favored when mates and/or pollinators occur at low densities (7). Partial or complete reversals from the nonradiate back to the radiate condition have been described (8), some of which appear to involve interspecific hybridization events (9). One explanation for such evolutionary gains and losses is that key regulatory genes control-

¹Department of Cell and Developmental Biology, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK. ²School of Biology, University of St. Andrews, St. Andrews KY16 9TH, UK. ³Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK. ⁴Centro Nacional de Biotecnología/CSIC, Darwin 3, Campus UAM, 28049 Madrid, Spain. ⁵Department of Plant Biology, University of Georgia, Athens, GA 30602, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: enrico.coen@bbsrc.ac.uk.

ling the trait can be modified and exchanged between species. To test this idea, we analyzed a well-documented case of interspecific exchange in *Senecio*.

Senecio vulgaris (Groundsel) (Fig. 1A) is an allotetraploid nonradiate species, native to Europe and occurring throughout temperate zones. Radiate forms of *S. vulgaris* (Fig. 1B) arose in the United Kingdom after the introduction of *S. squalidus* (Fig. 1C), a diploid radiate species originating from Sicily. *S. squalidus* was brought to the Oxford Botanic Garden about 300 years ago (10), from where it spread. As *S. squalidus* became dispersed throughout the United Kingdom, it crossed with *S. vulgaris*, yielding triploid hybrids (11). Although such triploids have low fer-

tility [seed set <0.02% (9)], some viable progeny occur as a result of backcrosses with *S. vulgaris*. Further rounds of backcrossing are thought to have led to introgression of the radiate trait into some populations of *S. vulgaris* (12, 13). The resulting polymorphism for the radiate condition in *S. vulgaris* is controlled by a single chromosome region or genetic locus, here termed *RAY* (14). Thus, the hypothesized introgression would have involved transfer of the *RAY* locus from *S. squalidus* into *S. vulgaris*.

The *RAY* locus affects floral symmetry. Disc florets have fivefold radial symmetry, whereas ray florets are bilaterally symmetrical (zygomorphic), having enlarged ventral (abaxial) and reduced dorsal (adaxial) petal lobes (Fig. 1D). *CYCLOIDEA*

(*CYC*) is a primary gene controlling floral symmetry in *Antirrhinum majus*, a species with entirely zygomorphic flowers (15). *CYC* encodes a DNA-binding protein belonging to the TCP family (16). Proteins from this family contain a conserved basic helix-loop-helix region that binds DNA (the TCP domain) and have a range of regulatory roles in plant development (16, 17). On the basis of *Antirrhinum* mutant phenotypes, it has been proposed that *CYC*-like genes might also control the development of ray florets in the Asteraceae (18). Supporting this theory, ectopic expression of a *CYC*-like gene from *Gerbera hybrida*, *GhCYC2*, has differential effects on ray and disc floret development in this horticultural species (19).

To determine whether *CYC*-like genes are involved in the *RAY* locus, homologs were isolated from *S. vulgaris*. RNA in situ hybridizations on radiate plants revealed that two of these genes, termed *RAY1* and *RAY2*, were specifically expressed in ray floret primordia (Fig. 2, A and B). *RAY1* and *RAY2* were expressed in a similar pattern in radiate (*R/R*) and nonradiate (*N/N*) genotypes (Fig. 2, A to D). However, the signal appeared to be stronger in *N/N* compared with *R/R*. This difference was confirmed by the expression levels in RNA from young flower heads (Fig. 2E). Stronger expression of the *N* alleles was also seen in RNA from *N/R* heterozygotes, suggesting that it reflects cis-regulatory changes (Fig. 2E). Phylogenetic analysis showed that *RAY1* and *RAY2* belong to a subfamily of TCP genes that include genes known to control flower asymmetry (clade in orange, Fig. 2F). *RAY1* and *RAY2* arose by a duplication event ~30 million years ago (20) that occurred early in the evolution of the Asteraceae, before divergence of *Helianthus*, *Gerbera*, and *Senecio* but after divergence of the Asteraceae from the Lamiales (Fig. 2F). *RAY2* appears to be orthologous to *GhCYC2* from *Gerbera*, which is also expressed preferentially in ray florets (19).

Fig. 1. Flower head of non-radiate *S. vulgaris* (A), radiate *S. vulgaris* (B), and *S. squalidus* (C). Scale bars, 3 mm. (D) Section through a flower head and two individual florets taken by optical projection tomography. Disc floret petals are outlined in orange, whereas ray floret petals are outlined in red (dorsal) or yellow (ventral).

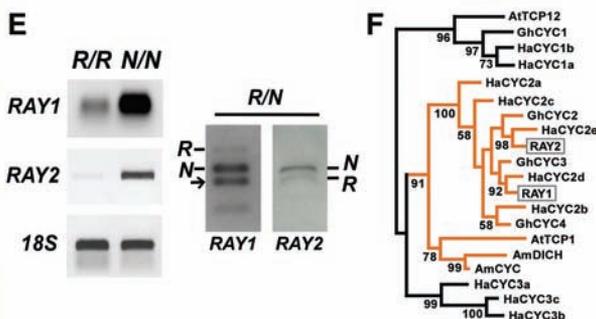
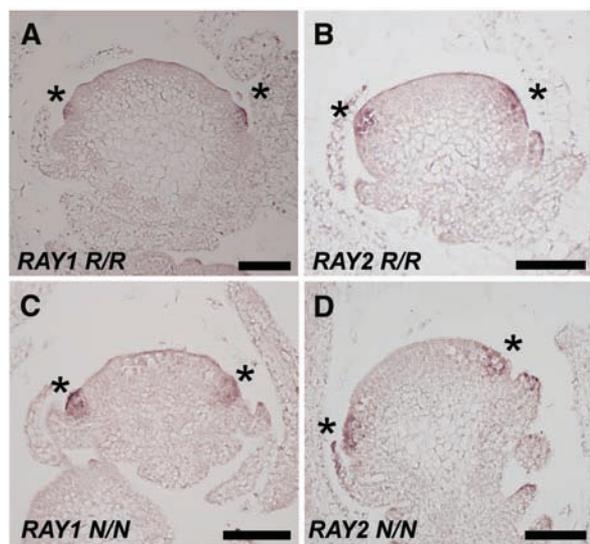
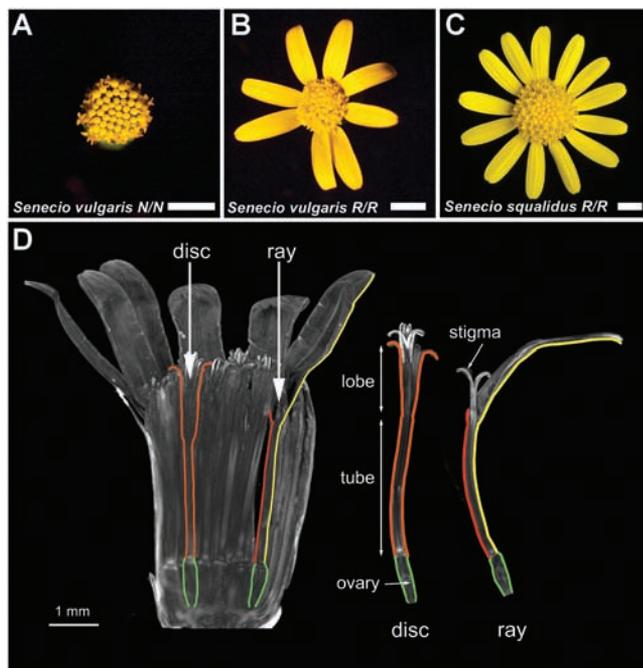


Fig. 2. (A) Expression pattern of *RAY1* in a longitudinal section of a developing radiate (*R/R*) flower head. (B) Expression of *RAY2* in radiate form. (C) Expression of *RAY1* in nonradiate (*N/N*) form. (D) Expression of *RAY2* in nonradiate form. In all cases, *RAY1* and

RAY2 are expressed in the outer floret primordia (marked by *). Scale bars, 100 μ m. (E) Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) showing *RAY1* and *RAY2* expression in young flower heads of *R/R*, *R/N* and *N/N* genotypes. A common band for *R* and *N* is indicated with an arrow in the *R/N* genotype. 18S RNA control is also shown. (F) Phylogenetic relationships between *RAY1*, *RAY2*, and a sample of other genes from the TCP family on the basis of a maximum likelihood analysis of amino acid sequences. *RAY1* and *RAY2* belong to a clade with *CYC* and *DICH*, which control floral symmetry in *Antirrhinum* (15). Bootstraps of 500 replicates (where greater than 50%) are shown. Species abbreviations and GenBank accession numbers are given in (30).

Fig. 3. (A) *RAY1*: a 412-bp band that cosegregates with *R* and a 238-bp and a 174-bp band with *N* in an F2 population. **(B)** *RAY2*: a 540-bp and a 156-bp band that cosegregate with *R* and a 696-bp band with *N*. PCR products of *RAY1* and *RAY2* coding regions were digested with *TaqI* and *EcoRI*, respectively. **(C)** Variable sites at *RAY1* and *RAY2* in and around the coding regions for the four haplotypes *N*, *NI*, *R*, and *R1*. Polymorphisms that are diagnostic for *N/NI* versus *R/R1* haplotypes are shown surrounded by black and white, respectively. All other polymorphisms are highlighted in gray. Nucleotide polymorphisms that cause amino acid changes are indicated with asterisks. Positions of deletions of TAAGGAAATCCAAACCCCA and ATAGAAA in the *RAY2-R1* haplotype are marked with arrows.

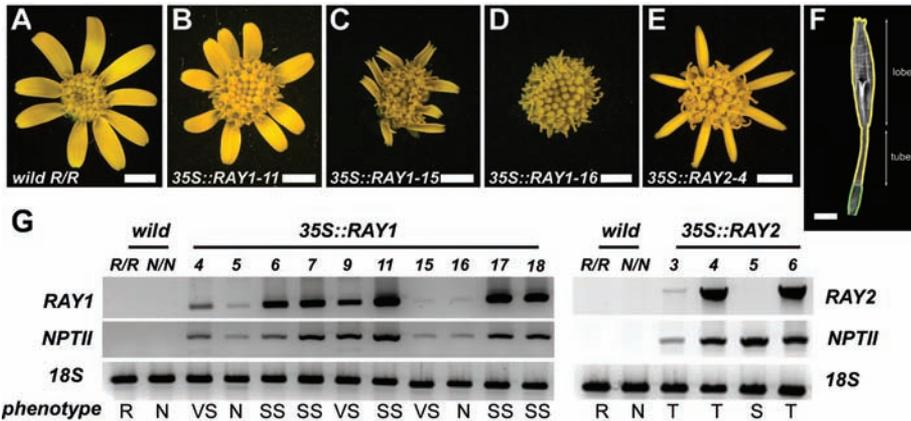
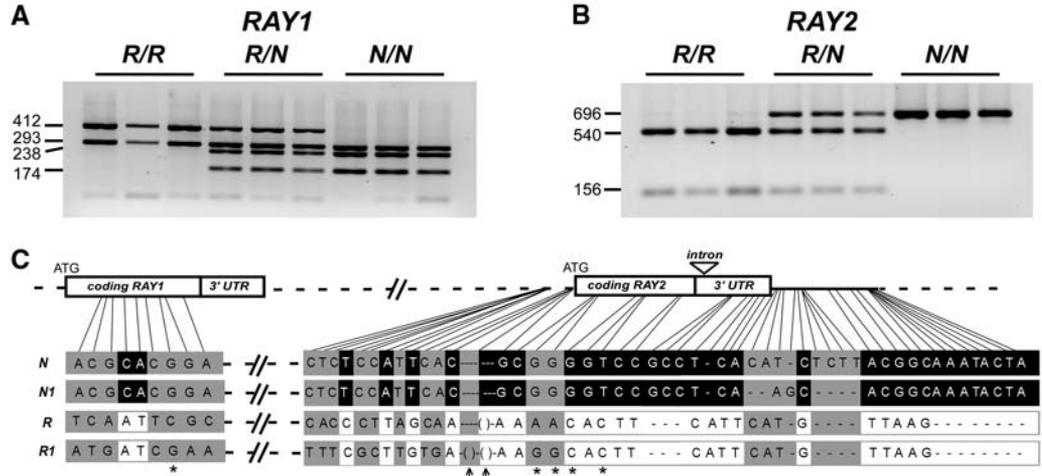


Fig. 4. (A) Flower-head phenotypes of *RR* nontransgenic control plant. **(B)** Flower head with slightly short rays from a transgenic plant overexpressing an internal fragment of *RAY1 N* allele coding sequences in a radiate (*R/R*) *S. vulgaris* background. **(C)** Flower head from a *RAY1* transgenic, as in (B), with very short ray florets. **(D)** Flower head from a *RAY1* transgenic, as in (B), giving no ray florets. **(E)** Flower head from a transgenic overexpressing the *RAY2 N* allele coding sequences in a radiate (*R/R*) *S. vulgaris* background, giving tubular ray florets. **(F)** Section through a ventralized ray floret, color-coded as in Fig. 1D. All transgenics are T1 generation, obtained by self-pollinating the primary transformants. **(G)** Semiquantitative RT-PCR showing expression levels of *RAY1* and *RAY2* in the transgenics, together with controls for 18S RNA and the kanamycin resistance gene (*NPTII*). R, normal radiate head; N, nonradiate or discoid head; SS, slightly short rays; S, short rays; VS, very short rays; T, tubular rays with ventralized petals. Scale bars, 2 mm [(A) to (E)] and 1 mm (F).

To determine whether *RAY1* or *RAY2* map to the *RAY* locus, their sequence was determined for parental radiate (*R/R*) and nonradiate (*N/N*) plants, allowing genotype-specific CAPS (cleaved amplified polymorphic sequences) to be designed (Fig. 3, A and B). Genotyping an F2 population derived from these parents revealed that both *RAY1* and *RAY2* segregated with flower-head phenotype, and we observed no recombinants in more than 700 plants. Linkage between *RAY1* and the *RAY* locus was further confirmed by bulk segregant analysis on *R/R* and *N/N* genotypes, and no recombinants were observed out of 2800 chromosomes. CAPS were also used to genotype accessions of radiate and nonradiate forms from various locations in the United Kingdom (table S1). In all cases, the *RAY1* and *RAY2* geno-

types matched the phenotype, confirming a tight association with each other and with the *RAY* locus. This was further confirmed by sequencing the *RAY* genes from several U.K. *S. vulgaris* accessions: All sequences from *R/R* genotypes were identical, while two minor variants were found among *N/N* genotypes, termed *N* and *NI* (Fig. 3C). Thus, both *RAY1* and *RAY2* are tightly linked and associated with *RAY*, and we were able to define three haplotypes: *N*, *NI*, and *R*. Because the radiate condition in *S. vulgaris* is thought to have originated from *S. squalidus*, *RAY1* and *RAY2* were also sequenced from various U.K. accessions of *S. squalidus*. This revealed two haplotypes, one identical to the *R*-haplotype of *S. vulgaris* and another that was a variant of *R* and was termed *R1* (Fig. 3C and table S1). These results provide

molecular proof that the radiate form of *S. vulgaris* arose through hybridization with *S. squalidus* plants and show that the *R*-haplotype was introgressed through this process.

Comparing the sequences of the haplotypes revealed several differences between *N/NI* and *R/R1* (Fig. 3C and fig. S1A). No diagnostic amino acid substitutions were found for *RAY1*, whereas two amino acid substitutions (S to F, D to E) were associated with the *N/NI* alleles of *RAY2*. The substitutions found in the *N* and *NI* alleles of *RAY2* were also found in the radiate species *S. vernalis* and *S. glaucus* (fig. S1B), making it unlikely that they are responsible for the nonradiate condition. Several diagnostic differences were also found in the 5' and 3' noncoding regions. As these represent only a limited sample of flanking sequence, it is likely that further differences would also be found in regions extending further out from the genes. Thus, the *N/NI* and *R/R1* haplotypes have accumulated multiple nucleotide differences since they diverged from their common ancestor and it is likely that the functionally important changes lie outside the *RAY1* and *RAY2* coding regions.

The rapid spread of the radiate trait in *S. vulgaris*, despite the strong reproductive barrier between *S. vulgaris* and *S. squalidus*, suggests that the introgression of the *R* haplotype may have been driven by selection, presumably acting on differences outside the *RAY1* and *RAY2* coding regions. However, testing for selection by analyzing sequence variation at the *RAY* locus is not straightforward because most selection tests assume a single interbreeding population (21), whereas introgression of *R* involved exchange between two divergent species separated by a major reproductive barrier.

As a further test of whether *RAY1* and *RAY2* play a role in ray floret development, we transformed radiate *S. vulgaris* with two constructs, both of which are driven by the constitutive 35S promoter (the radiate background was chosen because *N* is semidominant and is thought to

represent the derived condition). Expression of an internal fragment of the *RAY1* coding region (*N* allele) that includes the conserved TCP and R domains, yielded 10 independent transformants. Five of these plants produced slightly shorter ray florets (Fig. 4, A and B), three produced very short ray florets (Fig. 4C), and two had only disc florets (Fig. 4D), resembling nonradiate plants. These results suggest that overexpression of *RAY1* is repressing ray floret development, consistent with the higher levels of *RAY1* expression observed in *N/N* genotypes. However, the level of transgene expression did not correlate in a simple manner with the severity of the phenotype; transgenics with slightly short ray florets had higher levels of expression than the discoid transgenics (Fig. 4G). There was also no correlation with the endogenous levels of *RAY1* gene expression, because these levels were similar in transgenics with different phenotypes (fig. S1D). The variation in transgenic phenotype may reflect differences in the pattern of transgene expression, posttranscriptional interactions with the internal *RAY1* fragment used in the transformations, or perhaps promotive as well as inhibitory effects of *RAY1* on ray floret development. Whatever the explanation, the results indicate that *RAY1* plays a critical part in controlling ray versus disc floret identity.

Expression of the entire *RAY2* coding region (*N* allele) in the radiate background produced tubular ray florets in three independent transgenics (Fig. 4E). All petal lobes in these florets resembled the long ventral (abaxial) petal lobes of normal ray florets (Fig. 4, E and F), which suggests that *RAY2* is involved in promoting ventral identity in ray florets. Unlike ectopic expression of *GhCYC2* in *Gerbera hybrida* (19), disc floret development was not modified by expression of *RAY2*. This difference may reflect the fact that the innermost florets in *Gerbera hybrida* are not fully radially symmetrical (19) and may have some raylike character even in untransformed horticultural varieties.

We conclude that the *RAY* locus comprises a cluster of *CYC-like* genes that have played a key role in the evolution of the radiate condition.

Radiate development in the Asteraceae can be compared to the functionally analogous process controlling the development of individual flowers. For both systems, peripheral expression of regulatory genes is involved in establishing the identity of the attractive organs—*CYC* genes for radiate heads and MADS box genes for the flower (22–24). The main difference is that for radiate heads, peripheral expression is organized with respect to the inflorescence apex, whereas for floral organ identity it is organized in relation to the floral apex. Dorsoventral asymmetry within individual flowers is also established in relation to the inflorescence apex (18, 25). Thus, the ability of *CYC* genes to respond to a basic prepattern, centered on the inflorescence apex, could have led to their co-option and involvement in a key evolutionary innovation in the Asteraceae: radiate development.

The subsequent loss of the radiate condition in lineages of the Asteraceae most likely reflects tradeoffs involved in the evolution of breeding systems. Self-fertilization allows reproductive assurance under conditions where mates and/or pollinators are absent or occur at low densities (7) and may be favored, therefore, in colonizing and weedy species, such as *S. vulgaris* (26, 27), despite barriers imposed by inbreeding depression (28) and pollen discounting (29). However, a self-fertilization strategy may impose long-term limitations on responding to changing environmental conditions. Reintroduction of genes that promote outcrossing may therefore allow a self-pollinating species to revert and prevent extinction in the longer term. Our results therefore highlight the interplay between regulatory genes, development, and life history, and show how gene transfers between species may play an important part in the evolution of key ecological and morphological traits.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/322/5904/1116/DC1
Materials and Methods

Fig. S1

Table S1

References

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