Large-scale diversification without genetic isolation in nematode symbionts of figs


Diversification is commonly understood to be the divergence of phenotypes accompanying that of lineages. In contrast, alternative phenotypes arising from a single genotype are almost exclusively limited to dimorphism in nature. We report a remarkable case of macroevolutionary-scale diversification without genetic divergence. Upon colonizing the island-like microecosystem of individual figs, symbiotic nematodes of the genus *Pristionchus* accumulated a polyphenism with up to five discrete adult morphotypes per species. By integrating laboratory and field experiments with extensive genotyping of individuals, including the analysis of 49 genomes from a single species, we show that rapid filling of potential ecological niches is possible without diversifying selection on genotypes. This uncoupling of morphological diversification and speciation in fig-associated nematodes has resulted from a remarkable expansion of discontinuous developmental plasticity.

**RESULTS**

**Extreme disparity of forms in an island-like microecosystem**

Initial observations indicated that *Pristionchus* nematodes associated with figs show a high degree of morphological variation among adult forms, especially in their feeding structures. To determine the extent of this variation, we examined hundreds of nematode individuals and uncovered five distinct morphotypes associated with each of the three fig species mentioned above. Morphs associated with a single fig species differed qualitatively in their mouthpart complexity, or the total number of spatially independent structures or “cups” arising from the nematode stoma (17), including the presence and number of moveable teeth, serrated plates, and labial appendages (Figs. 1 and 2A). Furthermore, the shape and size of mouthparts for different morphs were strikingly disparate. Specifically, principal components analysis (PCA) of the geometric morphometrics of mouth shape and form (the latter including landmark-derived shape data + log centroid size) (18, 19) showed the five morphs in each fig species to occupy different parts of Procrustes morphospace with no or little overlap in shape, form, or both (Fig. 2B). Overall, the degree of discontinuous morphological variation that we observed between individuals from figs exceeded that observed between nominal genera of the same family (17) and among all other examined species of *Pristionchus* combined (Fig. 3), with some morphs exhibiting fantastic structures that, to our knowledge, have no analogs in other known nematodes (Figs. 1, C and E, and 2A).

**Morphological diversity from single genotypes**

Despite the disparity of adult nematode morphotypes discovered in individual figs, we surprisingly found that all *Pristionchus* nematodes isolated from a given fig species always had identical 18S ribosomal RNA (rRNA) sequences. This suggested that the diverse participants identified in each microecosystem belonged to a single species. To confirm species identity, we sampled the putative genetic diversity of multiple individuals of all morphotypes for each *Pristionchus* species in question. To do this, we sequenced the individual genomes of 49 specimens of *P. borbonicus*, and we sequenced rapidly evolving mitochondrial genes (20) for *P. sycomori* and *P. racemosae*. The genetic distance within each species was smaller than that between isolates of its selfing...
for corresponding regions in among different morphotypes, we inferred a genotype network of more
test whether genetic differences corresponded to any population structure
P. sycomori
drial DNA (mtDNA) maximal sequence divergence between isolates of
for individual lineages of (shown here), in the African and Oriental tropics. 

congener Pristionchus pacificus: nucleotide diversity for P. borbonicus
(\( \pi = 0.0002 \)), in particular, was an order of magnitude lesser than
for individual lineages of P. pacificus (\( \pi = 0.002 \)) (21), and mitochondri-
dna maximal sequence divergence between isolates of P. sycomori (\( \hat{\theta} = 0.01 \)) and P. racemosa (\( \hat{\theta} = 0.027 \) was lesser than that
for corresponding regions in P. pacificus (\( \hat{\theta} = 0.054 \) and \( \hat{\theta} = 0.064 \)). To test whether genetic differences corresponded to any population structure
among different morphotypes, we inferred a genotype network of more
than 10,500 single-nucleotide polymorphic sites for individuals of P. bor-onicus and mitochondrial haplotype networks for both P. sycomori and P. racemosa. No grouping of individuals by morphotype was observed,
and different morphs instead had similar or identical haplotypes (Fig. 2, C to E). These results indicate the absence of reproductive isolation between
different morphotypes within each species and that these morphotypes represent extreme intraspecific variation, possibly a polyphenism.

Condition dependence of diverse morphotypes
We next investigated the possibility that alternative morphotypes were
the result of a polyphenism, specifically of condition-dependent induc-
tion. Examination of nematode populations in figs of F. mauritiana
and F. sycomorus yielded four observations supporting polyphenism.
First, surveys of nematodes by fig phase revealed differences in ratios of
Pristionchus morphs between early and late interfloral figs. In figs that
had recently received their pollinators, only a single minute, micro-
vorinous morph of the associated Pristionchus species (morph V)
emerged (Fig. 4A). Sequencing of individual nematodes present on the
bodies of wasps that were captured exiting figs had confirmed that nematode
species observed in figs, including Pristionchus spp., were transmitted as
developmentally arrested (dauer) juveniles by the wasps (table S1).
Therefore, morph V of P. borbonicus and P. sycomori represents post-
dispersal adults that are brought to young, receptive figs. Second, in gener-
ations of nematodes following this colonization event, morph V was
never present, and instead, individuals of the four other morphs, includ-
ing three types of putative carnivores (morphs I to III), were found (Fig.
4A and table S2). Third, observations of very late interfloral-phase figs
revealed the occurrence of dauer larvae as progeny of morphs I to IV.
After passage through the fig-wasp vector, these dauer larvae form
phenotypes of morph V in early interfloral figs. Finally, we observed differ-
ences in ratios between sexes, suggesting that these morphs are induced from a single genotype in a sexually dimorphic manner (Fig. 4A), as
described for the mouth polyphenism of other Pristionchus species (22).

Evidence for resource polyphenism
To test whether different morphs were possible from the same nem-
atode line, we developed a protocol to successfully rear one generation of fig-associated nematodes in laboratory culture. Indeed, we found that
postdispersal adults (morph V) gave rise to an alternative nonpredatory
morph of unknown function (morph IV) under laboratory conditions.
Because nematodes could not be cultured further outside of the fig,
environmental cues inducing the other morphs could not be identified.
Taken together, surveys of nematode populations by fig phase and lab-
oratory experiments indicate that the multiple morphs of fig-associated
Pristionchus are the result of polyphenism and not genetic polymorphism.

Evidence for resource polyphenism
To test putative feeding specializations of individual morphs, we first
inspected the behavior of nematode morphs in the laboratory. We
found morphs I to III to be able to attack, kill, and feed on specimens
of Acrostichus sp. and Teratodiplogaster sp., which frequently co-occur
in the same figs (table S3). In addition, we screened the 49 genomes
of individual specimens of P. borbonicus for the presence of non-
Pristionchus nematode sequences. The presence of such sequences, pre-
sumably from the guts of sequenced individuals, would be an additional
indicator of predatory behavior in the wild. Indeed, several individual
genomes of morphs I and II showed an abundance of sequence reads
for Acrostichus sp. and Teratodiplogaster sp., whereas such reads were
not detected at high levels in morphs III to V (Fig. 4B). Thus, our meta-
genomic analysis distinguished morphs I and II as predators of other
nematodes. A more detailed analysis of the feeding specializations of
fig-associated Pristionchus awaits future studies of these minute animals
in their tropical settings.
Mcroevolution via alternative phenotypes

Having uncovered a novel polyphenism of unprecedented complexity, we then explored the implications of such plasticity for macroevolution, or change following speciation events. Geometric morphometrics and PCA revealed that the addition of multiple morphs in fig-associated nematodes was accompanied by rapid divergence among morphs. Not only did some added morphs fall completely outside the range of phenotypes in other Pristionchus species, some morphs were irreconcilable with any of those of other species (for example, morphs I and V of P. borbonicus and P. sycomori and morph ɛ of P. racemosae) (Fig. 3A). Thus, diverging selection has occurred so rapidly that homologies of most morphs between African and Vietnamese lineages, which

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Fig. 2. Genetic and phenotypic diversity in fig-associated Pristionchus nematodes. (A) Discrete adult morphs of P. borbonicus, P. sycomori, and P. racemosae. Homologous mouthpart regions are color-coded across morphs and species. (B) Projections of the first two principal components of mouth shape and form (PC1, principal component 1; PC2, principal component 2). Colors indicate morphs, as coded by labels in (A). Ovals represent a 95% confidence interval of the mean. (C) Genotype network for 49 individuals of P. borbonicus based on 10,500 single-nucleotide polymorphic sites. (D) Haplotype network for 77 individuals of P. sycomori based on sequences of ND1, Cytb, and COI genes. (E) Haplotype network for 104 individuals of P. racemosae based on sequences of the COI gene. (C to E) Each circle represents a unique genotype or haplotype and is colored according to morphs, as coded by labels in (A); if greater than 1, pairwise differences between joined haplotypes are denoted by hatch marks and vertices, with the latter also showing theoretical intermediate nodes introduced by the algorithm; the size of circles is proportional to the number of individuals sharing the same haplotype.
are both associated with a relatively narrow clade of host trees/wasps (23), can no longer be determined in terms of either morphometrics or presence of qualitative structures (Figs. 1, 2A, 3A, and 5). Given the degree of disparity between morphs of dubious homology, it is possible that diverging selection among morphs has been supplemented by the loss and gain of new or recurring alternative morphotypes, resulting in intraspecific divergence within lineage diversification (Fig. 3B).

**DISCUSSION**

**Multiplication of morphs and morphological diversification without speciation**

Here, we have revealed an extreme case of morphological diversification without genetic divergence or isolation. This intraspecific disparity was observed particularly in adult mouthparts, which in a phylum of animals relatively limited in external morphology are widely regarded as the primary trait for determining their position in the food web (24). Even before a complete account of the natural history of this unusual system has been achieved, based on morphological, behavioral, and metagenomic evidence, we speculate that the diversity of feeding forms reflects large-scale ecological diversification. For example, the small microbivorous morph (V) of *P. borbonicus* and *P. sycomori* may develop fast to compete for an ephemeral bacterial food source, a common strategy in nematodes with simple, tube-like mouths (25). In contrast, predatory morphs emerge in the following generations, coincident with the proliferation of cohabiting wasp-transmitted nematodes in the fluid-filled cavities retained within the figs. In the examined African figs, other nematode species include plant parasites (*Schistonchus* spp. and *Bursaphelenchus* spp.), wasp parasites (*Parasitodiplogaster* spp. and *Bursaphelenchus* spp.), and a range of other species such as *P. borbonicus* and *P. sycomori*.

**Fig. 3. Macroevolutionary disparity in fig symbionts relative to other *Pristionchus* species.** (A) PCA of the mouth shape of 18 species of *Pristionchus* (PC1, principal component 1; PC2, principal component 2). Colors indicate morphs. (B) Phylogeny of *Pristionchus* spp. inferred from 18S and 28S rRNA genes and 27 ribosomal protein genes. Lineages that have radiated following a presumptively single fig colonization event are shown in green. Bars show disparity between morphs of selected polyphenic species, which was measured as maximal Euclidean pairwise distance in the morphospace between morph means. The color of bars is proportional to the number of morphs observed for a given fig-associated species. St, Eu, and Mg represent the two to three possible morphs in other *Pristionchus* species. Asterisk represents node support of 100% posterior probability.
moreover, this intraspecific character displacement that has allowed niche partitioning within each nematode species is enabling *Pristionchus* nematodes to exploit the fig microecosystem in apparently novel, unknown ways, as indicated by at least one unprecedented form (morph ɛ) in the Vietnamese fig-associated *P. racemosae*. What makes this diversification remarkable is that the various morphotypes of all species were expressed at the same life stage, suggesting a hierarchy of developmental switches, in contrast to the ecological divergence that is typically limited to different developmental stages, as is common in zooplankton, holometabolous insects, and other nematodes.

**Conditions for diversification without genetic isolation**

The ability to produce any of five morphotypes at a single developmental stage, as evidenced by divergent feeding morphologies, is surprising in the absence of genetic isolation. The apparent uniqueness of this system therefore demands some explanation. First, a significant (albeit not exclusive) feature of *Pristionchus* fig symbionts is the ancestral presence of a feeding dimorphism (17) and its associated developmental switch (22), which may have disposed alternative morphs to relaxed selection on conditionally expressed phenotypes (26, 27). Indeed, the macroevolutionary maintenance of this dimorphism apparently led to increased rates of morphological evolution in this nematode family (17) and thus may also have facilitated the genetic variation needed to add successive switches to the mouth polyphenism. Alternatively, an ancestral polyphenism may have facilitated the recurrence of forms, allowing the rapid re-establishment of morphotypes (6) to result in a multiplied polyphenism. For example, morphs α and β of *P. racemosae* show characters (that is, stomatal rugae) that are absent from all other *Pristionchus* and its closest outgroups but are present in distant lineages of Diplogastridae (17), indicating the possibility of recurrence, if not uncanny convergent evolution. A second distinguishing feature is the bottlenecked vertical transmission of nematodes by their insect vectors. This mode of transmission presumably precludes the reliable transmission of multiple genetically distinct morphs to new figs, which may have promoted the maintenance of polyphenism over genetic assimilation of alternative phenotypes. Third, the small physical dimensions and spatially overlapping niches within figs may simply preclude genetic isolation in sympatry. In principle, character displacement between "allopatric" species (that is, species coevolved with fig and wasp...
hosts) with multiple phenotypes, which in this case would result from host switching by the wasp vector, could ultimately lead to the genetic assimilation of alternative forms in competing species (28). However, the repeated occurrence of five morphs suggests that any possible competition between otherwise allopatric species is not prevalent enough in this system to have led to the assimilation of alternative morphotypes in different species.

Conclusions
In summary, given the “empty niche” conditions predisposing an ecosystem to the trophic diversification of its colonists (in particular, low ecological complexity, previously limited immigration of species, and underutilized resources) (2), a pre-existing polyphenism, and vertically transmitted founder populations could have together facilitated macroevolutionary-scale diversification in fig-associated nematodes without lineage splitting. Despite the idiosyncrasies of the fig microecosystem, our findings support the prediction that “stem plasticity” in a lineage allows diversifying selection into multiple ecological roles (6,29). Thus, our findings reveal an alternative possibility to the principle that substantial phenotypic evolution only follows periods of speciation (1,30) and that developmental plasticity can lead to the multiplication of discontinuous novelties from a single genotype.

Taxonomy
*P. borbonicus* Susoy, Kanzaki, Herrmann, Ragsdale, and Sommer sp. nov. (Phylum Nematoda Potts, 1932; Order Rhabditida Chitwood, 1933; Family Diplogastridae Kreis, 1932).

**Etymology.** The species epithet is the Latin adjectival demonym for Île Bourbon, the former name for the island of the type locality of this species.

**Holotype.** Adult female of morph I is deposited in the University of California Riverside Nematode Collection (UCRNC).

**Paratypes.** Ten paratypes are deposited in the UCRNC, whereas 10 paratypes are deposited in the Swedish Museum of Natural History (Stockholm, Sweden).

**Locality.** Type specimens were collected at Grand Étang, La Réunion Island.

**Type host and vector.** Type specimens were isolated from within sycones of *F. mauritiana* Lamarck, 1788 (Moraceae), in association with *C. coecus* Coquerel, 1855 (Hymenoptera: Agaonidae).

**Description.** Body habitus, pharynx, female reproductive tract, male testis, and positions of ventral body openings are typical of *Pristionchus* nematodes (Fig. 1, C and D, Fig. 2A, and Fig. 5, A to E). Cuticle with fine annulation and thick, offset longitudinal alae are found on the entire body diameter, extending from the labial region to the tail, except for morph V, in which ridges are indistinct. Adults consist of five morphs. Because these morphs are of uncertain homology with respect to the eurystomatous and stenostomatous morphs of other *Pristionchus* species, we adopt a neutral nomenclature (that is, morphs I to V) for the new species:

**Morph I:** Body larger than those of other morphs, head widening just anterior to the labial region; lips large, lateral lips higher and wider than subventral and subdorsal lips; pre-gymnostomatal cheilostom twice as high as gymnostom, with anteriorly tapering rugae separated from each other for most of their length; gymnostom thick, barrel-shaped, overlapping cheilostom for most of height, finely serrated at anterior margin; pro-mesostegostom projecting into one dorsal and two subventral anteriorly serrated lobes; metastegostom with claw-like dorsal tooth, claw-like right subventral tooth, and large, coarsely serrated left subventral ridge extending dorsally past most of the dorsal tooth; postdental region (telostegostom) deeper ventrally than dorsally, sclerotized.

**Morph II:** Head narrowing just anterior to the labial region; with six offset lips, lateral lips smaller than subventral and subdorsal lips; labial region with conspicuous “beard” (a circumstomatal ring of thin cuticular filaments originating from anterior cheilostom); pre-gymnostomatal cheilostom same height as gymnostom, with closely spaced, tapering rugae, each tipped with several filaments and extending past the stomatal opening; gymnostom with thick walls, overlapping cheilostom for most of height, finely serrated at anterior margin; pro-mesostegostom projecting into one dorsal and two subventral anteriorly serrated lobes; metastegostom with claw-like dorsal tooth, claw-like right subventral tooth, and large, coarsely serrated left subventral ridge extending dorsally past most of the dorsal tooth; postdental region (telostegostom) deeper ventrally than dorsally, sclerotized.

**Morph III:** Head tapered, with small but distinct lips; stoma small; cheilostom with sharp anterior taper, with minute, tightly packed rugae not extending past the stomatal opening; gymnostom thin, barrel-shaped,
overlapping cheilostom for most of height, with fine punctuation; small projecting lobes in mesostegostom indistinct or absent; metastegostom with small dorsal and right subventral claw-like teeth and left subventral plate with two or three denticles, telostegostom as in morph I.

Morph IV: With six offset, separate, and thin-walled bulging lips, subdorsal and subventral lips larger than lateral lips, with pointed labial papillae; cheilostom large, vacuous, undivided; gymnostom thin, smooth, narrower than and barely overlapping cheilostom; stegostom simple, smooth, except for a small dorsal bulge with thickened cuticle; telostegostom narrow, sclerotized.

Morph V: Body thinner, smaller than other morphs, sometimes less than a third of the size of other morphs (that is, I and IV); stoma tube-like; cheilostom undivided, toroid-shaped; gymnostom slightly taller than cheilostom, barely overlapping the latter, anteriorly tapered; stegostom simple, smooth, except for a small dorsal bulge with thickened cuticle.

Eight pairs of genital papillae are arranged as <v1, v2, (C, v4), ad, (pd, Ph, v5 to v7)>, such that v3d is absent, v1 and v4 are each separated from v2 by ca. one anal body width, ad is equidistant between v4 and v5 to v7, and pd is clearly anterior to v5. Spicules have large manubrium, twisted lamina, and calomus anteriorly, with raised ventral ridge. Tail is filiform and long in males and females (ca. seven to nine cloacal/anal body widths) of morphs I to IV; tails of both sexes are short and conical in morph V.

Diagnosis. The new species is distinguished from all other Diplodogastridae, except for P. sycomori sp. nov. and P. racemosae sp. nov., by the presence of five morphs and by having morphs of laterally symmetrical and laterally asymmetrical stomatal structures in the same species. It is distinguished from all species, except for P. sycomori sp. nov., by the presence of a form (morph I) with a “bearded” inner stomatal opening, the lateral sides of which are axially longer than the dorsal and ventral margins. The new species is distinguished from P. sycomori sp. nov. by morph V having a thin versus posteriorly widened cheilostomatal toroid and by its unique 18S rRNA barcode (ca. 850-bp fragment defined by primers SSU18A and SSU26R; GenBank accession number KT188856), which differs from that of P. sycomori sp. nov. by 15 unique sites.

P. sycomori Susoy, Kanzaki, Kruger, Ragsdale, and Sommer sp. nov.

Etymology. The species epithet is the Latin genitive for that of its fig host, F. sycomorus.

Holotype. Adult female of morph I is deposited in the UCRNC.

Paratypes. Ten paratypes are deposited in the UCRNC, whereas 10 paratypes are deposited in the Swedish Museum of Natural History (Stockholm, Sweden).

Locality. Type specimens were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria (Pretoria, South Africa).

Type host and vector. Type specimens were isolated from within sycones of the syncerephorine fig F. sycomorus Linnaeus, 1753 (Moraceae), in association with C. arabisicus Mayr, 1906 (Hymenoptera: Agaonidae).

Description. Body habitus, pharynx, female reproductive tract, male testis, and positions of ventral body openings are typical of Pristionchus nematodes (Fig. 2A and Fig. 5, F to J). Cuticle with fine annulation and thick, offset longitudinal alae are found on the entire body diameter, extending from the labial region to the tail, except for morph V, in which ridges are indistinct. Adults consist of five morphs.

The nomenclature of morphs follows that used for P. borbonicus sp. nov. (that is, I to V), with which homologies of morphs could be assigned:

Morph I: Body larger than those of other morphs, head not tapering anterior to the labial region; lips large, lateral lips higher and wider than subventral and subdorsal lips; labial region with conspicuous beard (a circumstomatal ring of thin cuticular filaments originating from anterior cheilostom); pre-gymnostomatal cheilostom twice as high as gymnostom, with anteriorly tapering rugae separated from each other for most of their length; gymnostom thick, barrel-shaped, overlapping cheilostom for most of height, finely serrated at anterior margin; pros-mesostegostom projecting into one dorsal and two subventral anteriorly serrated lobes; metastegostom with claw-like dorsal tooth, claw-like right subventral tooth, and coarsely serrated left subventral ridge slightly overlapping the dorsal tooth; telostegostom deeper ventrally than dorsally, sclerotized.

Morph II: Head narrowing just anterior to the labial region; with six offset lips, lateral lips smaller than subventral lips; pre-gymnostomatal cheilostom with same height as gymnostom, with closely spaced, tapering rugae, each tipped with several filaments and extending past the stomatal opening; gymnostom thick, overlapping cheilostom for most of height, with fine anterior serration and with heavy punctuation on medial surface; pro-mesostegostom with small projecting lobes; meta- and telostegostom as in morph I.

Morph III: Head tapered, with clearly offset, squared lips; stoma small; cheilostom with sharp anterior taper, with minute, tightly packed rugae not extending past the stomatal opening; gymnostom thin, barrel-shaped, overlapping cheilostom for most of height, with fine punctuation; small projecting lobes in pro-mesostegostom indistinct or absent; metastegostom with small dorsal and right subventral claw-like teeth and left subventral plate with two to three denticles; telostegostom as in morph I.

Morph IV: With six offset, separate, thick-walled bulging lips, subdorsal and subventral lips larger than lateral lips, with pointed labial papillae; cheilostom large, vacuous, undivided; gymnostom thin, smooth, narrower than and barely overlapping cheilostom; stegostom simple, smooth, except for a small dorsal bulge with thickened cuticle; telostegostom narrow, sclerotized.

Morph V: Body thin, smaller than other morphs, sometimes less than a third of the size of other morphs (that is, I and IV); stoma tube-like; cheilostom undivided, toroid-shaped; gymnostom slightly taller than cheilostom, barely overlapping the latter, anteriorly tapered; stegostom simple, smooth, except for a small dorsal bulge with thickened cuticle.

Tail is filiform and long in males and females (ca. seven to nine cloacal/anal body widths) of morphs I to IV; tails of both sexes are short and conical in morph V.

Diagnosis. The new species is distinguished from all other Diplodogastridae, except for P. sycomori sp. nov. and P. racemosae sp. nov. (see below), by the presence of five morphs and by having morphs of laterally symmetrical and laterally asymmetrical stomatal structures in the same species. It is distinguished from all species, except P. borbonicus sp. nov., by the presence of a form (morph I) with a bearded inner stomatal opening, the lateral sides of which are triangular in section; gymnostom clearly taller than cheilostom, barely overlapping the latter, anteriorly tapered; stegostom simple, smooth, except for a small dorsal bulge with thickened cuticle.

Tail is filiform and long in males and females (ca. seven to nine cloacal/anal body widths) of morphs I to IV; tails of both sexes are short and conical in morph V.
**Materials and Methods**

**Nematode isolation**

To sample nematodes, we collected figs from *F. mauritiana* (La Réunion), *F. sycomorus* (South Africa), and *F. racemosa* (Vietnam). Individual figs were dissected, and all nematodes that were present in the cavity of the fig were collected. Nematodes that were identified as *Pristionchus* were individually transferred into a sterile buffer, which was developed to allow short-term culture of fig-associated nematodes. The composition of the buffer approximated the fig environment and was based on the chemical composition of raw figs (31). We then screened *Pristionchus* nematodes for their mouth phenotypes using a light microscope or stereomicroscope before proceeding with further experiments.

**Genome sequencing of individuals of *P. borbonicus***

We isolated adult individuals of *P. borbonicus* from freshly collected figs of *F. mauritiana*. Living nematode individuals were kept in a sterile buffer for 10 to 15 min to minimize possible surface contamination, after which the nematodes were individually transferred into a lysis solution and thereafter frozen until DNA extraction. The DNA of individual nematodes was isolated using the MasterPure DNA Purification Kit (Epicentre), following the manufacturer’s protocol for DNA purification from tissue samples. The DNA was sheared using a S220 focused ultrasonicator (Covaris) under the following operation conditions: 10 dc, 4 i, 200 cpb, and 80 s. Genomic paired-end libraries were prepared using Low Input Library Preparation Kit (Clontech Laboratories). Size selection was performed on final libraries using BluePippin and 1.5%
agarose gel cassettes (Sage Science). Paired-end libraries were sequenced using a HiSeq 2000 system (Illumina). mRNA libraries that were used for scaffolding (see below) were prepared from TRizol-lysed individuals. Total RNA was isolated from single specimens using the PureLink RNA micro kit (Invitrogen), after which complementary DNA (cDNA) was prepared using the SMARTer Ultra Low Input cDNA synthesis kit (Clontech Laboratories). cDNA was sheared using the following operation conditions: 10 dc, 4 i, 200 cpb, 40 s. Paired-end libraries were prepared and sequenced as described above.

Genome assembly and analysis of the molecular diversity of *P. borbonicus*

A draft genome assembly for *P. borbonicus* was generated from the DNA-sequencing data of a single individual using the SOAPdenovo assembler (32). Genomic and transcriptomic data from other individuals were used for additional scaffolding and gap closing (33, 34). After discarding contamination [contigs with more than half of the sequence showing a >95% identity to non-Diplogastrid sequences in the National Center for Biotechnology Information (NCBI) nucleotide database], the final assembly comprised 157,322 contigs spanning 155 Mb with an N50 contig size of 9.4 kb. Genomic data for 49 individuals were aligned to the *P. borbonicus* draft genome using a Burrows-Wheeler aligner (35). Single-nucleotide polymorphism (SNP) calling and diversity analysis were performed as previously described (21). Variable sites with coverage in all samples were extracted to reconstruct a TCS genotype network (that is, a phylogenetic network estimation using statistical parsimony) (36) using PopART 1.7 (http://popart.otago.ac.nz).

Haplotype diversity of *P. sycomori* and *P. racemosae*

We extracted DNA from living or DESS (a solution containing dimethyl sulfoxide, disodium EDTA, and saturated NaCl)–preserved individuals. Fragments of ND1, Cyth, and COI mitochondrial genes were amplified and sequenced for *P. sycomori*, and sequences of the COI gene were obtained for individuals of *P. racemosae*. The sequences were quality-checked and assembled using Geneious 6.1.4 and aligned using Muscle 3.8 (37). The concatenated alignment of ND1, Cyth, and COI genes for 77 individuals of *P. sycomori* contained 52 variable and 36 parsimony-informative sites. The alignment of the COI sequences of 104 individuals of *P. racemosae* had 98 variable and 54 parsimony-informative sites. Maximal mtDNA sequence divergence between isolates of the same species was estimated by p distance using MEGA 6 (38). For comparison, we estimated sequence divergence in corresponding regions of the mtDNA of 104 *P. pacificus* strains (21). TCS haplotype networks were inferred from those alignments using PopART 1.7.

Geometric morphometrics and analysis of disparity

To quantify the mouth morphology of different morphs and species of *Pristionchus*, we used a geometric morphometrics approach adapted from a previous study (17). Briefly, 13 two-dimensional landmarks were assigned to homologous structures of the nematode stoma (mouth), as informed by fine structural anatomy (39, 40). In addition to 11 landmarks used previously (17), two landmarks that marked the position of dorsal and ventral labial sensilla were recorded (fig. S1). Landmark coordinates were identified twice for each specimen, digitalized using tpsDig2 (41), and averaged for each specimen using MorphoJ (42). In total, our data set included 18 species and 450 individuals of *Pristionchus*. PCA of shape was performed on Procrustes-superimposed landmark coordinates, and PCA of stomatal form was performed on superimposed landmark coordinates + logarithm-transformed centroid size (18, 19). The proportion of total variance explained by the first three principal component axes for all analyses is given in table S4. Disparity between different morphs of polyphenic species of *Pristionchus* was calculated as the maximal Euclidean pairwise distance between morph means in Procrustes shape space using the MATLAB package MDA (43). Principal components that explained more than 10% of the total variance were included in the analysis.

Testing mode of transmission of nematodes

To confirm that *Pristionchus* nematodes were transmitted by wasps pollinating the figs, we brought ripe figs of *F. mauritiana* into the laboratory. The figs were kept at room temperature until a new generation of pollinator wasps (*C. coccus*) emerged from them. Emerging female wasps were collected in a trap and then preserved at −25°C until further examination. Ten wasps were placed in 2 ml of water with 0.1% Triton X and kept on a shaker for 1 hour to allow dispersal (dauer) juvenile nematodes to detach from the body surface of the wasp. Eighty-four nematode dauers were collected, and the DNA from individual dauers was extracted. We then amplified and sequenced a portion of the 18S rRNA gene, specifically an 830-bp diagnostic fragment developed for nematode identification (44, 45). The sequences were compared to those of nematodes previously isolated from figs of *F. mauritiana*.

Induction of alternative phenotypes

We collected figs of *F. sycomorus* of early interfloral phase and confirmed that only males and females of morph V of *F. sycomorus* were present. We then collected 20 gravid females from different figs, transferred them into a 3-cm Petri dish filled with the buffer described above, and provided them with a microbial food supply that enabled their reproduction on plates (that is, mixed bacteria extracted from host figs, 1 mg of *Escherichia coli* strain OP50, or 1 mg of *Erwinia typographi* strain P11-1, all of which resulted in the same induced phenotype). The females were allowed to lay eggs, which in 8 days developed into adult males and females. The mouth phenotypes of the offspring were then scored.

Metagenomic analysis of predation

Genomic data of 49 individuals of *P. borbonicus* were screened for 18S rRNA sequences using PhyloSift 1.0.1 (46). Paired reads that had similarity to eukaryotic 18S rRNA reference genes were extracted and assembled using Geneious 6.1.4. Resulting contigs were searched against the NCBI database using BLAST, and contigs that matched nematode 18S rRNA genes were identified. Those nematode 18S rRNA genes were used as references to which genomic reads of individual *P. borbonicus* nematodes were mapped, and the normalized coverage for non-*Pristionchus* nematode and *Pristionchus* 18S rRNA was estimated. Normalized coverage was calculated as follows: (coverage of non-*Pristionchus* 18S rRNA/coverage of *Pristionchus* 18S rRNA) × 3000, where 3000 is the average coverage of the 18S rRNA gene for *P. borbonicus* individuals.

Phylogeny of *Pristionchus*

We inferred the phylogeny of 28 described and 7 new species of *Pristionchus*. In addition to the three species described herein, we included species isolated from four other species or populations of *Ficus* (*Sycomorus*, section *Sycomorus* [*Ficus sycomorus* in South Africa (*Pristionchus* sp. 35), *F. racemosa* in Australia (*Pristionchus* sp. 36), and *Ficus variegata* in
Composition of buffer for short-term maintenance of fig nematodes

The buffer was composed of the following: potassium chloride, 55 mM; dipotassium phosphate, 3.6 mM; calcium chloride dihydrate, 8.7 mM; magnesium sulfate, 6.65 mM; ascorbic acid, 57 µM; sorbitol, 1.1 mM; ribitol, 1.3 mM; mannitol, 1.1 mM; citric acid, 0.5 mM; malic acid, 0.75 mM; BME Vitamin Solution (100×) B 6891 (Sigma-Aldrich), 10 ml/liter; trace metals solution (1.86 g of disodium EDTA, 0.69 g of FeSO₄·7H₂O, 0.2 g of MnCl₂·4H₂O, 0.29 g of ZnSO₄·7H₂O, and 0.025 g of CuSO₄·5H₂O, in 1 liter of H₂O), 10 ml/liter. The pH of the buffer was adjusted to 7.5 with potassium hydroxide, and the buffer was sterilized by filtering (Nalgene 295–4545).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/1/e1501031/DC1

Fig. S1. Positions of 13 two-dimensional landmarks recorded for the stoma of fig-associated Pristionchus species.

Table S1. Nematae dauerers transmited by wasps (C. coecus) emerging from ripe figs of F. mauniana.

Table S2. Ratio of morphs in a single fig sycony for males and females of P. sycomorri.

Table S3. Nematae species isolated from sycones of F. mauniana, F. sycomorri, and F. racemosa.

Table S4. Proportion of variance explained by the first three principal component axes in the PCA of mouth form and shape for fig-associated Pristionchus species.

REFERENCES AND NOTES


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