The Role of DAF-21/Hsp90 in Mouth-Form Plasticity in *Pristionchus pacificus*

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Abstract

Phenotypic plasticity is increasingly recognized to facilitate adaptive change in plants and animals, including insects, nematodes, and vertebrates. Plasticity can occur as continuous or discrete (polyphenisms) variation. In social insects, for example, in ants, some species have workers of distinct size classes while in other closely related species variation in size may be continuous. Despite the abundance of examples in nature, how discrete morphs are specified remains currently unknown. In theory, polyphenisms might require robustness, whereby the distribution of morphologies would be limited by the same mechanisms that execute buffering from stochastic perturbations, a function attributed to heat-shock proteins of the Hsp90 family. However, this possibility has never been directly tested because plasticity and robustness are considered to represent opposite evolutionary principles. Here, we used a polyphenism of feeding structures in the nematode *Pristionchus pacificus* to test the relationship between robustness and plasticity using geometric morphometrics of 20 mouth-form landmarks. We show that reducing heat-shock protein activity, which reduces developmental robustness, increases the range of mouth-form morphologies. Specifically, elevated temperature led to a shift within morphospace, pharmacological inhibition of all Hsp90 genes using radicicol treatment increased shape variability in both mouth-forms, and CRISPR/Cas9-induced *Ppa-daf-21/Hsp90* knockout had a combined effect. Thus, Hsp90 canalizes the morphologies of plastic traits resulting in discrete polyphenism of mouth-forms.

Key words: **plasticity, canalization, robustness, heat-shock proteins, *Pristionchus pacificus*.**

Article

Introduction

Developmental (phenotypic) robustness, often referred to as canalization, describes the property of organisms to produce an unchanged phenotype despite environmental perturbations (Wagner 2005). Heat-shock proteins of the Hsp90 family have been implicated in developmental robustness and were shown to act through Piwi proteins (Gangaraju et al. 2011). They are thought to have two potential functions in this context: first, they might suppress stochastic phenotypic variation and second, they are thought to facilitate unchanged development in the presence of mutations, rendering such mutations cryptic (Rutherford 2003; Siegal and Leu 2014). Hsp90 was shown to buffer phenotypic variation in diverse organisms, such as *Drosophila*, *Arabidopsis*, and cavefish, indicating that chaperone function is conserved throughout multicellular organisms (Rutherford and Lindquist 1998; Queitsch et al. 2002; Rohner et al. 2013). Despite the fact that heat-shock proteins are recognized to provide a molecular mechanism for developmental robustness, many conceptual issues are still controversially discussed in literature (Siegal and Leu 2014). In the following, we mainly use the term "developmental robustness" and investigate its relationship to plasticity.

On the surface, it appears that developmental robustness is an opposing evolutionary constraint to phenotypic variation. However, it has been proposed that developmental robustness facilitates morphological evolution because buffered phenotypic variation may be subsequently revealed and become a substrate for natural selection, a theory that is still contentious. This concept is similar to theories regarding the contribution of phenotypic plasticity as a pulse of morphological adaptation (Susoy et al. 2015). In phenotypic (developmental) plasticity, a single genotype can produce distinct phenotypes in response to environmental conditions. This ability is argued to facilitate evolutionary change by allowing more flexible adaptation and providing additional substrate for selection (Pigliucci and Hayden 2001; West-Eberhard 2003). When conditions ultimately favor the fixation of one morph over the other, built-up genetic variation allowed by having two options is released to one morph, leading to rapid evolution as seen in nematodes (Susoy et al. 2015).

The conceptual difficulty in studying the interplay between developmental robustness and plasticity lies in plasticity being viewed as sensitivity and robustness as insensitivity to the environment. Still, both developmental robustness and plasticity are essential systemic features of organisms and both have been speculated to accelerate evolutionary change (West-Eberhard 2003; Wagner 2005). Here, we use an example of discrete plasticity (polyphenism) in clonally propagating

nematodes to overcome existing conceptual and practical limitations for addressing the relationship between the two phenomena.

The nematode *Pristionchus pacificus* is a distant relative of *Caenorhabditis elegans* and has been developed as a laboratory model for comparative and evolutionary studies (Sommer 2015). It shares with *C. elegans* its hermaphroditic mode of reproduction resulting in isogenic lines and the availability of forward and reverse genetic, genomic and transgenic tools (Sommer and McGaughran 2013). In addition, *P. pacificus* is an omnivorous feeder that predates on other nematodes and generates feeding structures consisting of moveable teeth that occur in two alternative morphs (Bento et al. 2010): adult animals develop into either "narrow-mouthed" stenostomatous (St) or "wide-mouthed" eurystomatous (Eu) morphs after an irreversible decision in postembryonic development. St animals have a narrow stoma and a flint-like dorsal tooth, whereas the ventrosublateral tooth is replaced by a cuticular ridge with a minute denticle (fig. 1*A* and *B*) . In contrast, Eu animals have a broad stoma with a claw-like dorsal tooth and a hooked right ventrosublateral tooth (fig. 1*C* and *D*). Although Eu animals can kill and feed on nematode prey, St animals are strict microbial feeders under laboratory conditions (Wilecki et al. 2015).

Importantly, the two described phenotypes in *P. pacificus* are discrete. Varying the levels of environmental factors, such as applying a gradient of pheromone concentrations, never results in formation of intermediate mouth-forms, but instead shifts the ratio between the numbers of Eu and St individuals (Bose et al. 2012). This indicates that the developmental switch leading to the formation of one or the other morph operates in a threshold-dependent manner and that the polyphenism has a discontinuous reaction norm (see hypothetical representation in fig. 2). Consequently, the Eu:St ratio is subject to apparent stochasticity. Under standard laboratory conditions, the proportion of Eu animals in the wild-type strain RS2333 varies between 70% and 90% (Ragsdale et al. 2013; Serobyan et al. 2013; Susoy and Sommer 2016). Together, the discreteness and simultaneous production of both morphs make mouth-form polyphenism in *P. pacificus* a unique study system to investigate whether the same mechanism that guards development against stochastic perturbations is involved in maintaining polyphenisms.

Thus, we set out to determine whether heat-shock proteins, which were shown to act as capacitors of morphological evolution, play a role in the execution of correct mouth-forms in *P. pacificus*. In theory, four different scenarios are possible (fig. 2). First, as a null hypothesis, interference with heat-shock proteins might not affect mouth-form plasticity. Second, elimination of developmental robustness might lead to the occurrence of intermediate mouth-forms, resulting in one continuous distribution of morphologies and thus, the disappearance of the polyphenism (fig. 2*C* and *D*). Such a finding would suggest that the presence of the alternative mouth-forms requires developmental robustness. Alternatively, the elimination of developmental robustness might third, shift (fig. 2*E*) or fourth, expand the respective distribution of morphologies (fig. 2*F*), without eliminating the polyphenism. To distinguish between these partially overlapping scenarios, we applied geometric morphometrics approaches to detect such changes in the mouth shape of *P. pacificus*, which was subjected to conditions known to affect developmental buffering, ranging from a generic stress, such as exposure to elevated temperatures, to knock-out of a specific gene encoding Hsp90. We observed two kinds of predicted changes, shift and expansion of the distribution of morphologies, and conclude that Hsp90 activity canalizes mouth-form plasticity in *P. pacificus*.

Results

Investigation of the robustness of a polyphenic trait requires formal proof that shape variation of individual morphs is limited and that they are indeed discrete. To validate if mouth morphology in *P. pacificus* satisfies these criteria, we used geometric morphometric analysis of 20 landmarks in the stoma (fig. 1*E*) (Ragsdale and Baldwin 2010; Susoy, et al. 2015). In short, their coordinates were measured and then centered, rotated and scaled, resulting in Procrustes alignment that was used to conduct principal component analysis (PCA) (Dryden and Mardia 1998; Mitteroecker et al. 2004). Ordination of sets of landmarks representing individual *P. pacificus* RS2333 wild-type animals resulted in distinct distributions of morphologies for Eu and St animals without any overlap, thus representing a baseline to study the relationship between plasticity and robustness (fig. 1*F*).

To examine a potential role of heat-shock proteins in the execution of the Eu and St morphs, we analyzed the effect of elevated temperature on mouth morphology, as such treatment compromises the heat-shock machinery (Rutherford and Lindquist 1998). Animals reared at 28 °C, the highest temperature at which *P. pacificus* RS2333 continuously reproduces (Leaver et al. 2016), displayed evidently abnormal mouth morphology, represented as a shift within morphospace in relation to control conditions (fig. 3*A*). Morphological disparity estimated as sum of variances increased in comparison to the control group, but the change was small and, in case of Eu animals, not statistically significant (fig. 3*B*). Importantly, both mouth-forms were still clearly distinguishable, and thus the discreteness of the polyphenism remained clearly visible. Even though elevated temperature presumably produces a very general stress, this result provided the first indication that heat-shock proteins may be involved in limiting the distribution of mouth morphologies in *P. pacificus* and justified application of a more specific treatment.

Next, we used pharmacological inhibition with radicicol to reduce Hsp90 function in a targeted manner, as it was previously applied in *Drosophila*, *Arabidopsis* and cave fish (Rutherford and Lindquist 1998; Queitsch et al. 2002; Rohner et al. 2013). Like in heat-stress treatment, many animals exhibited abnormal mouth morphology. However, the most pronounced effect detected through morphometric analysis was not a shift within morphospace, as seen in temperature treatment, but an increase in shape variability, evident from almost 2-fold significant increase in morphological

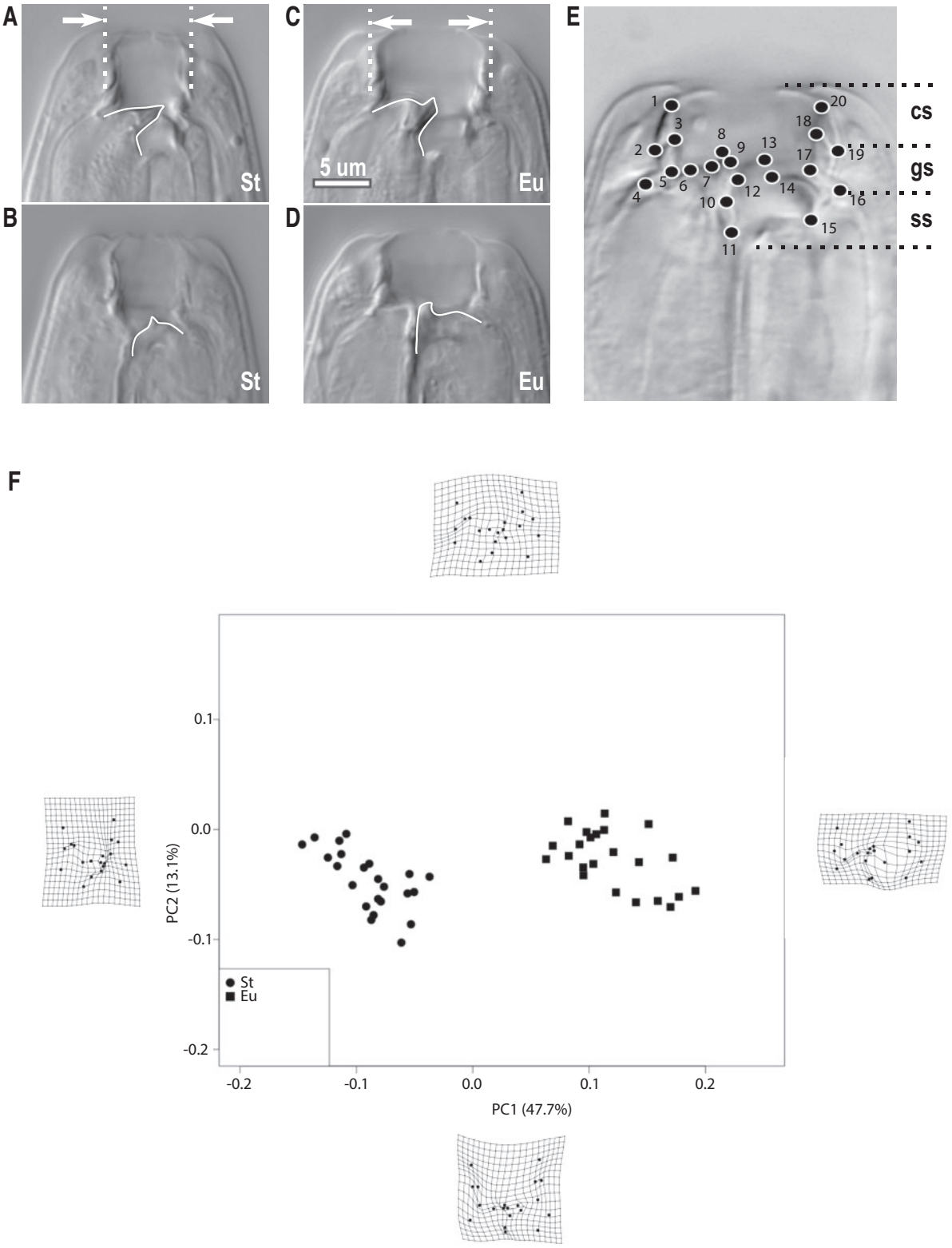

Fig. 1. Mouth dimorphism in *Pristionchus* nematodes. (*A*) St morph, view in sagittal (median) plane. Stoma is narrow and dorsal tooth is flint-shaped. (*B*) St morph, view in right parasagittal plane. Cuticularization of the right ventrosublateral part of stegostom is represented by a ridge with a minute denticle. (*C*) Eu morph, view in sagittal (median) plane. Stoma is wide and dorsal tooth is claw-shaped. Scale bar equally applies to images A–D. (*D*). Eu morph, view in right parasagittal plane. The right ventrosublateral part of stegostom contains a hooked tooth. (*E*). Landmarks in the stoma, which were used for geometric morphometric analysis (see supplementary table 1, Supplementary Material online for their detailed description). cs, cheilostom; gs, gymnostom; ss, stegostom; (*F*) PCA ordination of sets of landmarks in Eu and St animals, which shows clear separation of the two morphs. Each point represents one individual. Deformation grids depict differences between the shape coordinates of the corresponding extreme ends of the PC1 and PC2 axes and the mean shape of all specimens.

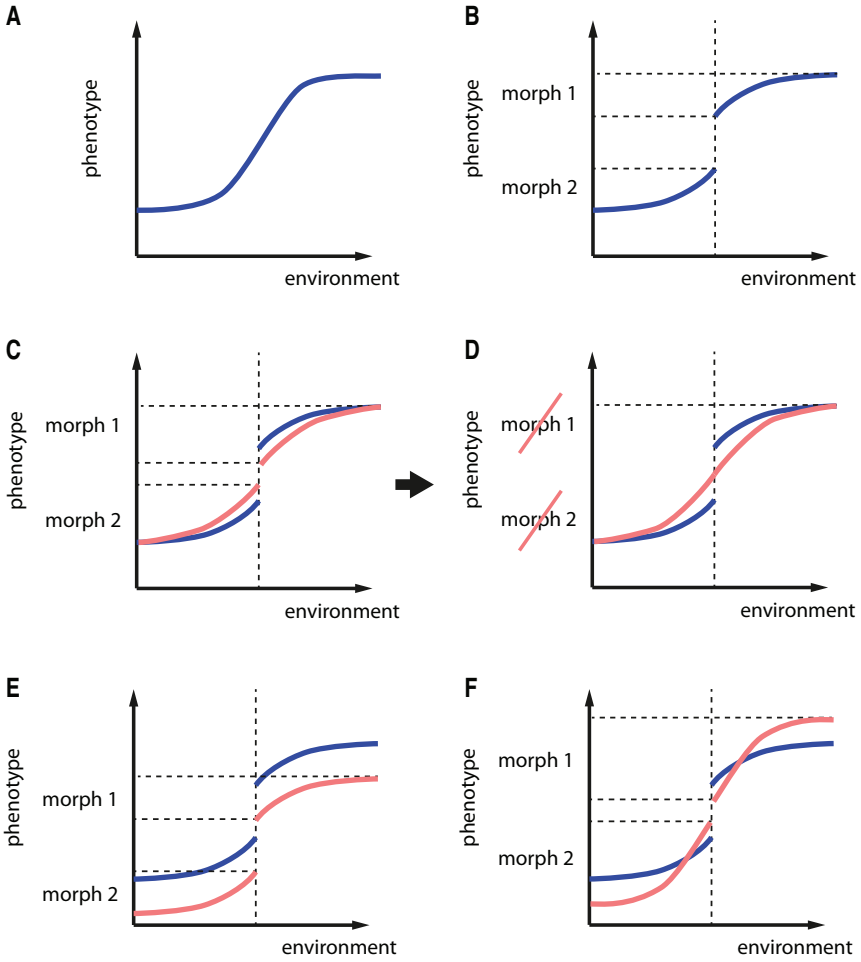

Fig. 2. Hypothetical scenarios of change in reaction norm of a dimorphic trait upon reduction of developmental robustness through suppression of heat-shock protein activity. (*A*) Reaction norm of a continuous trait. (*B*) Reaction norm of a polyphenic trait (i.e., plastic trait with discrete phenotypes). (*C*) Occurrence of individuals with intermediate phenotypes leading to narrowing the gap between the two morphs. (*D*) Extreme case of C, whereby the two reaction norms merge and polyphenism is negated. (*E*) Shift in reaction norms. (*F*) Expansion of reaction norms without loss of polyphenism.

disparity (fig. 3*A* and *B*). However, the distributions of morphologies in St and Eu animals are still fully separated. This finding suggested that Hsp90 proteins limit the range of possible mouth morphologies in *P. pacificus* from stochastic variation.

Next, we validated the previous finding by knocking out an Hsp90-encoding gene. In *C. elegans*, three genes encode Hsp90-type proteins, all of which have *P. pacificus* 1:1 orthologs (fig. 4*A*). *enpl-1* encodes an endoplasmic reticulum associated chaperone orthologous to GRP94, whereas *trap-1* is the ortholog of mitochondrial Hsp75/TRAP1 in vertebrates (Johnson 2012). The only Hsp90 gene that was identified in genetic screens in *C. elegans* is *daf-21* (Thomas et al. 1993; Birnby et al. 2000). It is required for larval development, chemosensory behavior and has a **da**uer **f**ormation **c**onstitutive (Daf-c) phenotype when mutated. DAF-21 has the highest sequence similarity to the *Drosophila* protein *Hsp83*, which has been identified as capacitor for morphological evolution (Rutherford and Lindquist 1998). Using CRISPR/Cas9 engineering we targeted exon 2 of *Ppa-daf-21* and were able to isolate a mutant strain (*tu519*) with a 10 bp insertion, representing a presumptive "loss-of-function" allele (fig. 4*B*). *Ppa-daf-21(tu519)* mutant animals are not Daf-c, but they are small, clear, locomotion-defective, vulvaless, and sterile (fig. 4*C* and *D*); therefore, cultures can only be kept as heterozygous carriers. These pleiotropic phenotypes of *Ppa-daf-21* mutant animals are consistent with the pleiotropy observed in flies (Rutherford and Lindquist 1998). The comparison between *Cel-daf-21* and *Ppa-daf-21* mutant phenotypes reveals multiple similarities, such as sterility, locomotion defects and abnormal gonad development, but also important differences, such as the absence of the Daf-c phenotype in *P. pacificus*. This observation follows the principal of developmental systems drift, which has previously been seen in vulva formation and dauer development between these two nematodes (Wang and Sommer 2011; Sommer and Mayer 2015).

With regard to mouth-form polyphenism, homozygous mutant animals exhibit severely distorted mouth morphologies (fig. 3*C*), an observation supported by a considerable shift within morphospace and a significant increase in morphological disparity (fig. 3*D* and *E*). Although a classification of

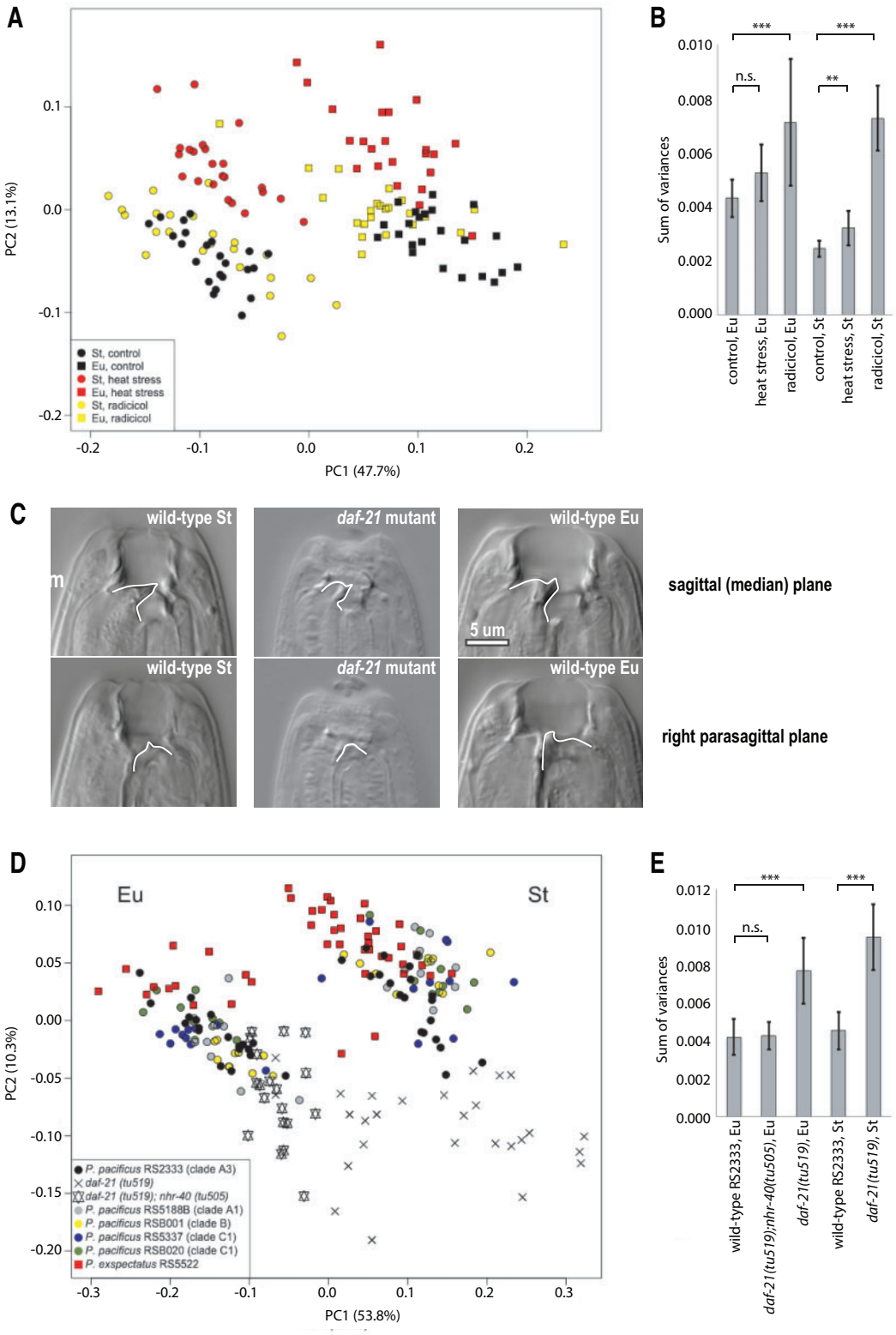

Fig. 3. Change in mouth morphology of *P. pacificus* upon impediment of function of heat-shock proteins. (*A*) PCA ordination of sets of landmarks representing control individuals (identical to the ones depicted in fig. 1*F*) and individuals exposed to heat stress and treatment by radicicol, a pharmacological inhibitor of Hsp90. (*B*) Morphological disparity in different groups shown in the PCA ordination in panel A. Error bars show SD. n.s., not significant (*P*-value $>$ 0.05); ***P*-value $<$ 0.01; ****P*-value $<$ 0.001. (*C*) An example of distorted mouth in a *Ppa-daf-21/Hsp90* mutant, alongside a St and a Eu wild-type animal. Scale bar applies to all six images. (*D*) PCA ordination of sets of landmarks from the laboratory *P. pacificus* strain RS2333, the *Ppa-daf-21* and *daf-21; nhr-40* mutants, four wild isolates of *P. pacificus* and a wild isolate of *P. exspectatus*. (*E*) Morphological disparity in wild-type *P. pacificus* and *daf-21* mutant. Error bars show SD. n.s., not significant (*P*-value $>$ 0.05), ****P* value $<$ 0.001.

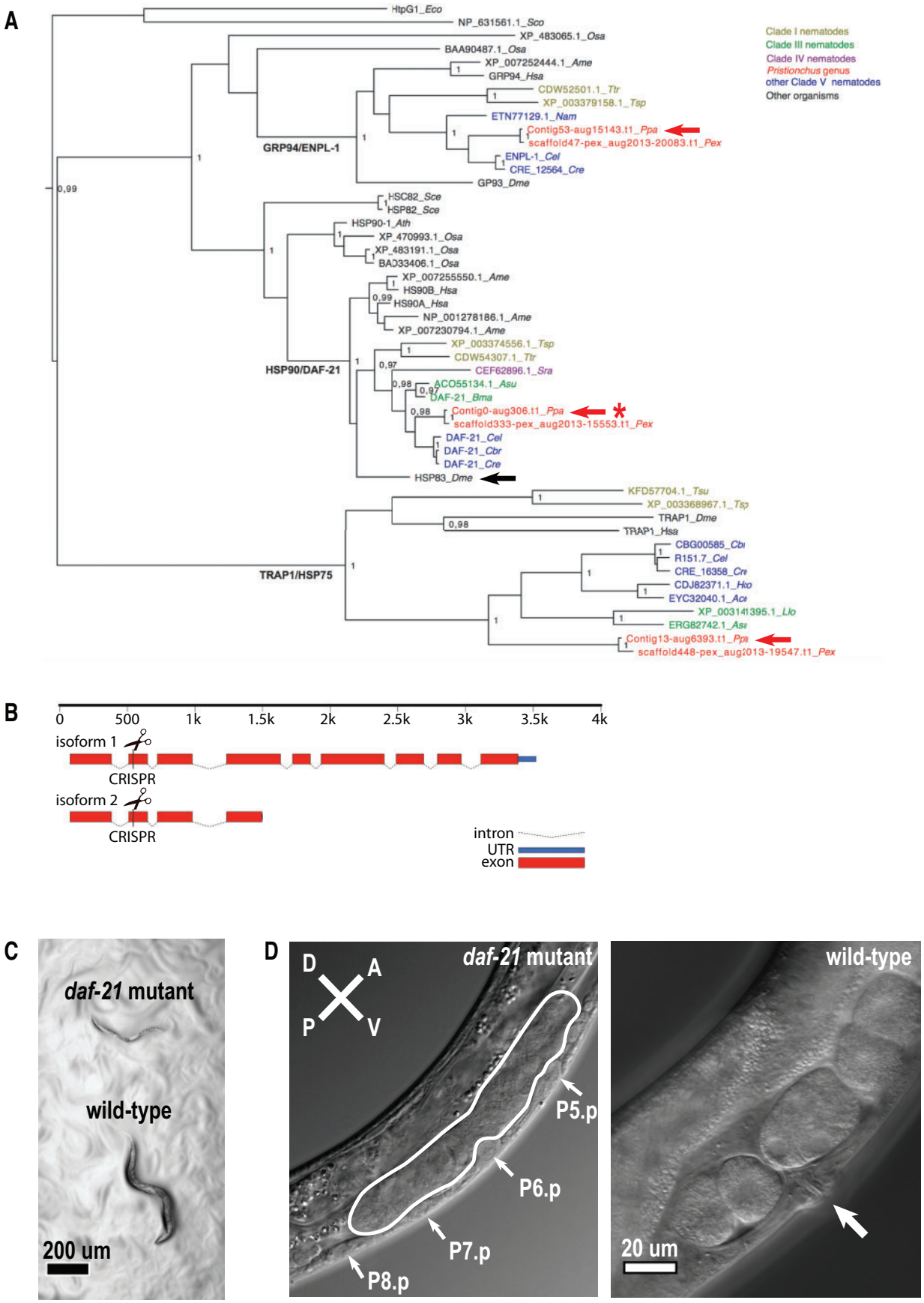

FIG. 4. *daf-21/Hsp90* knockout in *P. pacificus*. (*A*) Maximum likelihood tree of Hsp90 homologs in animals. Black arrow indicates the *D. melanogaster* protein used in previous studies (Rutherford and Lindquist 1998). Red arrows show predicted proteins in *P. pacificus*. Ppa-DAF-21 is marked with an asterisk. Branch support values under 0.97 are considered not significant and were thus removed from the tree.

animals into Eu and St morphs was still possible, morphologies were severely affected and some individuals displayed combined characteristics of different morphs to a certain degree (e.g., narrow mouth, like in St, and hooked right ventrosublateral tooth, like in Eu). The data shown in figures 3*D* and *E* indicate that the *Ppa-daf-21* knockout led to a shift within morphospace and an increase in shape variability and thus a combined effect when compared to temperature and radicicol treatments. Therefore, reducing the activity of HSP90-type proteins indeed affects the distribution of morphologies of both mouth-forms, indicating that developmental robustness is required for the discreteness of the polyphenism. Finally, it is worth noting that *Ppa-daf-21* mutant animals show altered ratios of Eu:St animals. Specifically, we found that 52% of *Ppa-daf-21* animals were Eu, while wild-type RS2333 had 90% Eu individuals ($n = 21$, $\chi^2 = 7.47$, $P = 0.006$).

Next, we wanted to know if the knockout of *Ppa-daf-21* still affects mouth morphology when one of the morphs is fixed and no dimorphism occurs. We crossed *daf-21(tu519)* with *nhr-40 (tu505)*, a mutant that produces only Eu individuals (Kieninger et al. 2016), and performed morphometric analysis on the *daf-21(tu519);nhr-40(tu505)* double mutant. The distribution of morphologies in such animals was still distinct from that of wild-type individuals (fig. 3*D*); however, mouth shape of the double mutant was less diverged from the wild type than mouth shape of the *daf-21* single mutant. Interestingly, no significant increase in morphological disparity was observed in double mutants, in contrast to the single mutant (fig. 3*E*). Together, these findings demonstrate that limiting a polyphenic trait to one morph also limits the amount of variation that can be released when Hsp90 activity is reduced.

Finally, capacitance theory suggests that a temporary decrease in buffering by heat–shock proteins (e.g., during stress) could expose cryptic morphological variants to selection, which would accompany microevolutionary processes and/or speciation (Rutherford and Lindquist 1998; Rutherford 2003; Hermisson and Wagner 2004; Paaby and Rockman 2014). Therefore, we set out to determine if the amount of morphological change induced by the mutation in *Ppa-daf-21* is similar to existing morphological divergence among various wild isolates of *P. pacificus* and between *P. pacificus* and its sister species *P. exspectatus* (Kanzaki et al. 2012; Rodelsperger et al. 2014). We compared *P. pacificus* RS2333 and *Ppa-daf-21* mutants with four wild isolates that cover the complete worldwide diversity of *P. pacificus* and with one strain of *P. exspectatus* (fig. 3*D*). Morphologies of most *P. pacificus* strains demonstrated considerable overlap, but some of them (e.g., Eu RSB020 and RSB001) showed apparent divergence. As expected, *P. exspectatus* appeared the most distinct from the rest of wild isolates, but it still retained some overlap with *P. pacificus*. Importantly, *Ppa-daf-21* mutants occupied a region within the morphospace as distant from wild-type *P. pacificus* RS2333 as regions occupied by *P. pacificus* RS2333 and *P. exspectatus* are from each other. This finding demonstrates that the amount of morphological change buffered by Hsp90 is on the same scale as the degree of morphological divergence across examined wild isolates. Thus, concealed morphological variation associated with diverging lineages is within the canalization function of Hsp90 proteins.

Discussion

We used alternative mouth morphologies in the isogenic nematode *P. pacificus* as a model to link developmental plasticity and robustness at the molecular mechanistic level. Although both plasticity and robustness have long been discussed as important principles of evolution, little was known about their relationship to each other. This has changed largely by the work of Lindquist and co-workers, who provided a molecular basis for developmental robustness by showing a link to Hsp90 proteins (Rutherford and Lindquist 1998). Still, it is inherently difficult to examine the interplay with plasticity because robustness and plasticity represent opposite systemic features of organisms and are often contrasted.

We predicted four possibilities for how reaction norm of a dimorphic trait may change once buffering from stochastic variation is released (fig. 2). We observed two kinds of change when heat-shock protein activity was impeded in *P. pacificus*. Specifically, incubation at an elevated temperature led to a shift within morphospace, pharmacological inhibition produced increased variation of both morphs, and mutation in the *daf-21/Hsp90* gene had combined effects. Thus, the main conclusion of our study is that heat–shock protein activity, which provides developmental robustness, canalizes the exact morphologies of mouth-forms in *P. pacificus*.

There are two possible reasons why the applied treatments affected morphology in different ways. First, these treatments interfere with a different number and types of molecular factors. Specifically, the mutation in *Ppa-daf-21* has a targeted effect, whereas the pharmacological inhibition is assumed to

Fig. 4 Continued

See supplementary table 1, Supplementary Material online for the list of accession numbers of sequences used and supplementary figure 1, Supplementary Material online for sequences of the *P. pacificus* proteins. (*B*) Inferred gene structure of *Ppa-daf-21*. CRISPR/Cas9 mutation was introduced in the second exon, which interferes with both identified isoforms. (*C* and *D*). Pleiotropic effects of the *Ppa-daf-21* knockout. (*C*) On the top, a homozygous mutant. Such animals are small and clear. On the bottom, a normally looking heterozygous or genetically wild-type individual from the same brood. (*D*) On the left, a seven-day-old homozygous mutant. Mid-body region, sagittal plane. The gonad is underdeveloped. Proximal part of the gonad is encircled (distal parts are not visible in this plane). Vulva is absent, as vulval precursor cells (marked with arrows) do not develop into the functional organ during postnatal development and can be still observed in adults. On the right, a 4-day-old wild-type individual. The gonad is fully developed and the uterus is filled with eggs. The slit of the vulva is marked with an arrow. The scale bar applies to both images. D, dorsal side; V, ventral side; P, posterior end; A, anterior end.

interfere with all proteins of the Hsp90 family, and the molecular effects of heat stress are arguably very diverse. Second, different treatments presumably influence Hsp90 proteins to a different extent, with a null mutation producing potentially the strongest effect. Generally, our data are consistent with the second and potentially sufficient explanation. Still, the possibility remains that the underlying mechanism involves complex machinery consisting of multiple molecular players.

In addition to this, the role of Hsp90 in mouth-form plasticity may go beyond its generic chaperone function (Arbeitman and Hogness 2000). This protein may be directly involved in the development of certain mouth structures, as any mutation in the gene network underlying mouth development may be expected to increase morphological variability of the mouth (Bergman and Siegal 2003). However, during the first genetic screens for mutations affecting mouth-form, only few mutants were identified, which had defects in the development of substructures of the teeth. In contrast, many mutants were found that alter mouth-form ratios (Ragsdale et al. 2013; Serobyan et al. 2016). It is worth noting that all these mutations did not increase shape variability of the whole mouth. Also, the nematode mouth is a heterogeneous structure formed by tissues of different developmental origin (Ragsdale and Baldwin 2010), so it is unlikely that a mutation affecting the development of one mouth component would increase the morphological variability of the complete organ. Nevertheless, the direct involvement of heat-shock proteins in mouth morphogenesis is possible and awaits future analysis.

We tested if interfering with Hsp90 function still has an effect on mouth shape when one of the morphs is fixed and no dimorphism occurs. The *daf-21; nhr-40* double mutants, which only produce Eu animals, were less diverged from the wild type than single *daf-21* mutants were and less variable than Eu individuals were of the single mutant strain. Together, this shows that once a polyphenic trait is constrained to one morph, the amount of variation released upon impediment of the Hsp90 system is also limited. This finding is consistent with the idea that simultaneous utilization of multiple developmental pathways by polyphenic traits provides space for additional morphological variants (West-Eberhard 2003).

The phenotypic effects described in this study were observed in an isogenic background. It is important to note that the isogenicity of *P. pacificus* allows a clear disentanglement of the role of Hsp90 as a suppressor of stochastic phenotypic variation from its involvement in buffering from mutations (Siegal and Bergman 2002). We speculate that the cause of increased phenotypic variation was either sensitization to microenvironmental changes or stochastic noise in gene regulatory networks. Given current information, it is impossible to determine which of the two possibilities is more likely. To our knowledge, this study is the first of its kind in self-fertilizing hermaphroditic animals, which arguably achieve a higher degree of isogenicity than gonochoristic species, such as *Drosophila* or cavefish.

Finally, there is a long-standing prediction that an erratic decrease in buffering capacity may release morphological variants that can subsequently undergo natural selection (Rutherford and Lindquist 1998; Rutherford, 2003). Strictly speaking, a proof that this has occurred in nature may be unachievable. Instead, we tested if the buffering capacity of Hsp90 system is sufficient to harbor the degree of variation that is actually seen across different strains and species of *Pristionchus*. We saw that mouth shape in the *daf-21/Hsp90* mutant is equally diverged from its background wild type strain *P. pacificus* RS2333 as the rest of strains and species tested are from each other. Therefore, our results indicate that morphological variation associated with diverging lineages and speciation is within the canalization capacity of Hsp90.

Materials and Methods

Nematode Cultures

For experiments with elevated temperature and radicicol, *P. pacificus* strain RS2333 was used. For comparison of wild isolates, *P. pacificus* strains RS5188B, RSB001, RS5337, and RSB020 and *P. exspectatus* strain RS5522 were used. Before each experiment, all *P. pacificus* strains were cultured for at least three generations at 20 °C on NGM plates (Stiernagle 2016) and fed *ad libitum* with *Escherichia coli* OP50. *Pristionchus exspectatus* did not produce Eu animals on *E. coli* OP50, so it was cultured on *Brevibacillus sp.* isolated from a contaminated culture where Eu *P. exspectatus* were seen.

Geometric Morphometrics Analysis

Worms were mounted on microscope slides on 4% agar pads containing 0.3% NaN_3. Animals were turned right side up and covered with a cover slip. Correct positioning of the head was verified by checking if amphid openings were seen close to the central axis. Animals were examined using a 100x/1.4 oil objective. A stack image of the head region was taken, and coordinates of 20 landmarks in XY plane were recorded using FIJI software (Schindelin et al. 2012). Detailed descriptions of landmarks are available in supplementary table 1, Supplementary Material online. Procrustes alignment and PCA were done in R using geomorph package (Adams and Otarola-Castillo 2013; R Development Core Team 2013). Sum of variances was calculated using the MATLAB package MDA (Guide MUs 1998; Navarro 2003). Four first PC axes were retained for the calculations. Rarefaction was done to correct for differences in sample size. Bootstrapping with 10,000 replicates was performed to calculate means and SDs of sum of variances. Bootstrapping with 100,000 replicates was done to estimate two-tailed *P*-values in pairwise comparisons.

Incubation at Elevated Temperature

A non-starved plate with many eggs was taken and vermiform stages were washed away with S-medium (Stiernagle 2016). Then, 1 ml of S-medium was added to the plate and eggs were gently scraped off using a glass cell spreader. Egg suspension was collected in a 1.5 ml tube, centrifuged for 30 s in a table-top centrifuge and washed with S-medium two times. The resulting egg suspension was transferred to a

staining block. Eggs containing pre-comma stage embryos were selected using a stereomicroscope and one egg was transferred to each well of a 96-well plate containing 200 µl S-medium with 1% w/v frozen and thawed *E. coli* OP50 and 0.5% DMSO. The plate was sealed with a paraffin film and placed in an incubator set to 28 °C. Animals were collected for analyses upon reaching maturity (4 days).

Radicicol Treatment

Worms were grown in the same way as described earlier but at 20 °C. Radicicol (Sigma-Aldrich) was dissolved in DMSO and then added to S-medium to the final concentration of 15 µg/ml radicicol and 0.5% DMSO. Solution containing no radicicol and 0.5% DMSO was used as control.

CRISPR/Cas9 Knock Out

As a first step, we searched for homologs of Hsp90 in *P. pacificus* genome. Protein sequences were retrieved from Genbank and the http://pristionchus.org databases. Alignment was done using MUSCLE (Edgar 2004). Conserved sites were manually selected using SeaView (Gouy et al. 2010). Phylogenetic trees were inferred using PhyML (Guindon and Gascuel 2003) with the LG + G substitution model. Branch support values were assessed using the approximate likelihood ratio test (Anisimova and Gascuel 2006). Ppa-DAF-21 was identified as the closest homolog of *Drosophila melanogaster* Hsp83 protein, which was knocked out in earlier studies (Rutherford and Lindquist 1998). Exon 2 of the *Ppa-daf-21* gene was selected as CRISPR/Cas9 target due to high sequence conservation. To verify if the mutation in this locus would interfere with the function of the protein product, we performed Rapid Amplification of cDNA Ends experiment and identified two isoforms both containing the targeted exon. Therefore, we concluded that targeting exon 2 should produce a strong "reduction-of-function" or even a null allele. Upon designing a sgRNA, BLASTn was done to confirm the absence of off-target sites. We followed the protocol of Witte et al. (Witte et al. 2015) to induce a mutation in the genetic background of the *P. pacificus* RS2333 strain. Progeny of a heterozygous mutant animal were singled out, allowed to lay eggs and the mutated locus was sequenced. After repeating this for several generations, we did not obtain any fertile homozygous mutants. To eliminate the need to sequence each generation, we checked for a characteristic phenotype or a set of phenotypes associated with homozygous mutants. We singled out 40 offspring of a heterozygous animal, tracked their development for 5 days and then sequenced the *daf-21* locus. Although some heterozygous or genetically wild type animals stopped their development at an unidentifiable larval stage, most of them developed into wild-type looking animals. Only homozygous mutants developed a characteristic complex of Small, Clear, Sick and Uncoordinated phenotypes.

Comparison of *Ppa-daf-21* Mutant with Wild Isolates

Before the experiment, multiple adult offspring of a heterozygous *Ppa-daf-21* mutant were singled out on a NGM plate seeded with 400 µl *E. coli* OP50. As expected, around two-third of these animals produced some progeny with a complex of phenotypes characteristic of *Ppa-daf-21* homozygous mutants. These animals were used to conduct morphometric analysis as described earlier. However, oftentimes the mouth shape was distorted so severely that it was not possible to measure the coordinates of landmarks 1, 2, 19, and 20. Hence, these landmarks were excluded from the analysis. Wild isolates were grown on NGM plates with 400 µl of bacteria and young adults were used for morphometric analysis. Landmarks 1, 2, 19, and 20 were also excluded to make wild isolate individuals comparable to *Ppa-daf-21* mutants.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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