Molecular aspects of the genetic switch regulating phenotypic plasticity of feeding structures in *Pristionchus pacificus* and its relevance for evolution

Dissertation

der Mathemathisch-Naturwissenschaftlichen Fakultät

der EBERHARD KARLS UNIVERSITÄT TÜBINGEN

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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aus Waiblingen

Tübingen

2015

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der
EBERHARD KARLS UNIVERSITÄT TÜBINGEN.

Tag der mündlichen Qualifikation:	09.06.2016
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Ralf J. Sommer
2. Berichterstatter:	Prof. Dr. Rolf Reuter
3. Berichterstatter:	Prof. Dr. Siegfried Roth

Acknowledgements

First of all I want to thank Prof. Dr. Ralf J.Sommer for giving me the possibility to do my PhD studies in his department and his great support during my studies.

I like to thank Prof. Dr. Rolf Reuter for being my supervisor at university and for examining this thesis. Additionally, I thank my PhD defense committee members Prof. Dr. Ulrike Zentgraf and Dr. Patrick Müller for their willingness of being an examiner of my work.

Very special thanks go to Prof. Dr. Erik Ragsdale for his invaluable support and help. I enjoyed working with him and spending time discussing about the great mystery of plasticity. I wish you all the best Prof. Erik!

I thank my dear friend Vahan Serobyan for his support and great discussions, which have not always been related to the mouth-form. Thanks for all the coffees and the chats we had.

Great thanks go to Dr. Gilberto Bento for introducing me to the mouth-form. When I started working he gave me great support and confidence in myself.

Many thanks to the new mouth-form group Bogdan Sieriebriennikov, Suryesh Namdeo and Masa Lenuzzi.

I thank Dr. Christian Rödelsperger for all the bioinformatic analyses and his patience in explaining them to me.

I like to thank all members of Lab 1. Gabi, Cameron, Vahan, Suryesh and Tobias; you are simply great!

I thank Hanh Witte for all her help and advice. Many thanks to Heike Haussmann for her help with freezing and thawing worms. I am grateful to Metta Riebesell for her help and assistance concerning microscopy techniques.

I am grateful to Lorena, Kostadinka, and Karin for their administrative assistance.

I thank James, Martin, Jan, and Melanie for helping me with new techniques and protocols. Thank you, that you always had time for my questions.

Thanks to all members of Department 4 for your help, support and discussions. Also, I like to thank all the past members of the Sommer lab, especially Simone, Akira, Isabella, Dan, and Ruxandra for their tremendous support and encouragement.

All of you make and made the lab a great atmosphere to work in. I consider myself lucky that I could work with all of you and I will also never forget all the fun we had outside the lab.

I am thankful to the Max Planck Society for granting my work.

Special thanks goes to my friends outside and off the bench, Elisabeth and Thomas Bürkle, Claudia and Dominique Kiefner, and David Schäfer. During the sometimes turbulent times in my PhD studies your friendship was like a solid rock in the waves and created my own essential retreat whenever I got the feeling that things are going on the top of me.

Vielen lieben Dank an meine Schwiegereltern für ihre Unterstützung und Hilfe.

Ein großer Dank geht an meine Eltern für ihre Fürsorge und unentwegte Unterstützung.

Zuletzt, möchte ich mich bei meinem Mann, Martin und bei meinen zwei Töchtern, Lea und Tanea bedanken. Eure Liebe, Geduld sowie der Rückhalt, den ihr mir gegeben habt, waren für mich von größter Wichtigkeit. Danke dafür!

Contents

1 Summary

This dissertation provides the onset for the mechanistic and molecular understanding of phenotypic plasticity in the model organism *Pristionchus pacificus*. The mouth dimorphism in *P. pacificus* is composed of a eurystomatous and a stenostomatous mouth-form. Despite extensive phenotypic analyses a natural monomorphic strain was never observed. The baseline characterization of the mouth-form in a reference strain revealed the existence of a sexual mouth dimorphism, in which males are highly stenostomatous and an associated maternal effect of a stenostomatous mother is further intensifying this difference.

Repeated mutagenesis experiments conducted in different strains of *P. pacificus* and representing different clades, demonstrated a high number of genes strongly influencing the mouth-form decision. Eurystomatous-form defective (*eud*) mutants of clade A and eurystomatous-form constitutive (*euc)* mutants of clade C were phenotypically and molecularly characterized. In the course of this work, two major genes regulating plasticity were identified and both of these haploinsufficient genes located on the X chromosome are major constituents of a genetic switch mechanism. The potentially catalytic active sulfatase *eud-1* is a master regulator gene of the mouth-form. Its dosage-dependant switch mechanism is reflected in the quantitative *eud-1* expression in evolutionary diverged strains of *P. pacificus*. Indeed the *eud-1* gene is not only linked to microevolutionary divergence, but also macroevolutionarily conserved in function as shown in the sister species of *P. pacificus, Pristionchus exspectatus*. The second switch gene is *Ppa-nhr-40*. The nuclear hormone receptor *Ppa-nhr-40* acts downstream of *eud-1* and completely reverts the all stenostomatous *eud-1* phenotype to all eurystomatous.

This thesis provides evidence that phenotypic plasticity is indeed genetically traceable and the great number of mutants that were found in multiple mutant screens strongly emphasize that this developmental decision is vigorously regulated by many genes, some of which are part of a definite genetic switch mechanism. Genetic regulation and developmental switches of plasticity have been anticipated by theoretical evolutionary biologists for a long time and this thesis provides strong empirical evidence for their existence. In addition, *eud-1* as master regulator of plasticity was actually linked to evolutionary differences in natural isolates of *P. pacificus.*

2 Zusammenfassung

Diese Dissertation beschäftigt sich mit der Entwicklung und Regulation sowie des mechanistischen Verständnisses von phenotypischer Plastizität. Der Mundformdimorphismus bei *Pristionchus pacificus* zeigt eine eurystomate und eine stenostomate Form. Bei intensiven phänotypischen Analysen der Mundform von natürlichen Stämmen wurde kein Stamm gefunden, der monomorph ist. Jedoch konnte in einer grundlegenden Charakterisierung der Mundform eines Referenzstammes ein geschlechtspezifischer Mundformdimorphismus festgestellt werden, der zudem noch maternal beeinflusst wird. Hierbei sind die männlichen Nachkommen einer stenostomaten Mutter fast ausschließlich stenostomat.

Wiederholte Mutageneseexperimente, die verschiedene Stämme von *P. pacificus* betrafen und zudem noch unterschiedliche Clades repräsentierten, zeigten, dass bei der Mundformbildung viele Gene beteiligt sind. Trotz zahlreicher genetischer Faktoren führen auch einzelne Genmutationen zu einer starken Verschiebung in der Mundformverteilung. Eurystomatous-form defective *(eud)* und eurystomatous-form constitutive *(euc)* Mutanten konnten von einem Clade A Stamm und einem Clade C Stamm ausgehend isoliert, sowie phänotypisch und molekular charakterisiert werden. Mithilfe der Genkartierung konnten zwei Gene identifiziert werden. Die mutanten Allele beider Gene, die auf dem X Chromosom zu finden sind, weisen Haploinsuffizienz auf und beide Gene sind Hauptkomponenten innerhalb eines genetischen Schaltmechanismus der Mundformentwicklung. Die potentiell katalytisch aktive Sulfatase EUD-1 ist einer dieser Hauptregulatoren. Eine dosisabhängige Schaltwirkung von *eud-1* spiegelt sich in verschiedenen evolutionär divergierten Stämmen von *P. pacificus* wider. Wie Hybridexperimente mit *P. exspectatus* zeigten ist *eud-1* nicht nur mit innerartlicher Divergenz verknüpft, sondern auch makroevolutionär in seiner Wirkung auf die Mundform konserviert. Das zweite Schaltergen ist *Ppa-nhr-40*, ein nukleärer Hormonrezeptor. *Ppa-nhr-40* ist epistatisch zu *eud-1* und revertiert den komplett stenostomaten Phänotyp des *eud-1* Mutanten zu einem vollständig eurystomaten Phänotyp.

Diese Dissertation beweist, dass phänotypische Plastizität genetisch verfolgbar ist und dass diese enwicklungsgenetische Entscheidung durch viele Gene reguliert wird, von denen einige Teil eines Schaltmechanismus sind, was von theoretischen Evolutionsbiologen lange vermutet wurde. Desweiteren konnte *eud-1* als ein Hauptschaltergen für Plastizität mit evolutionäreren Unterschieden in Wildisolaten von *P. pacificus* in Zusammenhang gebracht werden.

3 List of Publications

A developmental switch coupled to the evolution of plasticity acts through a sulfatase.

Müller, M. R.*, Ragsdale, E. J.*, Rödelsperger, C., and Sommer, R. J. (2013). Cell *155*, 922-933. †

† this paper was published as Ragsdale et al.

*authors contributed equally

Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed.

Serobyan, V., Ragsdale, E. J., **Müller, M. R.**, and Sommer, R. J. (2013).
Evolution & Development *15*, 161-170.

Please note that these papers were published under my name at birth; Müller.

4 Introduction

4.1 Phenotypic plasticity

In response to different environments a single genotype can develop multiple phenotypes. This phenomenon is known as phenotypic plasticity (Bradshaw, 1965; West-Eberhard, 1989; Pfennig et al., 2010). The phenotype itself includes all properties of an organism other than the genotype. This means that plasticity can affect morphology, physiologic states and behaviour of organisms (West-Eberhard, 1989). In general, phenotypic plasticity highlights the notion that the phenotype is not only guided by the genotype, but is also influenced by the environment and internal conditions like the epigenetic state of the organism (Martin et al., 2014). However, in addition to the environmental and ecological perspective of plasticity, several authors have argued for the importance of plasticity in generating and facilitating diversity (West-Eberhard, 1989; Pfennig et al., 2010).

Plasticity is ubiquitous, which does however not mean that all plastic traits are adaptive. While still under intense scientific debate, there is indeed evidence that plasticity can be non-adaptive or noisy without having any influence on fitness. Noisy plasticity is caused by developmental instability or irregular perturbations in the environment (Via, 1994). Therefore, some authors argued that phenotypes are more likely to be plastic in a complex and changing environment (Scheiner and Lyman, 1989). Environmental heterogeneity might cause contrasting selection pressures, which can promote the evolution of an adaptive plastic trait (Bradshaw, 1965). Unexpected environmental heterogeneity, which is experienced by organisms for example during colonization events, can reduce the risk of extinction and enable the organism to survive and reproduce.

The diversity of phenotypes within a population might be maintained if the adaptive value of each morph changes with the environment and the fluctuating habitat (Moczek, 2010). Therefore, the ability to express multiple phenotypes enables the organism to respond very rapidly to new selection pressures (Réale et al., 2003). As a consequence, adaptive plasticity enhances the organism's ability to survive or reproduce and boosts successful colonization of new environments (Yeh and Price, 2004; Ghalambor et al., 2007; Pfennig and McGee, 2010).

Adaptive plasticity has to be reproducible and therefore, already Bradshaw suggested in 1965 that phenotypic plasticity must be genetically controlled (Bradshaw, 1965). Indeed, the ability to express multiple phenotypes was shown to be heritable (Schlichting, 1986). Considered in more modern, developmental terms, the defined expression of distinct phenotypes has to be associated with a differential regulation of the underlying developmental pathways and thus has to involve genes defining the phenotypes. Such genes may be different from those genes controlling the plastic trait itself (Scheiner and Lyman, 1989). However, until recently, it was still controversial if genes defining plasticity would exist and no research programme aimed to identify them. This thesis contains strong genetic and molecular evidence for genes controlling plasticity and thereby defines a molecular model system in which phenotypic plasticity can be studied.

A decade ago, Pigliuggi stated most strongly that there is only little understanding about the genes and the developmental mechanisms underlying plasticity. He and others argued that plasticity might be too complex for a clear identification (Pigliucci, 2001). Taking continuous plasticity into consideration, this might well be the case. One possibility would be to work with the most extreme phenotypes of continuous plasticity in forward genetic approaches. However, the necessity to undoubtedly distinguish mutant lines carrying a mutation affecting plasticity from wild type lines with the normal phenotypic range strongly limits the prospect of a successful screen. A powerful alternative exists in working with discrete polyphenisms. The term polyphenism describes discontinuous and discrete plasticity at the morphological level (West-Eberhard, 2003).

Morphological plasticity is special as it is often irreversible given that morphology cannot be changed throughout life. This is in contrast to behaviour, which depends on the organism's ability to learn. At the same time behavioural plasticity is often difficult to define, not only as a plastic feature distinguishing from an ordinary action-reaction feedback, but also in its range and flexibility through lifetime. In contrast, morphological plasticity is in most examples obvious to the observer. However, as it cannot be changed, the organism has to cope with the consequences of its developmental decision throughout its lifetime. Therefore, the cues inducing the developmental decision have to be a reliable predictor of the environmental conditions in order to decide for the development of the fittest morph. Such environmental cues can be multiple and they can be hierarchically ordered in spite of sophisticatedly predicting the encountered habitat. When working with plastic traits, it is crucial to define these cues, or at least those cues that have a strong effect (as minor effects might be indistinguishable from standard fluctuations and noise). Accordingly, the organism

has to sense the cues correlated to specific environmental conditions and has to convert the external signals to cellular and molecular signals. This is often mediated by hormones, metabolites and receptor molecules and involves the nervous system and multiple cell-cell interactions (Moczek et al., 2011).

Several experimental study systems have been investigated in phenotypic plasticity often using very distinct environmental cues. One important and often studied cue of plasticity is species interaction. For example, kairomones of predators can induce major phenotypic changes in *Daphnia pulex* (Black, 1993). In another species, *Daphnia melanica,* melanism has evolved in response to the radiation in alpine lakes. As an anti-predator defence mechanism, the production of melanin is reduced after introducing salmonoid predators (Scoville and Pfrender, 2010). While both species show an anti-predator defence in response to kairomones, *D. melanica* has to deal with two environmental cues, UV radiation and predation. Interestingly, the kairomones are a more effective cue than UV light as the kairomones rapidly induce a lower melanin production in the population. However, reduced UV radiation also results in decreased melanin production and was shown to be associated with higher reproductive success, suggesting plasticity induces costs.

The ability of being plastic causes restrictions to the organism and not surprisingly, there are longstanding discussions in the literature on the cost of plasticity (for review see Pigliucci, 2001). While plasticity costs cause reduced fitness, they are not easy to measure and different arguments are found in the literature. On the one hand, several studies came to the conclusion that plasticity is in evolutionary terms relatively cheap (van Kleunen and Fischer, 2005; van Buskirk and Steiner, 2009). Usually, expenses of plasticity are discussed to involve i) the expression of a maladaptive phenotype for a given environment and ii) the ability to retain plasticity over longer evolutionary time scales (Auld et al., 2010). On the other hand, DeWitt argued for several costs for plasticity (DeWitt, 1998). Maintenance cost is the energy needed for detecting the environment. The production cost of a plastic trait is the exceed cost in relation to a canalized trait. The risk of forming a non-optimal phenotype is the cost of developmental instability. There are also information-acquisition costs for obtaining the information about the surrounding environment and the genetic costs when loci of plasticity are linked to loci with low fitness. However, it is largely impossible to specify these costs relative to others (Pigliucci, 2001). Nonetheless, plasticity reaches its limits if a genotype competent for plasticity cannot express the same phenotype like a non-plastic genotype (DeWitt, 1998).

All this being said, it is important to note that the sensitivity of the responsiveness to environmental cues is also under strong selection meaning that the threshold at which the phenotypic change is induced is genetically variable (Calderone and Page Jr, 1988; Frumhoff and Baker, 1988). Studies on different model systems have shown that the threshold of inducing a phenotypic switch is actually rapidly changing (Moran, 1992; Moczek and Nijhout, 2002; Tomkins and Brown, 2004). This implicates that the threshold of gene expression defining one of the morphs can be even shifted to extremes and this might explain the frequent observation that phenotypic changes can be induced by stress condition but normally are not observed in nature. With this in mind, a loss of plasticity might be due to a shift of the inducing threshold rather than a loss or change of the underlying developmental pathways. On the other hand, plasticity genes that are only infrequently expressed are thought to be shielded from selection, and therefore, such genes should experience relaxed selective pressure in a non-inducing environment (Gibson and Dworkin, 2004; Le Rouzic and Carlborg, 2008; van Dyken and Wade, 2010). It has thus been speculated that mutations might accumulate faster in such genes (van Dyken and Wade, 2009).

Taken together, while phenotypic plasticity represents a well-established phenomenon, the significance of phenotypic plasticity is still controversial, even though several studies support its role in diversification leading to speciation (Schlichting and Pigliucci, 1998; Pigliucci, 2001).

4.1.1 Genetic accommodation and genetic assimilation

Plasticity is based on variation and variation initiates genetic accommodation (West-Eberhard, 2003). Genetic accommodation describes an evolutionary process of increased or decreased sensitivity to an environmental cue, finally leading to a phenotypic change.

Under laboratory conditions, genetic accommodation has been shown in artificial selection experiments in the model organism *Manduca sexta* (Suzuki and Nijhout, 2006). In these studies, the heritable colour change of a black phenotype at 20 °C and a green phenotype induced at 28 °C was artificially selected for and a change of the threshold temperature in these monomorphic lines was documented. A shift in the production of Juvenile Hormone (JH) and the response to JH is a well-known modulator of plasticity in insects. Modifications of this endocrine signalling pathway cause changes in melanization of the larval cuticle and provide one point of regulation through which genetic accommodation might act.

Thus, evolution refines the sensitivity to an environmental cue optimizing the phenotypic outcome. Genetic accommodation reduces pleiotropic effects leading to minor fitness and shapes the formation of novel traits by optimizing the expression pattern of trait-specific genes (Moczek et al., 2011).

Driving genetic accommodation to extremes, the induced phenotype can also lose its environmental sensitivity and a previously changeable trait becomes fixed. This process, which reduces plasticity, is known as genetic assimilation and was introduced by Waddington (Waddington, 1953; Waddington, 1956). Waddington artificially selected for a cross-veinless phenotype induced by heat shock in *Drosophila melanogaster*. After several generations of selection, the phenotype became fixed and was expressed even without heat shock (Waddington, 1953). Further, he showed in another experiment that genetic assimilation of a *bithorax* phenotype in *D. melanogaster* induced by ether is possible (Waddington, 1956). The environmentally induced phenotype is expressed even without this stimulus. Successful genetic assimilation was also repeated in *M. sexta*, where an artificially selected monomorphic black line lost its response to temperature induction after only seven generations (Suzuki and Nijhout, 2006).

A short evolutionary timescale in which genetic accommodation happens has also been reported in colonization studies of Island tiger snakes (*Notechis scutatus*) (Aubret and Shine, 2009; Aubret, 2015). While an advantageous greater head is achieved by plasticity in a population of short isolation time, canalized development of greater head size is observed in populations of isolation times of a few thousand years. Thus, the environment can force selection for a single phenotype by genetic assimilation. A complete loss of plasticity resulting in the fixation of phenotypes occurs if expression and maintenance of plasticity is disadvantageous.

Together, genetic accommodation (refinement of thresholds) and genetic assimilation are important mechanisms for diversification. It is important to note that this evolutionary change does not have to rely on a genetic mutation. Indeed, the different phenotypic responses already present in the population can drive genetic accommodation, which might lead to an optimization of this phenotypic response to the most frequently encountered environments. The environment has the great advantage that it can influence the phenotype of many individuals at once contrasting a mutation in a single individual (West-Eberhard, 2005). The genetic divergence of populations can be enhanced further by promoting differential fixation of alternative phenotypes in subpopulations (Pfennig and McGee, 2010). Hence, fixation of a phenotypic property can contribute to speciation.

In this context it is important to note that only some of the phenotypic properties of an organism actually show variability. Others are invariable and express phenotypic robustness despite environmental or genetic perturbations. Therefore, Waddington described the robustness of a phenotype as a canalized path in the developmental landscape (Waddington, 1957; Waddington, 1959). Such genetic canalization is expected for traits that are relevant for fitness and a change would cause low fecundity or death. Despite of robustness to environmental, genetic, or developmental perturbations, canalization also enables the accumulation of cryptic genetic variation and enhances evolvability (Kirschner and Gerhart, 2005). De-canalizing conditions can release the so far hidden variation, which might lead to rapid evolutionary change under favoured selection (Flatt, 2005). The accumulated variations can suddenly be expressed and natural selection rapidly leads to adaptation of new phenotypic properties, which might lead to speciation.

4.1.2 Role of plasticity in speciation

Ecological and mutation-order speciation are two different models explaining the process of speciation (Schluter, 2009; Thibert-Plante and Hendry, 2009). In the mutation-order speciation model, divergence arises through new mutations in a population. These mutations get fixed and while adapting to similar selection pressures, the population diverges from its original genotype. In contrast, ecological speciation is mainly impelled by contrasting selection pressure, which causes adaptation to different environments. Thus, ecological speciation is dependent on already existing standing natural variation in the populations. This previously cryptic variation together with divergent natural selection caused by environmental differences can rapidly lead to the evolution of reproductive barriers (Schluter, 2009; Thibert-Plante and Hendry, 2009). Natural selection acts on the genetic components responsible or associated with the phenotypic trait (West-Eberhard, 2003). The threespine stickleback (*Gasterosteus aculeatus*) is a well-known example of morphological diversity within a single species. Sticklebacks show assortative mating related to body size, which is influenced by the marine or freshwater environment (McKinnon et al., 2004). Studies indicate that standing genetic variation already existed in the ancestral marine species facilitating repetitive colonization of new freshwater habitats. Later, reproductive isolation proceeded by environmental differences and divergent selection that was reinforced by selective mating (Schluter and Conte, 2009).

Such studies indicate that adaptive plasticity can play an important role in speciation as it creates phenotypic variation within the species and preserves it (Whitman and Agrawal, 2009; Pfennig et al., 2010).

Adaptive plasticity is characterized by controlled switching between phenotypes. Depending on the environmental conditions one of the possible morphs is formed. Such phenotypic switching can promote divergence even without breeding isolation and might restrict morphs to different habitats whereupon populations get isolated (West-Eberhard, 1989). Speciation occurred if sufficient genetic divergence was generated causing reproductive isolation. Reproductive isolation is promoted if dissimilar phenotypes get fixed, which can induce further divergence. Under such scenarios, ecological and genetic differences arise and accumulate in alternative morphs and the subsequent offspring will be of lower fitness. Under these circumstances, selection will favour assortative mating. Accelerated evolution in sex-specific (Jagadeeshan and Singh, 2005) and tissue-specific genes (Hastings, 1996; Duret and Mouchiroud, 2000) seems to be common and promotes speciation even further.

Evolutionary theory on phenotypic plasticity summarized above has been laid down in multiple monographs and review articles. However, in 2003, Mary Jane West-Eberhard moved the argument even further. She hypothesizes that rapid evolution of speciation is a three-step process (West-Eberhard, 1989, 2003). In the first place, alternative phenotypes become fixed in different populations according to environmental differences. Subsequently, each phenotype adapts to divergent selection pressures. This adaptation is likely to happen through genetic assimilation. Thus, the formation of another morph is prevented by acquired insensitivity to an environmental cue inducing a plastic change. Finally, reproductive isolation can evolve as a by-product or reinforcement of adaptive diversification. Here, developmental plasticity promotes species divergence due to contrasting environmental influences, but plasticity can also slow the evolutionary process by shielding the genotype from selection (Ghalambor et al., 2007).

As selection can only directly act on genes that contribute to the expressed phenotype and its fitness, genes whose expressions are restricted to a certain morph are hidden from selective forces in other morphs and experience relaxed selection. The monograph of West-Eberhard (2003) was also unique in making a strong connection between evolution and development, a field that had been built in parallel by the successive understanding of developmental mechanisms and desires to place these findings in an evolutionary context, resulting in evolutionary developmental biology (evo-devo).

4.2 Evo-devo

Evolutionary biology tries to understand how and why organisms change (Pfennig et al., 2010). The evolutionary change of an organism is displayed by genomic changes and leads to modifications of developmental pathways. Genes have a specific role in development and defining their function is important to understand evolutionary processes (Carroll, 2008). In contrast, most developmental studies aim for a mechanistic understanding of observed phenotypes (Rudel and Sommer, 2003). The phenotype is regarded as the interaction of genes and environment through development. Van Valen (1973) was the first to notice that the environment influences development and thus plays a major role in evolution because environmental interactions are the driving forces of selection and adaptation (van Valen, 1973). Therefore, ecology is an important factor for the understanding of evolutionary processes (Sommer, 2009).

As a logical extension of evo-devo, eco-evo-devo therefore defines and tries to use model organisms to combine genomic studies, population genetics, ecology, and development (Sommer, 2009; Sommer and McGaughran, 2013; Gilbert and Epel, 2015). This integrative approach unites the studies for proximate (mechanistic) and ultimate (evolutionary and ecological) causation in biology.

An often observed concept in evo-devo is that highly diverged taxa show the occurrence of deep homology. Deep homology describes the usage of pre-existing genetic developmental pathways in some cases already present in early metazoans in order to form morphological structures (Shubin et al., 2009). Well-known textbook examples are the *pax* genes for eye development and the homeotic genes defining the body axis (Gilbert, 2006). The strong conservation of the function of the *pax6* gene is impressive since the vertebrate transcription factor induces ectopic eyes in *D. melanogaster* (Gehring, 1996). *D. melanogaster* has two pairs of *pax* genes, which control different processes in eye development. The *pax6* genes *eyeless (ey)* and *twin of eyeless (toy)* are involved in eye determination, whereas related *pax* genes *eyegone (eyg)* and *twin of eyegone (toe)* resulting from gene duplications promote the growth of the eye (Czerny et al., 1999; Dominguez et al., 2004). Another striking feature of eye development is the fact that all eyesight in the animal kingdom depends on opsin molecules (Shichida and Matsuyama, 2009). However, analyses clearly indicate that opsins have evolved many times independently (Porter et al., 2012).

In this context, it is important to note that it is often not straightforward to define were novelty begins and homology ends (Shubin et al., 2009). Several studies have shown homologous genes and signalling pathways are used to form non-homologous structures in the same or different pathways. Homologous genes are expressed at new places or at different time points to develop new traits, a phenomenon first described as co-option by Raff (Raff, 1996). Therefore, ancient regulatory gene circuits are commonly reused for forming novel traits (Shubin et al., 2009).

Beetle horns are one example of an evolutionary novelty whose development depends on homologous genes (Moczek and Rose, 2009). Just as appendages, beetle horns develop from epidermal discs. They are outgrowths of the cuticle, but differ from appendages as they lack muscles and nerves. Doubtlessly, beetle horns are evolutionary novelties as they lack homologous structures, but in their development they show a very similar expression pattern of genes (*distal-less, homothorax*) used for appendage development (Moczek et al., 2006b). The conserved developmental programme for outgrowth was co-opted and expressed at new sites to form novel anatomical structures. Nevertheless, larval horn-like structures are needed for breaking the head capsule during larval to pupal moult. These structures could have served as an inception for adult beetle horn development (Moczek et al., 2006a).

Another example comes from treehoppers (Membracidae), hemipteran insects that form a great diversity of 'helmets' on the first thoracic segment mimicking natural elements in their habitat (Prud'homme et al., 2011). Besides the expression of appendages genes, which define the proximo-distal axis, the wing patterning gene *nubbin* and the existing vein network in the helmet provide strong support that the helmets are serial homologs of wings.

Co-option of signalling pathways and their environmental cues for developing novel traits has also been shown in the mouth dimorphism of the nematode *Pristionchus pacificus* (Bento et al., 2010). The endocrine dafachronic acid/*daf-12* pathway involved in dauer formation is also used for inducing alternative feeding structures. As this nematode is at the centre of this thesis, the next paragraph describes the biology of this nematode in more detail.

4.3 *Pristionchus pacificus*

The nematode *Pristionchus pacificus* was introduced as a satellite model in evolutionary developmental biology in comparison to *Caenorhabditis elegans* in 1996 and more recently, expanded its field of study to evolutionary ecology (Sommer and Ogawa, 2011). *P. pacificus* belongs to the family of *Diplogastridae*, which is a monophyletic group (Kanzaki and Giblin-Davis, 2015). *P. pacificus* is a self-fertilizing hermaphroditic species producing a high brood size and occasionally developing X0 males by meiotic non-disjunction. In its four day life cycle, animals go through four juvenile stages, while the first larval stage occurs in the egg (Sommer et al., 1996).

P. pacificus shows a cosmopolitan distribution and can be found in Asia, America, South Africa, and the Mascarene Islands (Herrmann et al., 2010). Isolated strains of *P. pacificus* can be cryopreserved to protect the original genome of the wild strain from falsification caused by genetic divergence in the laboratory. The availability of genomic, genetic, and transgenic tools including forward and reverse genetics make *P. pacificus* an effective model for comparative genetic studies (Schlager et al., 2009; Witte et al., 2015). The genome of *P. pacificus* was sequenced by classical Sanger sequencing technology with an available full assembly and annotation (www.pristionchus.org; Dieterich et al., 2008). The presence of a bacterial artificial chromosome (BAC) map, a genetic linkage map, and morphological markers allow map based cloning of genes (Srinivasan et al., 2002; Srinivasan et al., 2003; Kenning et al., 2004). The *P. pacificus* genome size is about 169 Megabases (Borchert et al., 2010) and is predicted to contain more than 26.000 predicted genes (Dieterich et al., 2008). Surprisingly, about one third of the predicted genes are pioneer genes, which display no sequence similarities above the genus level (Rödelsperger et al., 2013). Nevertheless, transcriptomic and proteomic studies provide strong evidence for their expression and functional importance (Borchert et al., 2010; Sinha et al., 2012b; Sinha et al., 2012a).

4.3.1 Ecology of *Pristionchus pacificus*

In the wild, *P. pacificus* is predominantly found on scarab beetles as dauer larvae (Herrmann et al., 2007; Herrmann et al., 2010). Therefore, the dauer stage is of major importance for the ecological niche of this nematode species (Ogawa et al., 2009). *P. pacificus* shows a necromenic association with these beetles; that is, when the beetle dies,

P. pacificus exits the dauer stage and feeds on microbes present on the beetle carcass (Herrmann et al., 2007). Most interestingly, *P. pacificus* is omnivorous as it feeds on bacteria, protozoa, fungi, and other nematodes (Bento et al., 2010). The first beetle host for *P. pacificus* identified was the oriental beetle *Exomala orientalis* in Japan and the US (Herrmann et al., 2007). Meanwhile, it was shown that *P. pacificus* is associated with beetles of several genera such as *Oryctes, Adoretus, Maladera* and *Hoplia* on La Réunion Island (Herrmann et al., 2010). *P. pacificus* recognizes and distinguishes different olfactory cues to find its appropriate beetle host (Hong and Sommer, 2006). This was also shown experimentally, as the sex pheromone of *Exomala orientalis* strongly attracts *P. pacificus* (Hong et al., 2008). Consistent with the geographic mosaic theory of species interaction, different *P. pacificus* strains differ in their pheromone attraction profile (Thompson, 2005). This theory argues for specific host recognition mechanisms and offers the possibility to perform mechanistic and ecological studies for host perception in this system.

The life stage of *P. pacificus*, which is associated to the beetle, is the dauer stage. Dauer larvae are formed under stress conditions and are specialized for dispersal and survival (Sommer and Ogawa, 2011). Dauers are an alternative third larval stage representing an example of phenotypic plasticity in this species. Morphological characters of the dauer stage are a closed mouth, remodelling of the pharynx associated with a thicker cuticle, and the appearance of worms being smaller and thinner than non-arrested J3 larva (Ogawa and Sommer, 2009). As dauers cannot feed, they also show metabolic changes. These changes have been studied in detail in *Caenorhabditis elegans*. For example, the activity of the tricarbon cyle (TCC) is lowered just as the enzymes of the glycolytic and glyoxylate cycle. Lipids are the main energy source during the dauer stage, as indicated by increased ß-oxidation (O'Riordan and Burnell, 1990; Holt and Riddle, 2003).

This dauer stage is induced by several environmental factors, including starvation, high temperatures, and high population density. High population density is sensed through small molecules emitted by the worms in their environment which function as pheromones (Reuss et al., 2012). In *C. elegans,* the secreted pheromone mix induces dauer formation and is mainly composed of ascarosides (for review see Schroeder, 2015). Conversely, *P. pacificus* pheromones contain the dideoxysugars ascarylose and paratose coupled to a variety of lipid and amino acid derivates (Bose et al., 2012).

4.3.2 Population genetics in *Pristionchus pacificus*

The genus *Pristionchus* consists of more than 30 different species (Kanzaki et al., 2014; Kanzaki and Giblin-Davis, 2015) and more than 800 *P. pacificus* strains were sampled (Ragsdale et al., 2015). These comprehensive collections allow for a sophisticated investigation of the population structure of this species and its evolutionary (phylogenetic) context (Herrmann et al., 2007; Herrmann et al., 2010; Sommer and McGaughran, 2013). Of all known *Pristionchus* species, *P. pacificus* has the broadest distribution in the world (Herrmann et al., 2010). Therefore, microevolutionary changes of phenotypic traits can be investigated by population genetics (Sommer, 2009). In this context, the small genome of *P. pacificus*, cryopreservation and the ease to keep it in the lab make it an ideal model for population studies (Herrmann et al., 2010).

Genetic variation in populations and their associated processes of natural selection, adaptation and genetic drift can be studied best in a system with limited external influences like an island system. In this context, the island of La Réunion was shown to represent a unique study system for the analysis of *P. pacificus* population genetics as it harbours the complete world-wide diversity of this species. The island of La Réunion is located in the Indian Ocean eastern of Madagascar. La Réunion is a young island with an estimated age of 2-3 Ma (Gillot and Nativel, 1982). La Réunion is of volcanic origin and only about 2.500 square kilometres in size. All these characteristics and its variety of different habitats make this system ideal to study natural variation in populations leading to a detailed understanding of phenotypic change (Sommer and McGaughran, 2013).

The genetic changes caused by mutation and recombination provoke phenotypic differences in natural strains of *P. pacificus* and can be identified by recombinant inbred lines (RILs), high-resolution mapping and association studies in the genome. In strains and populations with a high-coverage genome sequence, it is possible to detect deletions in the genome induced through selection or drift. Whole-genome sequencing data of 104 natural strains of *P. pacificus* from La Réunion Island is available and many more sequences are about to follow soon (Rödelsperger et al., 2014). Genomic comparisons group all sampled natural strains of *P. pacificus* in four lineages (lineages A, B, C and D). These lineages are genetically highly diverse and are usually restricted in their geographic distribution (Morgan et al., 2012). However, all four global lineages of *P. pacificus* have been found on La Réunion Island, which can be explained by multiple independent colonization events (McGaughran et al., 2013). Indeed, population studies in *P. pacificus* on La Réunion have been complemented

with ecological studies on beetle host interactions, providing a powerful system for studying co-evolution. For example, the endemic scarab beetle *Oryctes borbonicus* shows the highest infestation rate for *P. pacificus* dauer larvae on La Réunion and co-evolution between this beetle and *P. pacificus* resulted in a lineage designated as clade C (Herrmann et al., 2010). Co-evolution of the two species may well account for the vast genetic diversity within clade C strains (Sommer and McGaughran, 2013). Unlike *O. borbonicus*, the beetle *Maladera affinis* has invaded the island a few hundred years ago (Cheke and Hume, 2010). Consistently, *P. pacificus* strains found on *M. affinis* mostly belong to clade A. Isolated clade A strains from La Réunion show much less genetic diversity compared to clade C strains isolated on this island. This confirms that the clade A strains of *P. pacificus* were introduced relatively recently on the island in comparison to clade C strains, whose highly diversified genomes argue for a much earlier colonization (McGaughran et al., 2013).

The discovery of *Pristionchus exspectatus* as a sister species to *P. pacificus* refines microevolutionary analyses (Kanzaki et al., 2012). This gonochoristic species was isolated in Japan. The genomic sequences of the two closely related species diverge evenly with about 10 % over the whole genome (Rödelsperger et al., 2014). Reciprocal crosses between *P. pacificus* and *P. exspectatus* form viable, but sterile F1 hybrids (Kanzaki et al., 2012). This result shows that the two species separated only recently, although *P. pacificus* evolved a hermaphroditic mode of reproduction only recently.

4.3.3 Mouth-form development

The central focus of this thesis is the developmental regulation of the mouth-form feeding plasticity in *P. pacificus*. Nematode feeding is highly diverse with accompanying changes in pharyngeal morphology (Lee, 2002).

The development of the nematode pharynx was studied most extensively in *C. elegans*, a species that belongs to the order Rhabditida. The pharynx consists of 80 cells and is formed from a ball of cells, which create a linear tube (Portereiko and Mango, 2001). The tube connects anteriorly to the buccal cavity, while in the posterior, it connects to the midgut. More specifically, pharynx development shows three different stages. The first stage is the reorientation stage. In this stage, the initial lumen of the pharynx is lengthened. In the following epithelisation stage, epithelial cells are formed and the nascent pharynx is connected to the buccal cavity. Finally, in the stage of contraction, the pharynx moves anteriorly and the epidermis of the mouth posteriorly. No cell migration or cell intercalation is

observed during pharynx development. All extension of the pharynx is executed by new epithelia formation (Portereiko and Mango, 2001).

Pharyngeal neurons and the branching pattern of the neurons are important for feeding behaviour and the functioning of the mouth structures. Just like in *C. elegans*, *P. pacificus* has 20 pharyngeal neurons (Bumbarger et al., 2013). The homology of neurons compared to *C. elegans* is based on the positions of the cell bodies and their branching patterns. Substantial rewiring of the connections of the synapses by comparing both species can be seen. Therefore, *P. pacificus* shows a more complex connectivity pattern compared to *C. elegans* (Bumbarger et al., 2013).

Phylogenetic studies reveal that the teeth-like denticles present in *P. pacificus* are an innovation restricted to the Diplogastridae family (Hirschmann, 1951; Weingärtner, 1955; Susoy et al., 2015). *P. pacificus* has developed specific feeding structures concomitant to the loss of the pharyngeal grinder present in *C. elegans*. In *C. elegans* and related nematodes, the grinder is the organ of mechanical break-up of bacteria and the teeth-like structures of *P. pacificus* substitute for their function in digestion of prey items (Bumbarger et al., 2013).

Finally, it is important to note that *P. pacificus* shows a mouth dimorphism in its feeding structures (see Figure 4.1). Each population consists of animals with any of the mouth-forms; eurystomatous and stenostomatous animals. Both mouth-forms are characterized by distinct morphological features. Eurystomatous animals first have a shallow and broad buccal cavity. Second, they have a dorsal tooth of roundish shape, which possesses a claw-like structure. Finally, eurystomatous worms have a big subventral tooth (Figure 4.1 B, D). In contrast, stenostomatous animals lack the subventral tooth (Figure 4.1 A, C). The dorsal tooth has a narrow shape and the buccal cavity is narrow and deep in stenostomatous animals (Hirschmann, 1951; Lieven and Sudhaus, 2000). The mouth-form is fully executed in the J4 stage and is irreversible then (Bumbarger et al., 2013). There are nearly no intermediates between eurystomatous and stenostomatous animals and it was shown that eurystomatous animals are adapted to predatory feeding (Serobyan et al., 2014). Similarly, the stenostomatous mouth-form was shown to be adapted to bacterial feeding (Wilecki et al., 2015).

These different feeding characteristics, such as changes in the pumping rate of the pharynx and tooth movement in bacterial and predatory feeding, are mediated through the nervous system of the pharynx. During predatory feeding, the dorsal tooth is predominantly active and functions to open the cuticle of the prey (Bumbarger et al., 2013). In contrast, *P. pacificus* feeding on bacteria show no dorsal tooth movement.

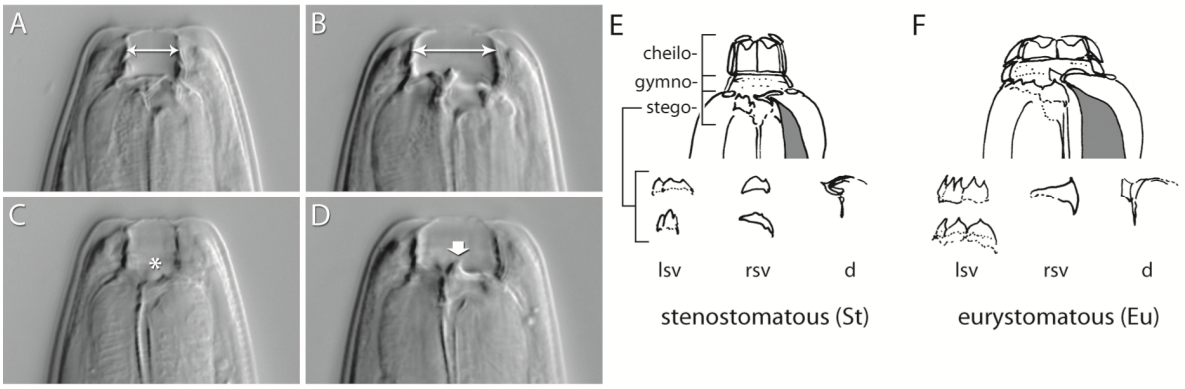


Figure 4.1: Mouth-form dimorphism in *P. pacificus*.
from (Sommer, 2015) by E. J. Ragsdale showing the mouth-form dimorphism of *P. pacificus*. **A-D** Nomarski images of the different mouth-forms of *P. pacificus*. **A and C** Dorsal and right subventral side of the stenostomatous mouth-form. **B and D** Dorsal and right subventral side of the eurystomatous mouth-form. **A** Stenostomatous: The arrow indicates the narrow buccal cavity. Below, the narrow dorsal tooth is present. **B** Eurystomatous: The arrow indicates the broad buccal cavity. Below the arrow, the dorsal tooth with its claw-like structure is seen. **C** Stenostomatous: The star indicates the subventral ridge without any tooth. **D** Eurystomatous: The arrow indicates the big subventral tooth only present in a eurystomatous animal. **E** and **F** show a schematic drawing of the two mouth-forms with the respective morphological characters. The mouth dimorphism involves differences in the stego-, gymno-, and cheilostom emphasizing that several cell types are involved in mouth-form development. **lsv** left subvental; **rsv** right subventral; **d** dorsal.

The eurystomatous mouth-form is thought to be the more sophisticated morph, because it develops more complex teeth-like structures (Figure 4.1 F). Most important, eurystomatous animals are advanced in predation (Serobyan et al., 2014). When relying on a prey diet, eurystomatous animals were demonstrated to show higher fitness than stenostomatous animals (Serobyan et al., 2014). Later experiments confirmed mouth-form dependent killing ability in multiple strains showing that eurystomatous animals are better in killing *C. elegans* larval prey than stenostomatous animals (Wilecki et al., 2015).

The morphology and development of teeth-like denticles in the wide eurystomatous and the narrow stenostomatous mouth-form was first investigated in the family of Diplogastridae in the species *Aduncospiculum halicti* (Baldwin et al., 1997). Stomatal structures were named after the nomenclature of Ley et al (1995) and consist of the buccal capsule and the buccal cavity (Ley et al., 1995). The buccal cavity reaches from the mouth opening to the lumen of the pharynx. The buccal capsule describes the cuticular lining of the buccal cavity. The cheilostom is the most anterior part of the buccal capsule and is separated from the posterior parts by a cell membrane ring. The cheilostom of *A. halicti* and *P. pacificus* is divided into six distinctive plates. Posterior of the cheilostom forms the gymnostom. The gymnostom is surrounded by arcade syncytia. The subsequent stegostom is the region surrounded by the pharynx and is the most posterior part of the buccal capsule (Lieven and Sudhaus, 2000). The stegostom can be further subdivided to Pro-, Meso-, Meta-, and Telostegostom. Each subdivision of the stegostom is defined by the associated radial cells (Ley et al., 1995).

Rhabdions are cuticular regions lining the buccal capsule (Baldwin et al., 1997). *A. halicti* shows a set of six rhabdions (R1-R6), which partially overlap. The associated cells can be muscle or epithelial cells. The rhabdion four (R4) and five (R5) both form the dorsal and the subventral tooth of *A. halicti*. About 60 % of the dorsal tooth is made of R4. R5 makes 40 % of the dorsal tooth. The anterior part of the dorsal tooth (R4) is associated with the muscle cell m_b. The posterior part of the dorsal tooth (R5) is connected to the muscle cell m_c (Baldwin et al., 1997).

The mouth dimorphism in the genus *Pristionchus* was first described by Hirschmann in 1951. Since the stenostomatous mouth does not noticeably differ from the larval mouth-form, Hirschmann suggested that the stenostomatous mouth-form is a neotenous morphology (Hirschmann, 1951).

4.3.4 Mouth dimorphism in *Pristionchus pacificus*

Selection line experiments performed with *P. pacificus* for ten generations confirmed that the discrete phenotypes of stenostomatous and eurystomatous animals indeed represent a plastic trait (Bento et al., 2010). Under stable laboratory conditions, the fraction of stenostomatous and eurystomatous animals in the population of a *P. pacificus* strain is constant and selection of a particular phenotype would not change the ratio of morphs in the subsequent generations.

Thus, the *P. pacificus* mouth dimorphism represents a special example of phenotypic plasticity as even under fixed environmental conditions both mouth-forms are formed. Such bistability is unusual for plastic traits, which are mostly induced by changing environments. Nonetheless, the average frequency of eurystomatous and stenostomatous worms among strains can be considerably different and was shown to be variable upon environmental perturbations (Bento et al., 2010).

Specifically, the environmental factors influencing the mouth-form frequency in the populations have been identified with a strain of *P. pacificus* showing 30 % eurystomatous animals under standard laboratory conditions. The environmental cues inducing a higher eurystomatous frequency are starvation and high population density. The frequency of eurystomatous worms increase significantly if the animals starve in early larval development. Further, high population density and starvation are also effective cues for dauer induction, which represents another example of a polyphenism.

P. pacificus can sense population density through pheromones, which are secreted by the worms and were shown to regulate direct versus dauer development (see subsection 4.3.1). Pheromones can be extracted from the supernatant of a liquid culture and application of the concentrated pheromone extract revealed a dose-dependent response of more eurystomatous animals in the population (Bento et al., 2010). Interestingly, eurystomatous mouth-form induction requires a much lower amount of pheromone than dauer induction.

Some compounds of the pheromone mix of *P. pacificus* were synthesized and were tested for dauer and mouth-form inducing capacity (Bose et al., 2012). These molecules were identified as ascarosides or derivates of ascarosides. For example, the dimeric ascaroside dasc#1 has no dauer inducing capacity but it induces the eurystomatous mouth-form development. Dasc#1 is the compound that shows the highest known mouth-form induction efficiency. Other components of the pheromone mix show only a weak induction of the eurystomatous mouth-form and only at high concentrations. These molecules were determined as the ascarosides pasc#9 and ascr#1, and the paratoside npar-1 (Bose et al., 2012). These results confirm that the two plastic properties in *P. pacificus*, dauer formation and mouth dimorphism are partially controlled through distinct and specific compounds.

Members of the eurystomatous mouth-form inducing signalling pathway downstream of the pheromone were identified to involve the steroid hormone Δ7-dafachronic acid (DA) and the nuclear hormone receptor *Ppa*-DAF-12 (Bento et al., 2010). DA/DAF-12 endocrine signalling is also regulating dauer formation (Ogawa et al., 2009). DA inhibits dauer formation by repressing DAF-12 activity. High DA concentrations drive the populations to a high stenostomatous frequency and DA acts at specific concentrations on the respective trait. Dauer formation is already strongly inhibited at a concentration of 250 pM, whereas a ten times higher concentration of DA is needed to observe a significant difference in the mouth-form frequency. Furthermore, time- and development-dependent application of DA shows that it acts on mouth-form development in the early J2 stage (Bento et al., 2010).

Ppa-daf-12 mutants are dauer-defective and do not form any dauers (Ogawa et al., 2009). From original studies in *C. elegans* it is known that DA prevents the transport of *Cel*-DAF-12 into the nucleus and thus from DNA binding. Three different alleles of *Ppa-daf-12* mutants show a low eurystomatous frequency in *P. pacificus.* However, *Ppa-daf-12* mutants still respond to starvation, indicating that starvation is also acting through a signalling pathway independent of *Ppa-daf-12*. Additionally, *Ppa-daf-12* mutants show no response to pheromone application (Bento et al., 2010). These results imply that the pheromone works entirely through the *Ppa-daf-12* signalling pathway. Together, these results

indicate that the environmental cues and also the endocrine switch mechanism itself were co-opted to create a novel plastic trait in *P. pacificus* (Bento et al., 2010).

The purpose of this thesis was a genetic analysis of the mouth dimorphism in *P. pacificus* in the context of the original observations on DA/*Ppa*-DAF-12. My findings described below strengthen the role of nuclear hormone receptors and steroid hormones and further, identified a sulfatase as a master regulator in this plasticity. Therefore, the remainder of this introduction will provide a short overview about sulfatases and nuclear hormone receptors.

4.4 Sulfatases and development

Sulfatases catalyse the hydrolysis of sulfate esters. Their natural substrates can be quite diverse, involving substrate-specific sulfatases that cleave sulfated glycoaminoglycans, glycolipids, or steroids at specific positions of sulfatation (Ghosh, 2007). A number of sulfatases with different natural substrates show enzymatic activity *in-vitro* for small aromatic substrates; hence the name arylsulfatase for these enzymes (Parenti et al., 1997). Sulfatases catalyzing desulfation of steroid molecules are known as arylsulfatase C (EC 3.1.6.2.). Their active sites are homologous to arylsulfatase A and arylsulfatse B since all three enzymes share nine residues important for catalysis (Reed et al., 2005).

Steroid sulfatases (STS) regulate the production of biologically active steroids because sulfated steroids, like estrogen sulfate, cannot bind to the estrogen receptor. Generally, sulfated steroids are considered as inactive precursors of steroids for transport or storage (Hobkirk, 1993; Falany et al., 2002). STSs were shown to be significantly increased in postmenopausal breast tumours and prostate tumours (Pasqualini et al., 1996; Nakamura et al., 2006). STS converts dehydroepiandrosterone sulfate (DHEAS) to dehyrdoepiandrosterone (DHEA) and adiol sulfate to adiol. Both, adiol and DHEA stimulate cell proliferation in breast tumours (Maggiolini et al., 1999). Also, STS is important for skin function. A lack of STS causes the human skin pathology X-linked ichthyosis and is characterized by large, brown skin scales and a thick stratum corneum (Shapiro et al., 1989).

In humans, 17 different sulfatases are known and seven of them are located in lysosomes and were shown to be involved in glycan degradation (Ghosh, 2007; Wiegmann et al., 2013). The lysomsomal disorder mucopolysaccharidosis (MPS) type VI leads to storage and excretion of high amounts of partially degraded dermatan sulfate and

chondroitin-4-sulfate (Litjens and Hopwood, 2001). The common name for this recessive disorder is Maroteaux-Lamy syndrome. MPS type VI is caused by deficiency of the N-acetylgalactosamine-4-sulfatase, also termed arylsulfatase B. Deficiency of this enzyme leads to skeletal malformations and heart defects (Isbrandt et al., 1994; Golda et al., 2013).

During its catalytic reaction the sulfate group from the substrate is stabilized by a cation, which was identified as a calcium ion (Ca^{2+}) *in vitro* (Bond et al., 1997). Conserved amino acid residues stabilize the ion and the sulfate ester in the active site of the enzyme. The conservation of the metal-coordinating amino acids indicates that most sulfatases require a cation, which is probably involved in stabilization during the enzymatic reaction. Sulfatases undergo a common posttranslational modification important for activity. A conserved cysteine residue near the N-terminus of the enzyme is converted to Cα-formylglycine (2-amino-3-oxopropionic acid residue) (Schmidt et al., 1995).

In humans, 16 single site mutations in the enzyme N-acetylgalactosamine-4-sulfatase were identified (Bond et al., 1997). Most of them show reduced enzymatic activity. Only the non-synonymous amino acid substitution R 95 Q disrupts the enzymatic activity of N-acetylgalactosamine-4-sulfatase completely.

Several studies provided evidence for the importance of sulfatases during development (Ratzka et al., 2010; Dawson, 2011; Sakuma et al., 2011; Butchar et al., 2012; Gorsi et al., 2014; Oustah et al., 2014; Tsai et al., 2015). Many of these sulfatases are involved in the remodelling of the extracellular matrix (Freeman et al., 2008; Arteaga-Solis et al., 2012; Dani et al., 2012; Mitsunaga-Nakatsubo et al., 2013). Glycosaminoglycans (GAGs) are components of the extracellular matrix and have been identified as targets for sulfatases (Bülow and Hobert, 2006). GAGs are polysaccharides consisting of disaccharide units. GAGs include hyaluronic acid, heparan sulfate, heparin, dermatan sulfate, chondroitin sulfate and keratan sulfate. Except hyaluronic acid, all GAGs are bound to proteins. GAGs that are covalently linked to a protein core are proteoglycans (PGs). The remodelling of PGs by sulfatases change their properties for binding growth factors or messenger molecules and are involved in mediating cell-cell interactions or interaction among other matrix proteins. Heparan sulfate is a glycoaminoglycan on the surface of all animal tissue and within the extracellular matrix (Bülow and Hobert, 2006). Cell surface heparan sulfate proteoglycans (HSPGs) and their remodelling by sulfatases are crucial in development, which has been shown in several model organisms.

In the sea urchin *Hemicentrotus pulcherrimus* most of its arylsulfatase shows no catalytic activity (Mitsunaga-Nakatsubo et al., 1998). The sulfatase is located on the apical

surface of epithelial cells. *Hemicentrotus* arylsulfatase (HpArs) is relevant for the extracellular matrix as is suggested by the presence of an N-terminal signal peptide (Sasaki et al., 1988). HpArs shows a strong affinity to sulfated polysaccharides in the extracellular matrix. In sea urchin development, Ars is expressed before epithelial cells start to move during morphogenesis (Rapraeger and Epel, 1981; Sasaki et al., 1988). Low expression of HpArs provokes retardation in gastrulation. There is indication that HpArs is a component of the extracellular matrix and defines areas in the extracellular matrix for further development (Mitsunaga-Nakatsubo et al., 2009).

This hypothesis is further supported by studies on another arylsulfatase in sea urchins. The arylsulfatase of the sea urchin *Lytechinus variegates* defines the status of the extracellular matrix and is involved in cell-cell crosslinking (D'Andrea-Winslow et al., 2012).

In zebrafish (*Danio rerio*), sulfated GAGs regulate the function of morphogens, growth factors, and receptors modifying their activity or distribution. It was shown that a sulfatase is involved in trunk and cartilage formation acting through the transforming growth factor beta (TGF-β) pathway (Moro et al., 2010). The sulfatases Sulf1 and Sulf2 are extracellular 6-O endosulfatases and remove specific sulfate groups from heparan sulfate chains. A knockdown of *sulf1* results in aberrant muscle development of the trunk, impaired pigmentation, and improper migration of lateral line primodium cells by modulated bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signalling (Meyers et al., 2013). Further, it has been shown that *sulf1* has to be up-regulated at certain intervals to ensure proper morphogen signalling of Sonic hedgehog (Shh). Sulf1 activates Shh and ensures development of neurons and glial cells in the ventral spinal cord (Oustah et al., 2014). The pattern of sulfatation in heparan sulfate chains regulated by Sulf1 also promotes the interaction with vascular endothelial growth factor A (VEGFA) at the cell surface. Further, a knockdown of *sulf1* induces dysmorphogenesis in arterial development. Therefore, Sulf1 is crucial for arterial specification by regulating VEGFA activity (Gorsi et al., 2014).

In avian model organisms the N-acetyl glucosamine sulfatase QSulf1 of quails was shown to be induced by Shh and to drive muscle development through desulfation of HSPGs promoting Wnt signalling (Dhoot et al., 2001). The endosulfatase QSulf1 removes 6-O sulfates from heparan sulfate chains, which promotes Wnt binding to the Frizzled receptor and initiates the Wnt signalling cascade (Ai et al., 2003). The enzymatic remodelling of HSPGs of QSulf1 also releases the BMP antagonist Noggin from the cell surface facilitating BMP cell response (Viviano et al., 2004).

HSPGs 6-O desulfation has also been shown to be important for several signalling pathways in *Drosophila melanogaster*. DSulf1 is one sulfatase of several homologous vertebrate Sulfs present in *Drosophila*. DSulf1 influences the epidermal growth factor receptor (EGFR) pathway (Butchar et al., 2012). Sulfatation of HSPGs in *D. melanogaster* is regulated by DSulf1 and the two sulfate transferases Hs2st and Hs6st. Loss or reduction-of-function of one of these enzymes is compensated by the remaining sulfate modelling enzymes to ensure proper FGF signalling (Kamimura et al., 2006). BMP and Wnt signalling pathways are further influenced by DSulf1 (Dani et al., 2012; Kleinschmit et al., 2013). DSulf1 influences the morphogen gradient by stabilizing Wingless (Wnt1 in vertebrates) and Wingless further activates DSulf1 expression (Kleinschmit et al., 2010). Another signalling pathway influenced by DSulf1 is the Hedgehog (Hh) pathway. DSulf1 modulates Hh distribution. DSulf1 weakens the interaction of Hh and HSPGs leading to lowered Hh concentrations at the apical membrane of the wing discs during wing development (Wojcinski et al., 2011).

All of these examples show that extracellular sulfatases modifying the identity of the extracellular matrix are important players in development of animals.

4.5 Nuclear hormone receptors

Nuclear hormone receptors (NHRs) are ligand-activated transcription factors. When the receptor binds to lipophilic hormones or a hormone-like substance that has diffused through the lipid bilayer of the cell membrane, the receptor changes its conformation (Gronemeyer et al., 2004). The induced conformational change in the hormone-receptor complex further leads to dissociation of co-repressors and recruits co-activators to enhance transcriptional activity (Chawla et al., 2001; McKenna and O'Malley, 2002). Inactivity of the NHRs in the absence of the hormone ligand is controlled by a number of inactivating factors including Hsp90 (Pratt and Toft, 1997). Nuclear import is facilitated when the hormone binds to the receptor and releases the inactivating factors and Hsp90, leading to the translocation of the NHR into the nucleus (Tsuji et al., 2014).

NHRs share three common functional domains: the N-terminal transactivation domain (NTD), the DNA-binding domain (DBD), and the ligand-binding domain (LBD). While the NTD is little conserved, the DBD is the most conserved domain among NHRs and contains two zinc fingers with four highly conserved cysteine residues, each coordinated with one Zn^{2+}

(Freedman et al., 1988). The hydrophobic core formed by two helices in the DBD binds specifically to the Hormone Response Element (HRE) in the major DNA groove (Luisi et al., 1991). HREs are composed of two groups of five to six nucleotide sequences separated by a spacer sequence and direct or inverted repeats (Truss and Beato, 1993). Estrogen and glucocorticoid receptors normally bind as dimers to the HREs (Carson-Jurica et al., 1990), whereas retinoid acid, thyroid hormone, and vitamin D receptors preferentially form heterodimers (Leid et al., 1992; Marks et al., 1992).

The LBD is the second most conserved domain with eleven to twelve helices located in the C-terminal half of the receptor (Mangelsdorf et al., 1995; Renaud et al., 1995). Close to the C-terminal end of the LBD several co-activators and co-repressors can bind. Those are important for inducing changes to the chromatin structure or are involved in binding components of the transcription assembly (Hong et al., 1996; Horwitz et al., 1996; Kamei et al., 1996; Li et al., 1997; Torchia et al., 1997; Ding et al., 1998). The structures of the DBD and the LBD are defined properly (Huang et al., 2010).

NHRs can bind as heterodimers, homodimers or also as a monomer to the promoter of downstream-target genes (Wada et al., 2008). The LBD binds hydrophobic molecules, such as steroids, retinoids, and lipid metabolites. It is important to note, however, that many NHRs are orphan receptors, whose native ligand is not known. Interestingly, few NHRs, like knirps in *D. melanogaster,* miss the LBD. For others, such as tailless, despite the presence of a LBD, the ligand itself has not been found. Often, such transcription factors act as transcriptional repressors rather than activators (Morán and Jiménez, 2006). Thus, while the structure of NHRs is generally conserved, the mechanisms of action can be quite diverse, including posttranslational modifications and ligand binding.

NHRs are ancient and already exist in cnidarians (Wiens et al., 2003; Reitzel et al., 2011). Expansion of the receptors happened early in the metazoan evolution and within the vertebrate lineage. In humans, 48 NHRs were identified (Maglich et al., 2001). In the literature, the number of NHRs in the genome of *D. melanogaster* is inconsistent and some studies report about 18 NHRs (King-Jones and Thummel, 2005; Boulanger and Dura, 2015; Jaumouillé et al., 2015). In contrast, others argue for 21 nuclear receptors (Maglich et al., 2001; Evans and Mangelsdorf, 2014). A dramatic exception of the duplication rate of receptors demonstrates the nematode lineage. The genome of *C. elegans* encodes 284 NHRs, whereas only 15 of them are conserved nuclear receptors among metazoans (Gissendanner et al., 2004). The spectacular burst of receptors is mostly originated in duplications of the Hepatocyte Nuclear Factor 4 (HNF-4)-related receptor in humans (Robinson-Rechavi et al.,

2005). Indeed, at least 250 of the NHRs in *C. elegans* descend from the expansion of the HNF-4 receptor and diverged only in nematodes. Most of the nuclear receptors contain nematode specific sequences and little is known about the function of these receptors. It is hypothesised that the great expansion of this gene family occurred in response to numerous xenobiotics in the environment making the worms resistant to several toxins (Lindblom et al., 2001).

The human HNF4α receptor is an orphan receptor that forms homodimers and exists in two conformational states (Dhe-Paganon et al., 2002). Both states are thought to contain an endogenous fatty acid ligand of unknown identity and it is still not completely clear if the fatty acid molecule is needed for its activity. Therefore, HNF4α is an orphan member of the nuclear receptor family and is expressed in the liver, kidney, gut, and pancreas.

Independent thereof, it is well established that HNF4α and several other nuclear receptors show acetylation as a posttranslational modification. Acetylated sites provide docking to co-repressors and co-activators (Wang et al., 2011). Acetylation of HNF4α increases DNA binding ability and acts in nuclear retention. Non-acetylated HNF4α is actively exported from the nucleus and remains in the cytoplasm. For example, the CREB (cAMP response element binding protein)-binding protein (CBP) possesses an intrinsic acetyltransferase activity and acetylates histones and non-histone molecules, like transcription factors. HNF4α is acetylated at four conserved lysine residues by CBP, which induces a conformational change and the nuclear export signal sequence is inaccessible for the nuclear export system (Soutoglou et al., 2000). If the four lysines acting as acetylation sites in HNF4α were mutated, all HNF4α is solely located in the cytoplasm (Shi et al., 2014).

Cel-nhr-40 is characterized as a HNF-4 related nuclear receptor in *C. elegans*. *Cel-nhr-40* is expressed in the pharynx, the body wall, the sex muscles, and in a subset of neurons. It is required in late embryogenesis and essential for proper body wall muscle formation. Accordingly, reduced expression of *Cel-nhr-40* demonstrates late embryonic and early larval arrest and further defects in elongation and morphogenesis (Brožová et al., 2006).

Important for this thesis, one factor targeting HNF4α is cholesterolsulfate (CS) in mammals (Shi et al., 2014). Sulfatation of cholesterol in humans is catalysed by the sulfotransferase SULT2B1b. Sulfotransferases transfer the functional sulfuryl group ($-SO_3^-$) from the obligatory sulfate donor 3'-phosphoadenosyl-5'-phosphosulfate (PAPS), which is a co-substrate in the reaction, to hydroxyl or amine groups to form sulfate esters or sulfamates (James, 2014). PAPS formation is catalysed in two coupled enzymatic reactions in the cytosol from ATP and inorganic sulfate (SO_4^-). In the first reaction, the enzyme ATP-sulfurylase

combines ATP and sulfate to form APS and pyrophosphate. In the subsequent reaction catalysed by APS-kinase, ATP and APS react to PAPS and ADP. Both reactions need Mg^{2+} as a cofactor. The conservation of the PAPS binding site in sulfotranferases reflects that sulfation does not happen in the absence of PAPS (Komatsu et al., 1994). Sources of inorganic sulfate in higher organisms are intestinal absorption and the sulphur-containing amino acids cysteine and methionine (Lucke et al., 1981; Mulder and Jakoby, 1990).

The most prominent and intensively studied nuclear receptor in nematodes is *Cel*-DAF-12. The closest homologs to *Cel*-DAF-12 in vertebrates are pregnane-X and Vitamin D receptors (Antebi et al., 2000). *Cel*- DAF-12 is expressed in all somatic cells and regulated by DA (Motola et al., 2006). *Cel*- DAF-12 is involved in larval transition of L2 to L3, longevity, fat metabolism, and dauer formation (Antebi et al., 1998; Ludewig et al., 2004; Gerisch et al., 2007). *Cel-daf-12* is located downstream in the dauer pathway acting as an endocrine switch mechanism (Riddle et al., 1981; Gottlieb and Ruvkun, 1994). Under beneficial environmental conditions, TGF-β and Insulin signalling are up-regulated and promote DA anabolism. DA binds to *Cel*-DAF-12 and consequently prevents dauer formation, whereas unligated *Cel*-DAF-12 induces dauer diapause (Fielenbach and Antebi, 2008).

4.6 Aims of research

This project was conducted to identify genes involved in the developmental regulation of a plastic trait, the mouth-form dimorphism in *P. pacificus*. Thus, the mouth-form dimorphism in *P. pacificus* was used as a model for studying the genetic regulation of phenotypic plasticity. Additionally, the phenotypic observation of a large number of evolutionary diverged strains in *P. pacificus* was used to ascertain the stable and uniform expression of the discrete mouth dimorphism.

I found that phenotypic plasticity is indeed driven by and can be assigned to genes, in particular to developmental switch genes. In a genetic screen for eurystomatous-form defective (*eud*) genes, I isolated the gene *eud-1*, which encodes a sulfatase and acts as a developmental switch. If the gene is mutated, all animals are stenostomatous. In contrast, if the gene is over-expressed, all animals express the eurystomatous phenotype indicating that this gene is associated with developmental switching. Developmental switching was long anticipated by evolutionary theory to represent a crucial factor for phenotypic plasticity, but this study was the first to identify such genes. In a second project, using a suppressor screen analysis, the nuclear hormone receptor *Ppa-nhr-40* was identified to act downstream of *eud-1*. Follow-up experiments revealed that *Ppa-nhr-40* is also part of the developmental switch regulating the mouth-form plasticity of *P. pacificus*. Therefore, this thesis provides the first, unbiased, genetic, molecular and mechanistic insight into genes regulating phenotypic plasticity in eukaryotes.

5 Results and Discussion

5.1 Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed

Serobyan, V., Ragsdale, E. J., **Müller, M. R.**, and Sommer, R. J. (2013). Evolution & Development *15*, 161-170.

5.1.1 Synopsis

The execution of a plastic trait often has strong ecological relevance as the individual morphs are best adapted to specific environmental circumstances affecting the fitness of an organism. This involves the importance of plasticity in the evolution of novel traits, whose mechanistic and developmental understanding is still in their infancies. The mouth dimorphism of *P. pacificus,* which is defined by a stenostomatous (narrow-mouthed) and a eurystomatous (wide-mouthed) form with several mouth-form associated and morph-specific characteristics, can be studied under laboratory conditions. This study represents a comprehensive investigation concerning the initial basis of the mouth plasticity in a reference strain of *P. pacificus*.

Spontaneous males, which develop by non-disjunction of the X chromosome, express a severe stenostomatous mouth-form bias compared to their hermaphroditic counterparts. Investigations of mouth-form defined crosses further revealed an influence on the mouth-form development in males through the mouth-form of the respective hermaphrodite used for the cross. More eurystomatous males are formed if the respective hermaphrodite is also eurystomatous. No cross-dependent or maternal influences could be detected for hermaphrodites. The maternal influence on the males in the respective generation suggests the implication of epigenetic mechanisms affecting the next generation. The fact that only males are affected implies an *in-utero* provisioning of a component becoming effective during male

sex determination. Further, stage-specific isolations of juveniles show a decrease in eurystomatous animals when the worms were isolated in J2 or J3. An isolation of later stages indicated a gradual decrease of sensitivity to environmental cues after the J3 stage. The two mouth-forms also differ in their developmental timing, when reaching adulthood. When bacteria as a food source are provided, stenostomatous hermaphrodites show an accelerated hatching to the J4 stage compared to eurystomatous animals. This implicates an advantage of the stenostomatous mouth-form for fast population growth in a non-predacious, bacterial-rich environment.

5.1.2 Own Contribution

I performed all experiments in collaboration with Vahan Serobyan and Dr. Erik J. Ragsdale. I was involved in the experimental design and analyses. My contribution of this publication is 20 %.

5.2 A Developmental Switch Coupled to the Evolution of Plasticity Acts through a Sulfatase

Müller, M. R.*, Ragsdale, E. J.*, Rödelsperger, C., and Sommer, R. J.
(2013). Cell *155*, 922-933. †

† this paper was published as Ragsdale et al.

* authors contributed equally

5.2.1 Synopsis

The genetic and molecular mechanisms associated with the environmental influence on phenotypes in the same genetic background are little understood. The nematode *P. pacificus* shows a mouth dimorphism represented by eurystomatous and stenostomatous morphs adapting the species to different diets. In a forward genetic approach we identified the master switch gene *eud-1* regulating the developmental decision in the mouth dimorphism in *P. pacificus*. The elimination of the *eud-1* gene results in a loss of the eurystomatous mouth-form, whereas *eud-1* overexpression transforms the phenotype to all eurystomatous. The expression of *eud-1* is sex-specific and the dosage-dependant phenotypic outcome explains the observed sexual mouth dimorphism. *eud-1* also affects the mouth-form of natural strains in a dosage-dependant manner as the mouth-form phenotype is correlated to the quantity of EUD-1 expressed in these strains. Overexpression induced a significant increase of eurystomatous animals in such populations. Further, we could confirm that *eud-1* is conserved above the species level, which was shown in hybrid experiments in the sister species of *P. pacificus, Pristionchus exspectatus*. *eud-1* codes for a sulfatase and was target of a recent gene duplication event in the *Pristionchus* lineage. The conservation of its coding region among *P. pacificus* strains indicates that the strain-specific mouth phenotype is mainly regulated by *eud-1* promoter remodelling. Different mutant alleles of *eud-1,* located at an important catalytic region within the protein and competitive inhibition experiments, which mimic the Eud phenotype argue that EUD-1 indeed is enzymatically active. We showed expression of *eud-1* in several somatic and pharyngeal neurons throughout juvenile development implicating function in sensation or hormone signalling. Epistatic analyses

reveal that the mouth-form affecting pheromone components and the Δ7-dafachronic acid (DA)/*Ppa*-DAF-12 hormone signalling pathway act upstream or in parallel of *eud-1*.

Together, a new gene, surprisingly coding for a sulfatase was identified as a master regulator in plasticity acting downstream in the mouth-form decision pathway. *eud-1* operates as a switch and was coupled to observed phenotypic diversity in micro- and macroevolution in *Pristionchus*.

5.2.2 Own Contribution

I performed all experiments together with Dr. Erik J. Ragsdale. The bioinformatics analyses were done by Dr. Christian Rödelsperger. I contributed to the experimental design and analyses and was involved in writing the manuscript. My contribution to this publication is 50 %.

5.3 The nuclear hormone receptor *Ppa-nhr-40* is a downstream target of *eud-1* and part of a developmental switch mechanism

5.3.1 Introduction

Mutations in the gene *eud-1* lead to the formation of an all stenostomatous mouth-form phenotype. Genetic analysis confirmed that *eud-1* is so far, the most downstream regulator of the mouth dimorphism. In order to find potential downstream targets of *eud-1*, which might enable us to assign this sulfatase to a known signalling pathway and could reveal *eud-1*'s endogenous substrate, I performed a suppressor screen. Here, I report the isolation and characterization of *Ppa-nhr-40* as downstream effector of *eud-1* and second constituent of the developmental switch mechanism.

5.3.2 *eud-1* suppressor screen

The *eud-1* allele *tu445* containing a premature STOP codon was used for the suppressor screen by EMS mutagenesis. After the mutagenesis, the F2 generation was screened for animals with a eurystomatous phenotype. In a screen of approximately 1500 gametes, I could isolate four mutant lines showing a eurystomatous-form constitutive (Euc) phenotype. Three of these lines could be backcrossed successfully. In the *eud-1* mutant background all three backcrossed mutant lines still showed a high eurystomatous phenotype. *tu505* was even completely eurystomatous. *tu466* showed a phenotype of 90 % eurystomatous and *tu464* of 45 % eurystomatous animals (see Figure 5.3.1). In total, each mutant was at least seven times backcrossed. The mouth-form phenotype was additionally confirmed by a screen using Nomarski optics.

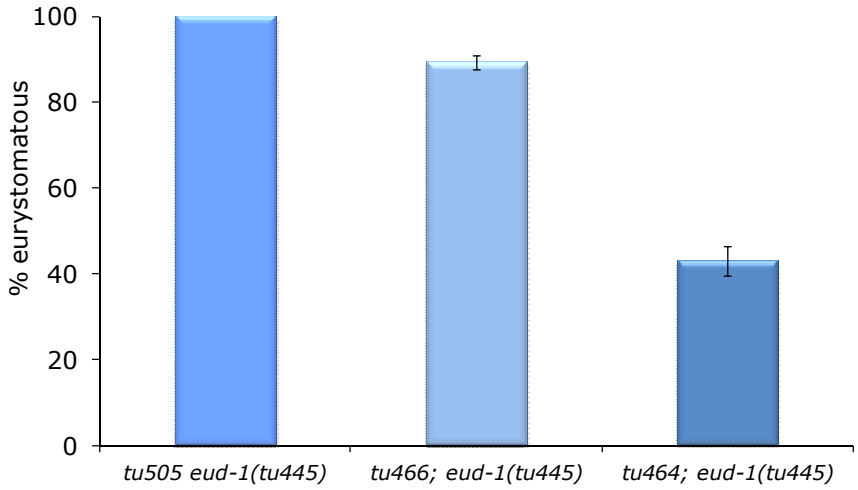


Figure 5.3.1: Three mutant lines were isolated and backcrossed in a *eud-1* suppressor screen. All mutant lines still contain *eud-1(tu445)*. *tu466* and *tu464* were backcrossed seven times. *tu505*, which shows a phenotype of all eurystomatous, was eight times backcrossed. The error bars represent 95% confidence intervals.

5.3.3 Mapping of the suppressor mutants

The genetic background of the *eud-1* mutant *tu445* is the clade A strain RS2333 (California). For the generation of mapping lines I used the highly stenostomatous clade A strain RS5200B, which is approximately 10 % eurystomatous. I decided to use a closely related clade A strain for mapping to avoid crossing incompatibilities between strains. A morphological dumpy (Dpy) mutant was isolated in the RS5200B background by EMS mutagenesis screen. The mutant *tu472* showed a strong and penetrant Dpy phenotype and was used for the creation of mapping lines. I prepared DNA libraries for mapping by RAD sequencing. In total, 32 positive mapping lines, which showed the mutant Euc phenotype and 32 negative mapping, which showed a high stenostomatous phenotype, were isolated of each of the three Euc mutants and used for RAD library preparation (see Figure 5.3.2). For data complementation, the three *euc* mutants and the *dpy* mutant *tu472* used for mapping were whole-genome sequenced. Figure 5.3.3 shows a summary of the RAD mapping results. The mutant allele *tu505* could be mapped to a broad region of chromosome X. Also the mutant *tu464* showed the most significant association to chromosome X, but close to the position of *eud-1*. Analysis of the parental mouth-form phenotype and the associated genotype revealed that the eurystomatous phenotype correlated to the RS5200B genotype. This implies that the observed mapping signal in *tu464* is actually an artefact, which could be caused by epistatic interactions with the functional *eud-1* gene of RS5200B. The most significant signal of the mutant *tu466* is seen on chromosome V.

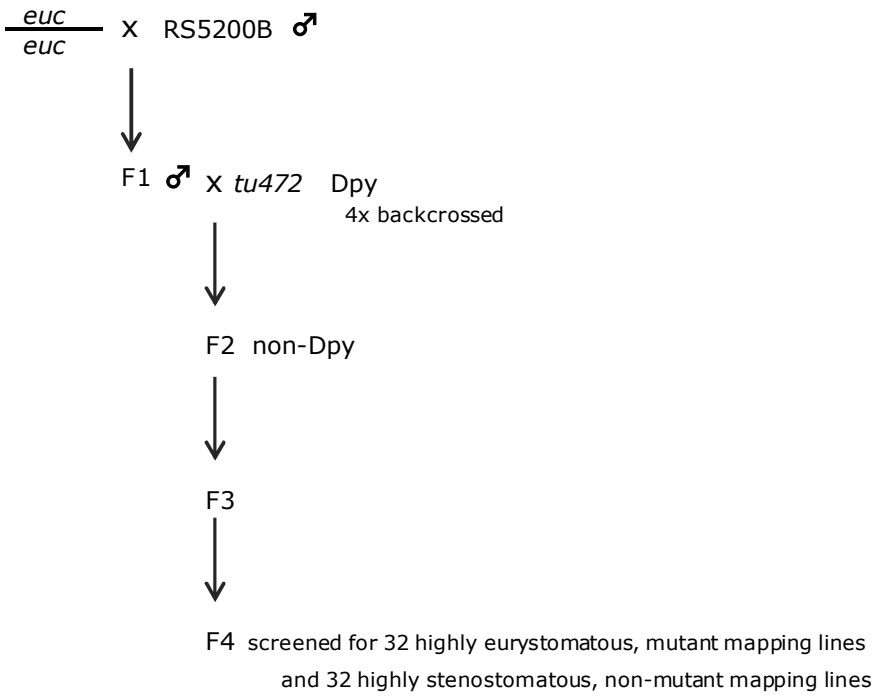


Figure 5.3.2: Crossing scheme for generation of mapping lines used for RAD sequencing.
For each mutant line 32 highly eurystomatous (positive) mapping lines carrying the mutation and 32 highly stenostomatous (negative) mapping lines without a mutant mouth-form phenotype were kept for RAD sequencing.

After mapping, the obtained whole genome data were further analysed to identify candidate genes. Since the mutant *tu464* suggested a potential rescue by epistatic interactions, this mutant was not analysed further. For the mutant *tu466*, no non-synomymous substitution was found in the candidate region on chromosome V.

However, the mutant *tu505* showed a clear association to chromosome X by RAD mapping and only one non-synonymous substitution was found in this candidate region. A guanine base was substituted by an adenine base on Contig 77 at position 555208 causing an exchange in the predicted amino acid sequence of Serin to Leucin. The predicted gene of this region is Contig77-aug18009.t1 (*P. pacificus* AUGUSTUS 2013 gene annotation) and codes for the nuclear hormone receptor 40 (*Ppa-nhr-40*).

RAD results

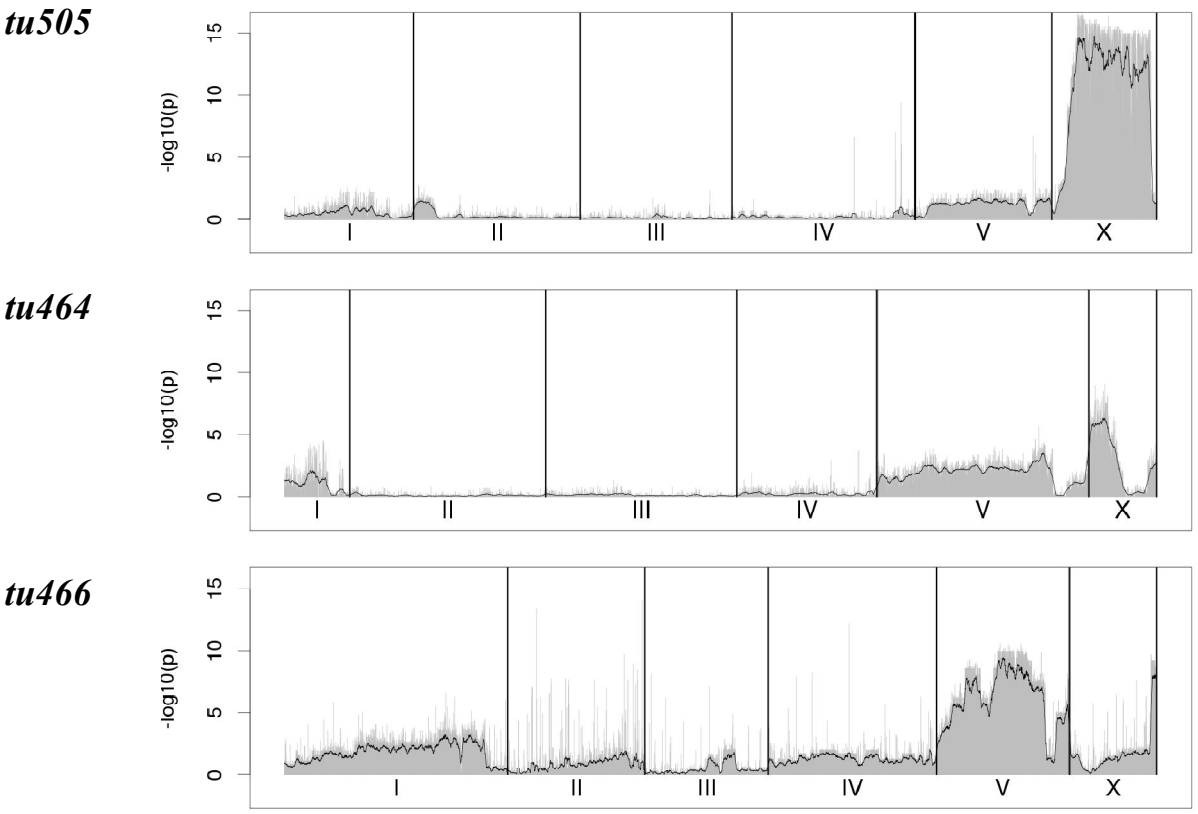


Figure 5.3.3: RAD mapping results of the *eud-1* suppressor mutants.
The three different plots show the RAD sequencing results after analysis for the three *eud-1* suppressor mutants *tu505, tu464*, and *tu466*. The x-axis represents the relative position of the genotyped markers on the *P. pacificus* chromosomes. The y-axis shows the significance of the association between phenotype and genotype. Gray bars show individual data points and the black line demonstrates a running average.

Table 5.3.1: Candidate gene list for the suppressor mutant *tu505* on chromosome X.
The suppressor mutation of *tu505* was assigned to chromosome X. The table shows all mutations in genes or close to genes on chromosome X found by whole-genome sequencing in the chromosomal region showing a high association signal detected by RAD mapping.

tu505 (Chr X)			
Gene	**Position**	**Exchange**	**Kind of mutation**
Contig10-snap.108	702404	G>A	intronic
Contig10-snap.11	711022	G>A	intronic
Contig10-aug5005.t1	1182092	G>A	intronic
Contig127-snap.5	219960	G>A	intronic
Contig135-snap.21	115824	C>T	intronic
Contig50-snap.110	531809	C>T	intronic
Contig50-snap.177	851722	C>T	intronic
Contig77-aug17978.t1	417717	G>A	synonymous
Contig77-aug18009.t1	555208	G>A	nonsynonymous S>L

5.3.4 The nuclear hormone receptor *Ppa-nhr-40* and its influence on the mouth dimorphism

As the mutation in the predicted gene Contig77-aug18009.t1 is the only one leading to a change in the protein, I concentrated on this gene as a potential candidate. Contig77-aug18009.t1 codes for the nuclear hormone receptor *Ppa-nhr-40*. *Ppa-nhr-40* is located in the operon Contig77-snapOP.127 containing two genes (Sinha et al., 2014). The second gene, Contig77-aug18008.t1 codes for a protein of unknown function. Interestingly, the gene *T03G6.1* is the orthologous gene of Contig77-aug18008.t1 in *C. elegans*, and also in *C. elegans, Cel-nhr-40* is located next to this gene. This shows a strong conservation of the whole operon and suggests an involvement of the following gene in function or regulation of *Ppa-nhr-40*.

In order to obtain the real exon-intron structure of Contig77-aug18009.t1, I performed several independent RACE experiments with different primers, according to the predicted gene structure. Both, 5' and 3' RACE were conducted. The sequence of the splice leader SL1 was found at the 5' end. SL1 splicing was already predicted for this gene by Sinha and co-workers (Sinha et al., 2014). The predicted first and fifth exons were not found in the RACE sequences. The gene starts a few bases upstream of the predicted second exon and the predicted fifth exon was actually shifted about 70 bases downstream. The predicted amino acid change from Serin to Leucin could be confirmed in the seventh exon (see Figure 5.3.4). Besides the predicted long isoform of *Ppa-nhr-40*, I also found a much shorter isoform of the *Ppa-nhr-40* gene. The short isoform of *Ppa-nhr-40* consists of the same first three exons and continues with its 3'UTR, which is about 250 bp in length. An overview of the gene structure is provided in Figure 5.3.4. The fact that the short isoform of *Ppa-nhr-40* was found in each RACE experiment when the appropriate primers were used, and that *Ppa-nhr-40* short encodes the whole DNA binding domain (DBD) of two zinc finger domains (type C4), suggest a potential functional importance of the short isoform in the worm. Besides the DBD, *Ppa-nhr-40* short encodes a short, but unique sequence of 37 amino acids, which is predicted to be disordered except for a sequence of six amino acids forming a β-sheet. The mutation found in the suppressor mutant *tu505* is only present in the long isoform of *Ppa-nhr-40*.

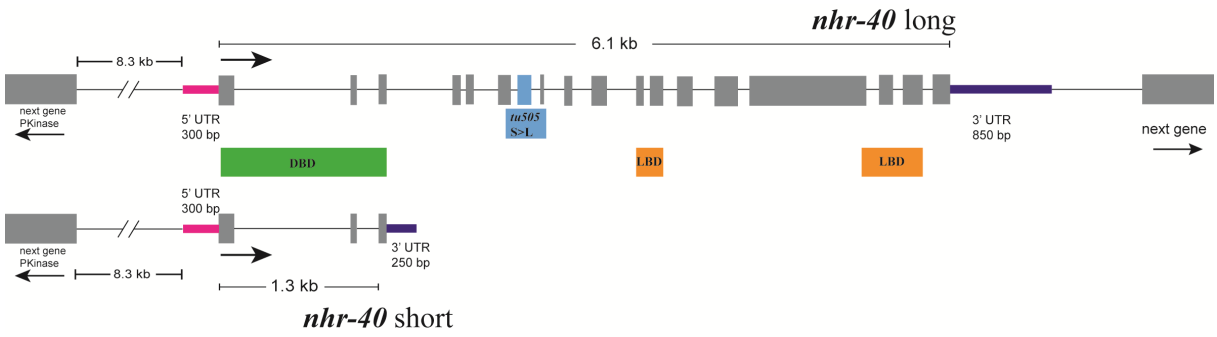


Figure 5.3.4: Gene structure of *Ppa-nhr-40*.

Both isoforms of *Ppa-nhr-40* are shown. The mutant allele *tu505* is only expressed in the short isoform of *Ppa-nhr-40*. The rectangles represent the exons. The thin line shows non-coding introns or intergenic sequence. DNA binding domain **(DBD)** is located in exon 1 to 3. The ligand binding domain **(LBD)** is located in Exon 11 and 12, and continues at the end of exon 15 until the end of exon 17.

5.3.5 The long isoform of *Ppa-nhr-40* acts downstream of *eud-1*

Transgenic microinjection experiments confirmed that the base substitution in *Ppa-nhr-40* is indeed causing the suppressor phenotype (see Figure 5.3.5). The genomic wild type construct of *Ppa-nhr-40* of the Californian strain was injected into the *tu505* mutant line, still containing the *eud-1* allele *tu445*. Independent transgenic lines showed a complete or nearly complete reversion of the all eurystomatous phenotype to all stenostomatous (see Figure 5.3.5).

Subsequent outcrossing of the *eud-1* mutation and screening of the mouth-form phenotype in the single mutant allele *tu505* showed that the all eurystomatous mouth-form phenotype of *Ppa-nhr-40* is not dependent on the *eud-1* mutant background. To investigate the influence on the mouth-form for both isoforms of *Ppa-nhr-40* separately, I performed microinjections of the two cDNAs with the *Ppa-nhr-40* promoter region already used for the genomic construct of *Ppa-nhr-40* in the above mentioned rescue experiment. Another rescue experiment in *tu505* without the *eud-1* mutation was performed with the previously identified long cDNA of *Ppa-nhr-40*. Several independent transgenic lines confirmed once again that *Ppa-nhr-40* is responsible for the Euc phenotype observed in *tu505* (see Figure 5.3.5). Interestingly, this rescue revealed that *Ppa-nhr-40* is part of the developmental switch mechanism, which was already observed in *eud-1*: Injections of the wild type long cDNA construct of *Ppa-nhr-40* do not only rescue the mouth-form phenotype to the wild type phenotype of 90 % eurystomatous, a high extrachromosomal copy number of *Ppa-nhr-40*

leads to a switch in the mouth-form decision and reverts the phenotype to nearly 100 % stenostomatous.

Injection of the short cDNA isoform of *Ppa-nhr-40*, which contains only the DBD of the nuclear receptor, could not rescue the mutant phenotype. These observations result in three major conclusions: First, *Ppa-nhr-40* acts as a developmental switch downstream of *eud-1*. Second, only the long isoform is of functional significance in mouth-form regulation. Third, I could not find any evidence for the short isoform to represent a repressor of *Ppa-nhr-40* long.

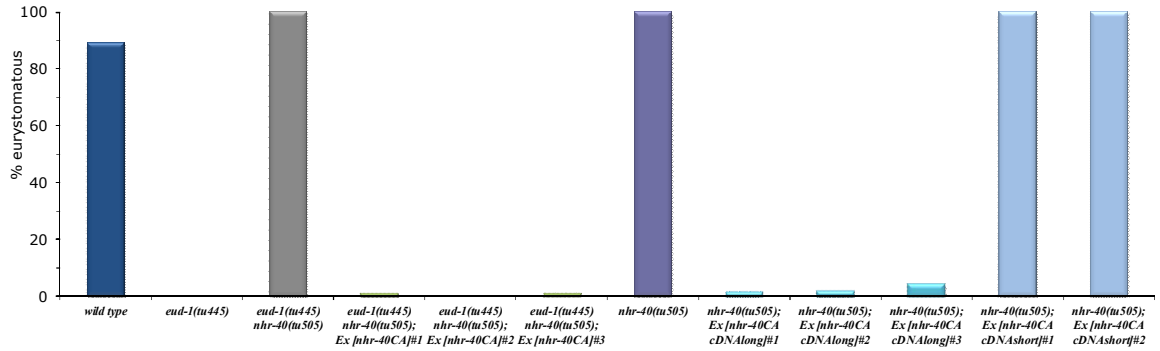


Figure 5.3.5: Mouth-form phenotypes of the Californian wild type strain, the mutants *eud-1(tu445)* and *Ppa-nhr-40(tu505)*, and transgenic rescue lines.
The wild type strain RS2333 showed a 90 % eurystomatous phenotype. This strongly contrasts the *Eud-1* phenotype, which was all stenostomatous. The *eud-1(tu445) Ppa-nhr-40(tu505)* double mutant and also the *Ppa-nhr-40(tu505)* single mutant were all eurystomatous. The double mutant and the *Ppa-nhr-40* single mutant Euc phenotype were completely or nearly completely reverted to all stenostomatous when the genomic or the long cDNA of the *Ppa-nhr-40* wild type gene was injected in high concentrations (10 ng/µl). Transgenic injections of the short cDNA of *Ppa-nhr-40* could not change the all eurystomatous phenotype of *Ppa-nhr-40*(*tu505*).

5.3.6 *tu505* is a dominant mutation

Already during backcrossing the dominance of the *tu505* allele was indicated as only few stenostomatous animals were formed in the F1 generation. In order to confirm the dominance of the *Ppa-nhr-40(tu505)* allele, I performed crosses with a transgenic wild type strain, which expressed the integrated marker *egl-20::RFP*. RFP expressing F1 animals were screened and not a single stenostomatous hermaphrodite was observed (see Figure 5.3.6). The crossed F1 hermaphrodites were all eurystomatous suggesting complete dominance of the allele. Previous transgenic rescue experiments with the wild type version of *Ppa-nhr-40* revealed that the dominant effect is actually caused by haploinsufficiency. Although, so far there is no further experimental evidence, this would support the hypothesis that *Ppa-nhr-40* acts like *eud-1* in a concentration-dependent manner.

Furthermore, I also screened the F1 male phenotype. Wild type males show a mouth-form phenotype of about 30 % eurystomatous (Serobyan et al., 2013). However, *Ppa-nhr-40(tu505)* mutant males are just like the hermaphrodites all eurystomatous. This shows that *Ppa-nhr-40* is also changing the male phenotype to 100 % eurystomatous (see Figure 5.3.6). The fact, that the *eud-1 Ppa-nhr-40* double mutant could be backcrossed by phenotype suggests that *eud-1* induced the formation of at least some stenostomatous animals in heterozygous *eud-1*/+ *Ppa-nhr-40*/+ animals.

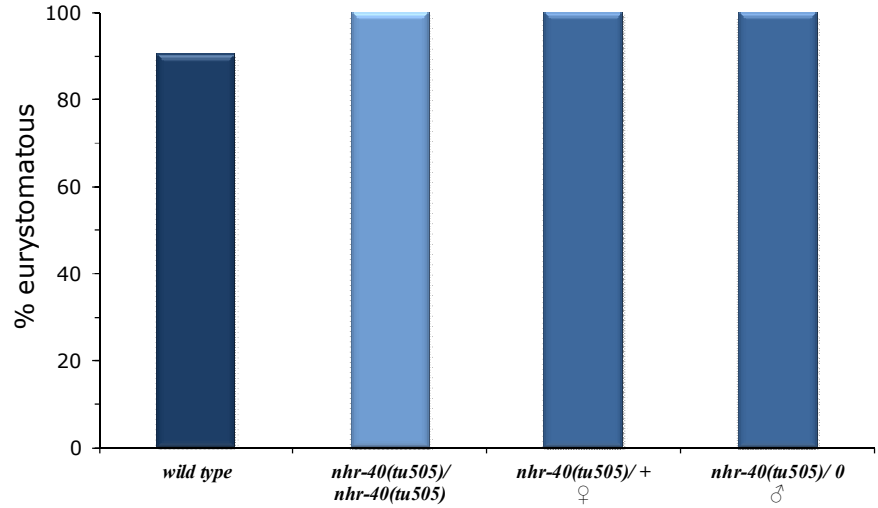


Figure 5.3.6: Dominance experiment.
Ppa-nhr-40(*tu505)* was crossed to RFP expressing wild type males. Heterozygous hermaphrodites show a penetrant mutant phenotype, which indicates complete dominance of *Ppa-nhr-40*. In the course of comprehensive phenotypic description, the mouth-form phenotype of males was also described. *Ppa-nhr-40* males are all eurystomatous and show the same phenotype as their hermaphroditic counterparts.

5.3.7 *Ppa-nhr-40* expression

Experiments discussed above indicate that *Ppa-nhr-40* is necessary and sufficient to regulate the phenotypic switch of mouth-form development. To gain a better understanding how and where *Ppa-nhr-40* is acting in the worm, I created a translational reporter construct to reveal which cells express *Ppa-nhr-40*. The reporter contained the same 6 kb promoter region that was also used for the rescue constructs. RFP was fused to the C-terminus of genomic *Ppa-nhr-40*. Although, I used a high concentration of the reporter construct in the injection mix (10 ng/µl) the RFP signal was not strong enough to get sufficiently recorded. Thus, I used antibody staining to amplify the RFP expression signal. The reporter showed a consistent labelling in the pharyngeal areas of the nematodes. Three independent reporter lines are shown below (see Figure 5.3.7). Due to fixation methods and the cellulase reaction used for antibody staining, the morphology of animals was partially disrupted and so far, a

clear identification of the cells expressing *Ppa-nhr-40* was not possible, so further analyses will be necessary. Preliminary analyses show that *Ppa-nhr-40* is expressed at three different locations. The first and second location is posterior of the first pharyngeal bulb where the nerve ring is located on the dorsal and the ventral side of the animal. These two locations showing *Ppa-nhr-40* expression are rather broad suggesting staining of multiple neuronal cell bodies and neuronal processes located in the nerve ring. The third position of *Ppa-nhr-40* expression is located besides the terminal bulb. Here, only one definite cell shows RFP expression. The expression pattern was consistent in young J3 until adulthood. Expression in younger animals could not be confirmed due to the low signal.

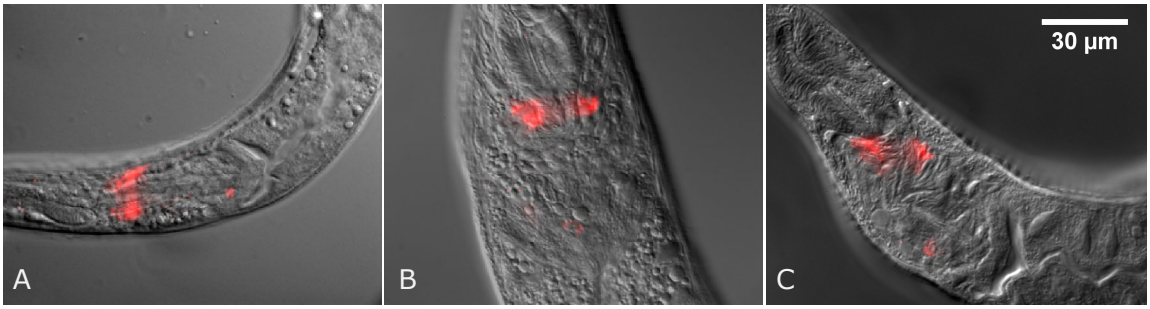


Figure 5.3.7: Antibody staining for the translational reporter lines.
Pictures show different and independent *Ppa-nhr-40(wild type)* reporter lines of the wild type strain RS2333. **A** J4 larva of the reporter line#1. **B** RFP expression of an adult hermaphrodite of the *Ppa-nhr-40(wild type)* reporter line #2. **C** The RFP expression in an adult hermaphrodite of the *Ppa-nhr-40(wild type)* reporter line #3 corresponds to the expression seen in other reporter lines. The inner structures of the nematodes are partially disrupted, which is caused by the preparation of the worms for antibody staining. Thus, the identification of the cells expressing RFP is rather difficult but a consistent staining is seen from stage J3 on. RFP expression in earlier stages is likely, but could not be confirmed due to the small size of the worms and the faint RFP staining arguing for a low expression of *Ppa-nhr-40*. Scale bar, 30 µm.

5.3.8 *Ppa-nhr-40* is epistatic to *Ppa-daf-12*

Mutations in the *Ppa-daf-12* gene, which codes for another nuclear receptor, have been shown to affect the mouth-form phenotype, but *Ppa-daf-12* is also necessary for dauer formation (Ogawa et al., 2009; Bento et al., 2010). *Ppa-daf-12* mutants show a mouth-form phenotype of less than 20 % eurystomatous animals. To study if *Ppa-nhr-40* is epistatic to *Ppa-daf-12* and to analyze if *Ppa-daf-12* is able to lower the all eurystomatous phenotype of the *Ppa-nhr-40* mutant, I generated a *Ppa-daf-12; Ppa-nhr-40* double mutant. Subsequently, the potential double mutants were verified by Sanger sequencing.

The *Ppa-daf-12(tu389); Ppa-nhr-40(tu505)* double mutant shows a completely eurystomatous phenotype (see Figure 5.3.8). This indicates that *Ppa-nhr-40* is epistatic to *Ppa-daf-12* and *Ppa-daf-12* does not lower the all eurystomatous *Ppa-nhr-40* phenotype. Therefore, I

conclude that *Ppa-nhr-40* acts downstream or in parallel of *Ppa-daf-12*. Together, these results show that *Ppa-nhr-40* is epistatic to *eud-1* and *Ppa-daf-12*, and is so far the most downstream gene identified in the phenotypic mouth-form switch.

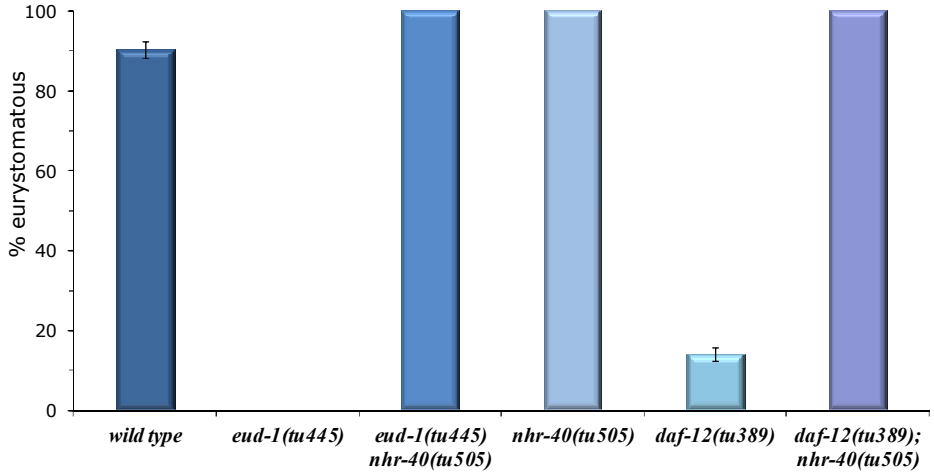


Figure 5.3.8: Mouth-form phenotype of epistatic crosses.
eud-1(tu445) and *Ppa-daf-12(tu389)* mutants show a highly stenostomatous mouth-form phenotype compared to their wild type strain RS2333, which is 90 % eurystomatous. The all eurystomatous phenotype of *Ppa-nhr-40(tu505)* is not affected by *eud-1(tu445)* or *Ppa-daf-12(tu389)*. Double mutants are still 100 % eurystomatous. *Ppa-nhr-40(tu505)* is epistatic to both mutant genes. Error bars represent 95% confidence intervals.

5.3.9 Predicted acetylation sites are necessary for *Ppa-nhr-40* function

Hepatocyte nuclear factor 4 *(hnf-4)* is the most closely related human gene to *Ppa-nhr-40*. The work of Soutoglou et al. found two lysines in the DBD to represent the most important acetylation sites and important for function (Soutoglou et al., 2000). Soutoglou and co-workers demonstrated that disruption of these acetylation sites greatly reduced nuclear retention of HNF-4 and therefore affecting the DNA binding activity of HNF-4. Bioinformatical analyses suggest the presence of several acetylation sites in *Ppa*-NHR-40. Two of them are located in the highly conserved DBD. Two lysines, which are potential acetylation acceptor sites are indeed conserved in human HNF-4 and in NHR-40 of different nematode species (see Figure 5.3.9).

To test for the importance of the predicted acetylation sites of *Ppa-nhr-40* in *P. pacificus,* the lysines at these sites were mutated to arginines by site-directed mutagenesis. Arginine is known to mimic deacetylation and preserves the electric charge of the original

lysine. Therefore, this kind of mutation is widely used in acetylation studies (Fu et al., 2003; Dormeyer et al., 2005; Lan et al., 2008; Yang and Seto, 2008). The acetylation sites of *Ppa*-NHR-40 are located in the third exon and are therefore present in both *Ppa-nhr-40* isoforms. As the short isoform does not show any effect on the mouth-form phenotype in the *Ppa-nhr-40* mutant, I investigated the predicted acetylation sites of the long isoform of *Ppa-nhr-40*.

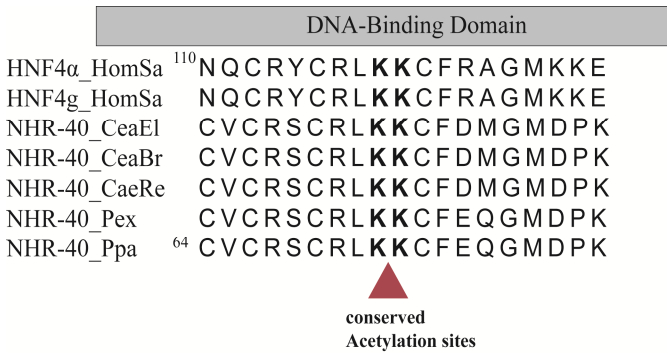


Figure 5.3.9: Protein alignment showing conserved acetylation sites.
The alignment shows the homologous human hepatocyte nuclear receptor HNF4α and γ and the NHR-40 proteins of different nematode species including *P. pacificus* and its sister species *P. exspectatus*. Both lysines were changed in *Ppa-nhr-40* of *P. pacificus* to arginine to disrupt the acetylation reaction.

Two independent transgenic lines carrying the long cDNA of *Ppa-nhr-40,* in which both lysines were mutated to arginine, do not show any rescue (see Figure 5.3.10). This implies that the acetylation sites are important for *Ppa-nhr-40* activity and necessary for the developmental decision of the mouth-form phenotype.

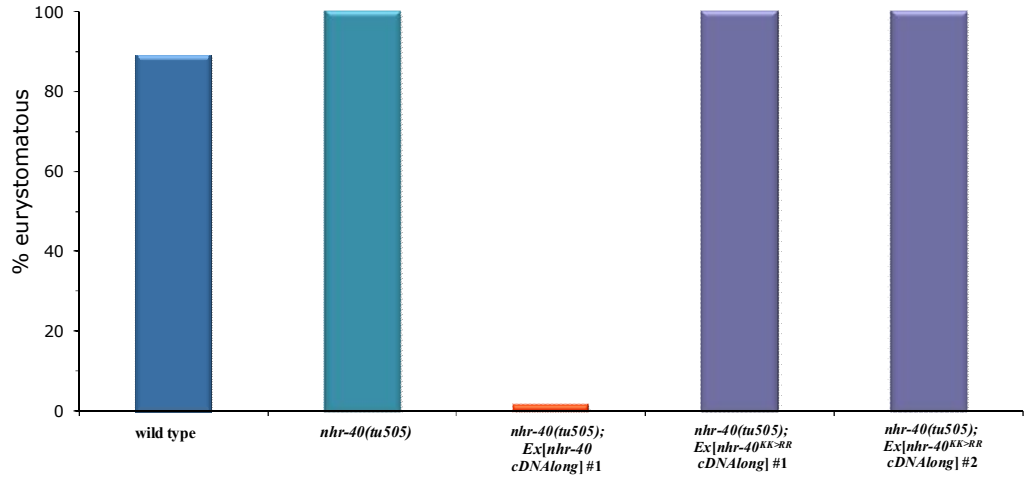


Figure 5.3.10: Mouth-form phenotype of transgenic lines with mutated *Ppa-nhr-40* acetylation sites.
Transgenic lines of *Ppa-nhr-40(tu505)* carrying the extrachromosomal array of *Ppa-nhr-40* cDNA long, in which the two lysines were mutated to arginines showed no rescue of the all eurystomatous phenotype. This supports the importance of the predicted acetylation sites.

5.3.10 Materials and methods

Culture conditions

All *P. pacificus* strains were kept on 6 cm Nematode Growth Medium (NGM) agar plates. Plates were spotted with 500 µl of the *Escheria coli* OP50 strain grown in L-Broth over night. For all crosses (e.g. backcrossing and generation of mapping lines) only 20 µl of OP50 was used and dried over night. All experiments were conducted at 20 °C.

Phenotypic analyses

The mouth-form type was defined by three reference characters. Briefly, were considered the width of the mouth opening, the form of the dorsal tooth, and the presence of a subventral tooth. In a stenostomatous animal the mouth opening is narrow, the dorsal tooth has a narrow shape and no subventral tooth can be found. The eurystomatous animal is defined by a broad mouth opening, a roundish dorsal tooth with a claw-like structure and the presence of a conspicuous subventral tooth. Only adults were used for phenotyping. If intermediates were found (<0.1 %), they were not included in the count. In high throughput screens the SteREO DicoveryV.12 stereomicroscope from Zeiss was utilised for phenotypic analyses. The magnification for screening was always 150. Approximately 100 animals of each mutant line were additionally screened by differential interference contrast (DIC) with a Zeiss Axioskop.

Suppressor screen

The *eud-1* mutant allele *tu445* with a premature Stop codon was used for mutagenesis. The mutant is 100 % stenostomatous. One well grown, mixed-stage plate was chemically mutagenized with Ehtyl methanesulfonate (EMS) according to the protocol of Pires da Silva (Pires da Silva, 2006). J4 animals were singled out and the F2 of these worms were screened for a eurystomatous mutant phenotype. Eurystomatous animals were singled out and the mutant lines were screened for several generations for their mouth-form phenotype. Additionally, the mutant lines were Sanger sequenced for the mutation in the *eud-1* gene.

For the generation of mapping lines the highly stenostomatous clade A strain RS5200B (10 % eurystomatous) was mutagenized. From this, morphologically striking dumpy (Dpy) mutants were isolated in the F2 and screened for their phenotypic penetrance for several generations. The dumpy mutant *tu472* (RS2708) was selected, backcrossed four times and subsequently used for generation of mapping lines.

Backcrossing of the suppressor mutants

Three of the four mutant lines were backcrossed at least seven times to remove background mutations. The all stenostomatous *eud-1*(*tu445)* was used for backcrossing. Suppressor mutant J4 hermaphrodites were crossed with *tu445* males. In crossing plates containing many males, 40 J4 and J3 hermaphrodites were picked from the F1. The following day, the mouth-form was checked and only stenostomatous animals were kept. The F2 was screened for its mouth-form frequency. In the F2, 40 animals in J3 and J4 stage from the plates showing the highest stenostomatous frequencies were picked onto single plates. The mouth-form was checked the next day and only eurystomatous animals were kept. In the F3, plates were screened for the mutant mouth-form phenotype. These plates were kept or used for another backcrossing cycle.

Outcrossing of *tu445* in the *eud-1(tu445) Ppa-nhr-40(tu505)* mutant

The backcrossed suppressor mutant RS2703 is a *eud-1(tu445) Ppa-nhr-40(tu505)* double mutant and was crossed to RS2333 males. Males were allowed to mate for two days before being killed. From successfully crossed plates containing many males, young hermaphrodites in J3 and J4 stage were isolated. After egg-laying hermaphrodites were analysed for heterozygosity at the *Ppa-nhr-40(tu505)* locus and the progeny of F1 heterozygous mothers were singled out. In the F2, the *Ppa-nhr-40(tu505)* and the *eud-1(tu445)* locus of the worms were analysed by Sanger sequencing after egg-laying. Out of 80 analysed F2 mothers, only three were wild type at the *eud-1(tu445)* locus and contained the mutant *Ppa-nhr-40(tu505)* sequence suggesting linkage between both loci. One of the single mutant *Ppa-nhr-40(tu505)* lines were chosen for cryopreservation. The line was designated RS2771 *tu505*.

Table 5.3.2: Used primers for genotyping crossed animals for *Ppa-nhr-40(tu505)* and *eud-1(tu445)*.

	Forward Primer 5'-3'Seq	Reverse Primer 5'-3'Seq
Ppa-nhr-40 **allele *tu505***	GATTTCTATCGAATGAGCCGAGT	ATAGGCTTCTGTTGGGTGGTG
eud-1 **allele *tu445***	1st PCR CATTGGAGAAGAAAGGGGAAG	1st PCR AAGTAGATGGTTTGGGTTATCGAA
	nested PCR GTATTTTCGGCCATTCCAGA	nested PCR ACTTGACCGAAAACGGTAAACAC

Ppa-daf-12(tu389); Ppa-nhr-40(tu505) double mutant

RS2771 *tu505* hermaphrodites were crossed with *Ppa-daf-12*(*tu389)* males. Males were again killed after 2 days. F1 J4 hermaphrodites were picked to individual plates from crossed plates with many F1 males indicating successful mating. After the F1 hermaphrodites laid eggs, they were checked for heterozygosity in the *Ppa-nhr-40*(*tu505*) mutant locus by Sanger sequencing. Several F2 hermaphrodites were singled out from plates of a heterozygous mother. After they laid eggs the hermaphrodites were checked for homozygous *Ppa-nhr-40(tu505)* and *Ppa-daf-12(tu389)* locus by Sanger sequencing. The double mutant *Ppa-daf-12(tu389); Ppa-nhr-40(tu505)* was verified for its mouth-form phenotype.

Table 5.3.3: Used primers for genotyping crossed animals for *Ppa-nhr-40* and *Ppa-daf-12*.

	Forward Primer 5'-3'Seq	Reverse Primer 5'-3'Seq
***Ppa-nhr-40 tu505* allele**	GATTTCTATCGAATGAGCCGAGT	ATAGGCTTCTGTTGGGTGGTG
***Ppa-daf-12 tu389* allele**	1st PCR CACATCGATTCTGTCCCGGTGGAGAGTC	1st PCR CGAGTGTGTGGAGATCATGCGACAGGATAC
	nested PCR GACCCATTGAGAGACCACCATGACGAGAC	nested PCR CACTTGTGAGAGTTGCAAGGCTTTCTTCAGA

Whole-genome sequencing

To complement data for mapping each backcrossed mutant line was whole-genome sequenced. In preparation for sequencing, five full grown plates containing only residual bacteria were washed in a 50 ml falcon tube with 0.9 % NaCl-solution. The worms were centrifuged at 1.300 rcf for 4 min at 4 °C. The supernatant was removed and the falcon tube was refilled with 0.9 % NaCl containing Ampicillin (50 µg/ml) and Chloramphenicol (25 µg/ml) and washed for 12 h at RT. The worm pellet was harvested and frozen at -20 °C.

DNA was extracted using the Epicentre MasterPure DNA purification kit. Libraries were prepared using the Illumina TruSeq Nano Library Prep Kit. Libraries were prepared following manufacturer's instruction protocol with 100 ng of DNA used for each sample. Libraries were quantified by Qbit dsDNA BR assay kit measurements and the fragment size was verified by Bioanalyzer measurements. All libraries were diluted to 10 nM in 0.1 % EB-Tween and pooled as 10 plex. The libraries were single-read sequenced on an Illumina Genome Analyzer.

Mapping lines

In preparation of RAD sequencing for mapping, mapping lines were created. The backcrossed mutant line was crossed to RS5200B males. Two Euc J4 hermaphrodites and three to five young males of RS5200B were picked to crossing plates and males were killed after two days. Males of the following generation were crossed to *tu472* Dpy J4 hermaphrodites and non-Dpy hermaphrodites were singled out. From those plates animals were picked once again on individual plates and screened in the next generation. For mapping, 32 lines showing the mutant Euc phenotype (positive mapping lines), but also 32 lines showing the highly stenostomatous phenotype (negative mapping lines) were kept for RAD sequencing.

RAD library preparation

One well-grown plate of each mapping line with limited amount of residual bacteria was washed with 0.9 % NaCl-solution in a 15 ml falcon tube. The worms were centrifuged at 1.300 rcf for 4 min at 4 °C. The supernatant was removed and the falcon tube was refilled with 0.9 % NaCl containing Ampicillin (50 µg/ml) and Chloramphenicol (25 µg/ml) and washed for 12 h at RT. The worm pellet was transferred to a 1.5 ml Eppendorf tube and stored at -20 °C. DNA of the worm pellet was extracted with the Epicentre MasterPure DNA purification kit following the manufacturer's instructions. The DNA pellet was resolved with 35 µl Elution buffer instead of TE buffer. DNA was quantified by Qbit dsDNA BR assay kit. For a 96 sample library preparation the DNA of each sample was normalized to 20 ng/µl in a 96 well plate. Restriction site-associated DNA (RAD) markers were generated by using MseI and PstI restriction enzymes. 96 plex libraries were prepared by the method described by Poland (Poland et al., 2012). The libraries were quantified by Qbit dsDNA BR assay kit measurements and the fragment size was verified by Bioanalyzer measurements. All libraries were diluted to 10 nM in 0.1 % EB-Tween. The libraries were single-read sequenced on an Illumina Genome Analyzer.

Transgenesis

Extrachromosomal arrays were created as described by Schlager (Schlager et al., 2009). Young adult hermaphrodites were injected with the injection mixture, containing the genomic construct or the reporter construct (genomic promoter fused to cDNA construct) (2-10 ng/µl), the transgenic marker *Ppa-egl-20*::TurboRFP (10 ng/µl) and genomic carrier DNA (60 ng/µl) from the recipient mutant or strain. DNA extraction was done by Epicentre MasterPure DNA Extraction Kit. The transgenic marker was digested with PstI and the construct was digested with XmaI or NotI. The genomic DNA was double digested with PstI and XmaI or NotI respectively depending on the construct.

Translational *Ppa-nhr-40* reporter construct

The translational reporter construct of *Ppa-nhr-40* contained the 6 kb upstream region of the gene as a promoter sequence, just like in the rescue construct. The STOP codon of *Ppa-nhr-40* was removed and the gene sequenced was directly fused to Turbo RFP together with the 3'UTR of *rpl-23*. The whole reporter construct was 14.3 kb in size. It was digested with XmaI and injected to the wild type strain RS2333 in a concentration equal to 10 ng/µl. The injection mix further contained the transgenic marker *Ppa-egl-20::*TurboRFP (10 ng/µl) digested with PstI and the genomic DNA of RS2333 (60 ng/µl), digested with PstI and XmaI.

RNA extraction and cDNA synthesis

Total RNA was extracted using Ambion™ TRIzol® reagent. Supercript® II Reverse Transcriptase from Invitrogen was used for reverse transcription following manufacturer's instruction.

RACE

5' and 3' RACE experiments were carried out for *Ppa-nhr-40* by SMARTer® RACE cDNA Amplification Kit from clontech. Instructions were followed according to the manufacturer's protocol. Gene-specific primers, shown in table 5.3.4 were designed and used for the RACE reactions.

Table 5.3.4: Gene specific primers used for *Ppa-nhr-40* RACE experiments.

Ppa-nhr-40 **RACE**	Primer	Oligo
5' RACE primers	C77-5'Exon3-1	CCGACGAGACATTTGCCTTCGAACTGAC
	C77-5'Exon4-2	ACTTCTTGAGACGACAGCTTCGGCAGAC
	C77-5'Exon15-1	TTGTAGGAGGAAGGGGAGGCATGTTGCT
	C77-5'Exon15-3	AGCCAGAAGAGAAGCTTGTTCCGTCAGC
	C77-5'Exon14-4	CAGGTGGGTTCATCCTCGAGAATCTCATC
3' RACE primers	C77-3'Exon15-1	CGTAAGCTGACGGAACAAGCTTCTCTTCTGG
	C77-3'Exon16-2	GACCTCTCTCCCAACAACTCTTCCTACGTTCC
	C77-3'Exon19-3	GCTCTCCGTAAGATGTTCACGATGACTTCC
	C77-3'Ex19-4	ACGACGAGCGAGTTCCCTCACCATC
	C77-3'Exon2-1	CGGGAGAAGATTCCGGAGGGAAC
	C77-3'Exon2-2	TCTGTGTCGTCTGTGATGATTCGGCCTCT
	C77-3'Exon2-3	CTGTCGCTTCGTGCAATGGATGCAAAAC
	C77-3'Exon8-2	GAGAGCTGATGCGAATCGAGAAGAAGG
	C77-3'Exon8-1	GCGAATCGAGAAGAAGGTTGTCGAAG

Antibody staining

The antibody staining was done according to Wilecki et al. (Wilecki et al., 2015). The primary antibody for RFP staining was RFP Rabbit Polyclonal Antibody from Invitrogen. The secondary antibody was the Alexa Fluor ® 555 anti-rabbit IgG antibody from Invitrogen. The images were taken with a Zeiss Axioskop microscope camera.

Site-directed mutagenesis

The phusion of the genomic *Ppa-nhr-40* promoter sequence of 6 kb and the long 4 kb cDNA plus 3'UTR of *Ppa-nhr-40* were cloned into pCR™2.1-TOPO® plasmid of Invitrogen. For site-directed mutagenesis the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies was used following manufacturer's instructions. Primers were designed according to manufacturer's instructions. The mutated constructs were verified by Sanger sequencing.

Table 5.3.5: Used primers for site-directed mutagenesis.

Mutagenesis primers	5'-3' Oligo Sequence
*Ppa-nhr-40*_KK_RR1F	GCCGAAGCTGTCGTCTC**agaaga**TGTTTCGAACAGGG
*Ppa-nhr-40*_KK_RR1R	CCCTGTTCGAAACA**tcttct**GAGACGACAGCTTCGGC

5.4 Phenotypic and molecular characterization of *euc* mutants isolated from a clade C strain of *Pristionchus pacificus*

5.4.1 Introduction

Previous results of mutagenesis experiments revealed a great number of eurystomatous-form defective (*eud*) mutants in a clade A strain (RS2333) of *P. pacificus*, which shows 70-90 % eurystomatous animals in the population dependent on culturing conditions (Serobyan et al., 2013). Furthermore, I have already shown in my diploma work that it is possible to isolate eurystomatous-form constitutive (*euc*) mutants from this strain (Müller, 2010). The four different clades in *P. pacificus* are highly diverse (Rödelsperger et al., 2014). This raises the question if the genes defining the mouth dimorphism in an evolutionary diverged clade are as numerous as in the clade A strain RS2333 and if these genes have the same relevance for mouth formation as they differ in their hierarchical order. Although, *euc* mutants have been isolated before in mutant backgrounds, it was still not clear if such a screen would be successful in a strain that is naturally highly stenostomatous. Besides clade A, clade C represents the most strain-rich clade and possesses a high genetic diversity (Rödelsperger et al., 2014). Here, I report results of forward genetic approaches yielding several *euc* mutants from the highly stenostomatous clade C strain RSB020 (~ 2 % eurystomatous) and their phenotypical as well as molecular characterization.

5.4.2 Mutant screen for *euc* mutants in RSB020

To identify genetic effectors responsible for the formation of a highly stenostomatous mouth-form phenotype, I used a forward genetic approach. The strain used for EMS mutagenesis was the highly stenostomatous clade C strain RSB020. Before the mutagenesis was conducted, the *P. pacificus* strain RSB020 was screened for several generations under standard laboratory conditions for its consistent and highly stenostomatous mouth-form frequency. RSB020 populations were always highly stenostomatous with an average of 2 % eurystomatous animals in the population (see Figure 5.4.1).

Mutagenized F2 animals were screened for a eurystomatous mouth-form. F3 populations showing a high eurystomatous phenotype were kept as a potential mutant line and multiple plates were screened for multiple generations. In a mutagenesis screen of

approximately 5000 gametes, I isolated 20 mutant lines showing a mouth-form phenotype of at least 50 % eurystomatous. The Euc phenotype of the mutant lines was additionally confirmed by Nomarski microscopy. I successfully backcrossed thirteen of these mutant lines with the highest eurystomatous phenotypes for subsequent mapping and cryopreservation (see Table 5.4.1). The consistency of the mutant mouth-from phenotype was again confirmed by screening various plates of several generations (see Figure 5.4.1). These results suggest that the highly stenostomatous phenotype of RSB020 is genetically determined and can be reverted by single gene mutations.

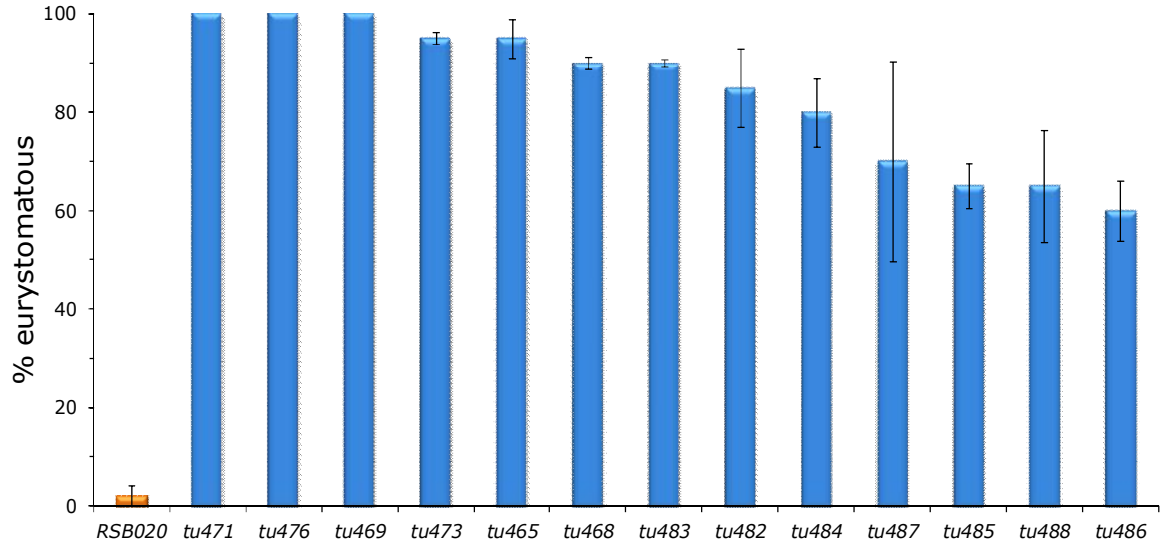


Figure 5.4.1: Euc mouth-form phenotype of the thirteen isolated and backcrossed mutant lines.
Three lines are completely eurystomatous. Not a single stenostomatous animal was observed in these three lines. All the other mutant lines are highly eurystomatous, though they are able to form stenostomatous animals. The RSB020 is 2 % eurystomatous. Error bars show 95% confidence intervals.

Table 5.4.1: List of cryopreserved mutant lines.
The table shows their RS numbers, *tu* numbers, their Euc phenotype in percentage of eurystomatous (Eu) animals, and the number of backcrossings.

RS number	*tu* number	% Eu	times backcrossed
RS2701	*tu465*	95	5
RS2704	*tu468*	90	5
RS2705	*tu469*	100	5
RS2707	*tu471*	100	4
RS2709	*tu473*	95	6
RS2712	*tu476*	100	3
RS2726	*tu482*	85	3
RS2727	*tu483*	90	3
RS2728	*tu484*	80	3
RS2729	*tu485*	65	3
RS2730	*tu486*	60	3
RS2731	*tu487*	70	3
RS2732	*tu488*	65	3

5.4.3 Mapping by RAD sequencing

Mapping lines were generated for each backcrossed mutant line. In order to prevent crossing incompatibilities, which would result in an elevated mortality rate of recombinant lines and to ensure uniform recombination along the chromosomes, I used the highly stenostomatous clade C strain RSB025 for mapping. RSB025 has a mouth-form phenotype of 10 % eurystomatous under standard laboratory conditions. However, since no morphological mutant of RSB025 was available, I isolated several mutants of this strain showing a dumpy (Dpy) phenotype by an additional EMS mutagenesis. The mutant *tu470* showing a penetrant, strong Dpy phenotype was used for the second backcross. A potential influence on the mouth-form phenotype was excluded by mouth-form screening.

Using next-generation sequencing for mapping, 32 positive mapping lines showing the highly eurystomatous, mutant phenotype and 32 negative mapping lines showing the RSB025 or RSB020 wild type mouth-form phenotype were singled out. The negative mapping lines were intended to control for potential chromosomal areas of varying recombination rates and therefore, ensure for a precise and high resolution in the RAD analysis to indentify, at best, a small region on a chromosome carrying the mutation responsible for its Euc phenotype. To complement the RAD sequencing analysis, all mutant lines including the phenotypic Dpy mutant *tu470* used for mapping, were sequenced by Illumina whole-genome sequencing. The results of the RAD libraries are summarized in separate panels for each *euc* mutant below (see Figure 5.4.2, RAD results).

In the RAD sequencing analysis, the 64 mapping lines generated for each of the 13 mutant lines were genotyped. Some of the mutants show a strong association to a single chromosome (e.g. *tu469*). However, some of the mutants show peaks at several genomic loci (e.g. *tu471*) or barely any significant association at all (e.g. *tu482*).

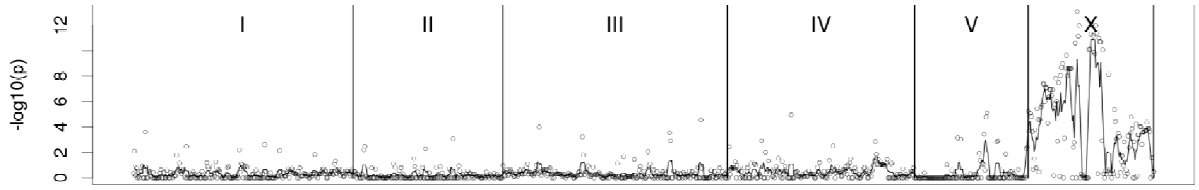
Why RAD sequencing analysis could not assign some mutations to a single chromosome remains unclear. The phenotype of the mapping lines was reassured several times. Therefore, wrong phenotypic classification can be excluded. However, while the used mapping strain RSB025 was selected according to its phenotype and close phylogenetical distance, I cannot completely exclude the possibility of effects on the mouth-form phenotype due to epistatic interactions between these two strains. Also, it is important to note that the Californian strain RS2333, for which the genome draft is most accurate, is only distantly related to the clade C strains. Therefore, large-scale insertions, deletions, rearrangements, and copy number variations might influence this mapping analysis of RSB020 and RSB025. A

low association value could imply that mutations are located on contigs, which are not assigned to a chromosome and the flanking genomic regions show a high recombination rate or are wrongly linked to different chromosomes. The false assignment of contigs to chromosomes would also explain the findings where RAD sequencing data show several peaks for different chromosomes.

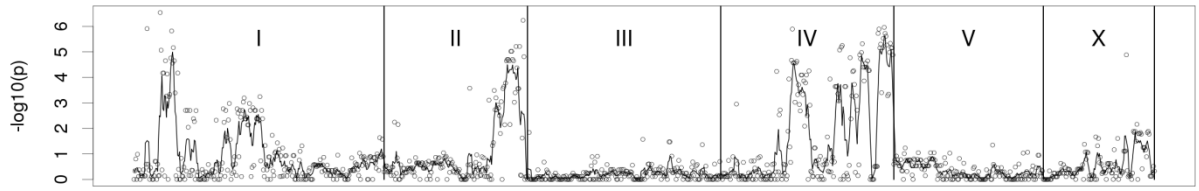
Therefore, the RAD results shown below provide just a first and rough mapping and further analysis is needed to narrow down the mapping interval in most of the *euc* mutants. Mutant lines showing a clear association to a single chromosome were analysed further. A list of all mutations in the chromosomal regions with a high RAD score was generated based on the whole-genome sequencing data.

RAD results:

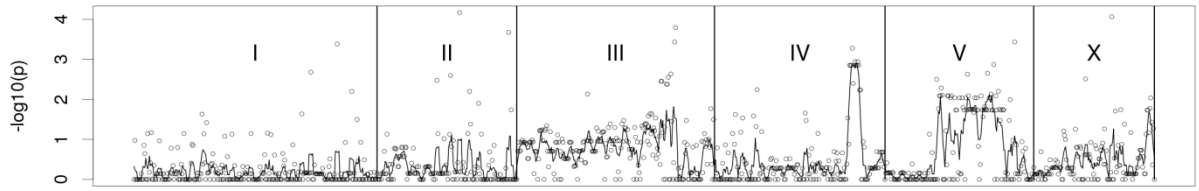
tu469



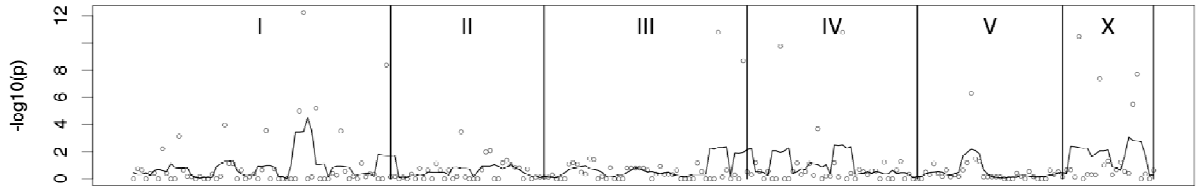
tu471



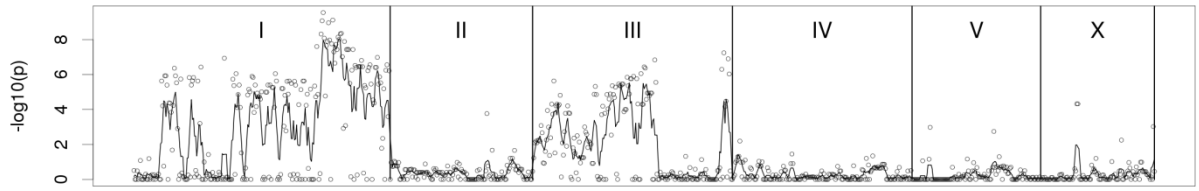
tu476



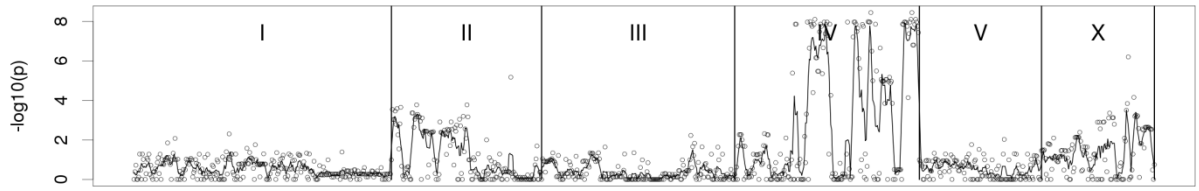
tu473



tu465



tu468



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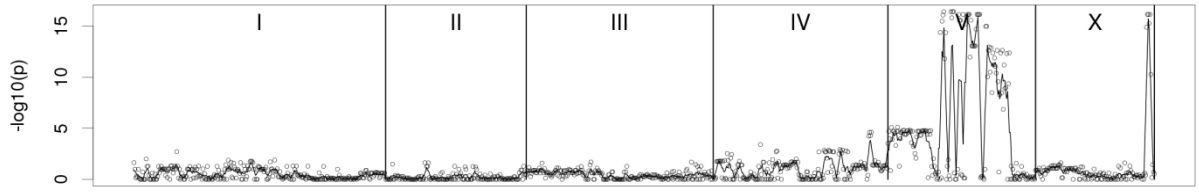

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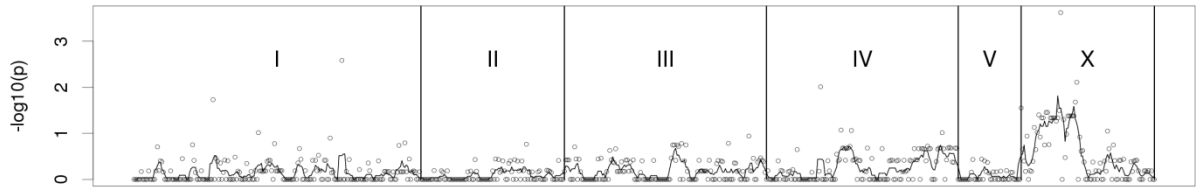

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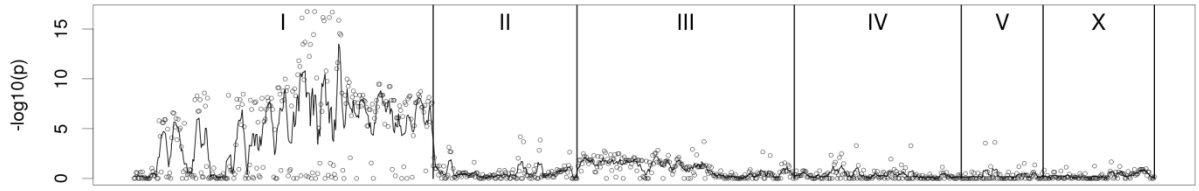

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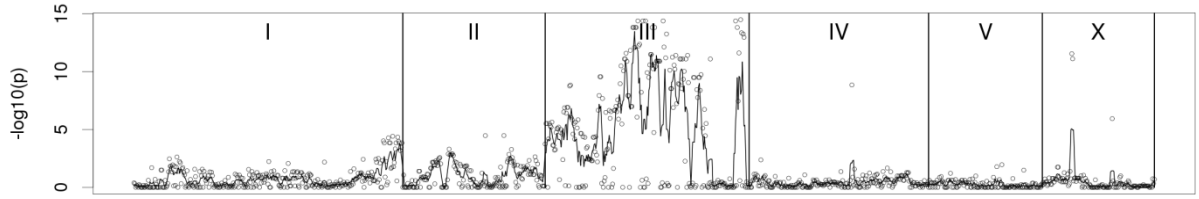

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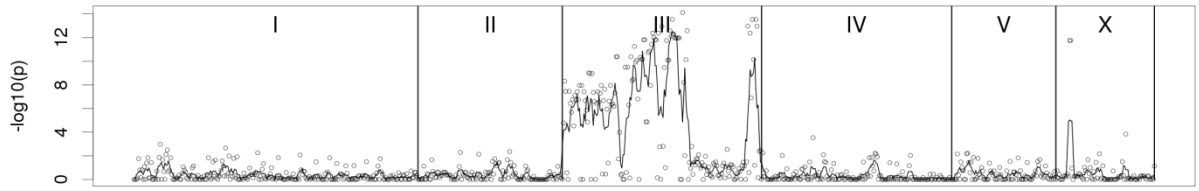

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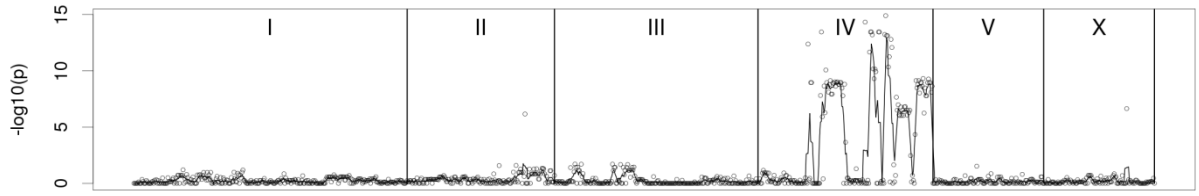

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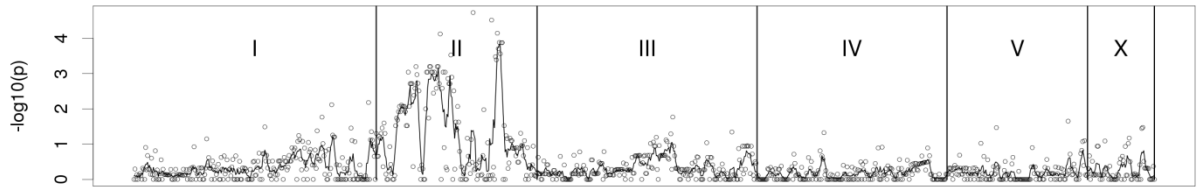

Figure 5.4.2: RAD Mapping results of *euc* mutants.
Each panel shows the RAD mapping results of the respective *euc* mutant. The x-axis displays the relative positions of the genotyped markers available. The y-axis demonstrates the significance of the association between genotype and phenotype. These plots along the chromosomes were achieved by positioning *P. pacificus* contigs, based on their marker positions on the genetic linkage map. Consequently, fragmented peaks could be explained by unresolved orders on the linkage map, large structural variations, or different genetic loci affecting the mouth-form phenotype.

5.4.4 Candidate mutations responsible for the Euc phenotype

Candidate gene lists of all mutant lines, which could be assigned to a single chromosome by RAD analysis, were created (see Table 5.4.2-5.4.8). These lists contain all the mutations on the assigned chromosome in or close to genes, including intronic regions. Intergenic regions containing mutations and located in gene-dense chromosomal regions were not designated to a specific gene, but included in the candidate gene list. All the genes of the gene list are bioinformatically predicted, thus mutations located at intergenic or intronic positions could still be inside a coding gene sequence. On the other hand, the Euc phenotype could also be provoked by mutations inside a promoter region or inside an intron, regulating gene expression or leading to a non-functional splice variant.

Table 5.4.2: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu469* by whole-genome sequencing in the genomic region of Chromosome X showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu469				
Gene	**Position**	**Exchange**	**Kind of Mutation**	
Contig50-snap.59	Contig50:283725	C>T	nonsynonym	R>K
Contig50-snap.112	Contig50:548426	C>T	intronic	
	Contig50:96300	C>T	noncoding	
Contig8-snap.206	Contig8:1579778	G>A	intronic	
	Contig8:1950207	C>T	noncoding	
	Contig8:1997193	C>T	noncoding	

Table 5.4.3: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu483* by whole-genome sequencing in the genomic region of Chromosome V showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu483				
Gene	**Position**	**Exchange**	**Kind of Mutation**	
Contig120-snap.45	278009	C>T	intronic	
Contig120-snap.46	279325	C>T	nonsense	STOP
Contig21-snap.74	427433	C>T	nonsynonym	L>F
Contig21-snap.181	1152404	C>A	nonsynonym	C>F
Contig22-snap.115	777201	C>T	intronic	
Contig22-snap.215	1347772	G>T	nonsynonym	V>F
Contig25-snap.185	1212989	C>T	nonsynonym	A>T
Contig3-snap.90	632912	G>A	nonsynonym	G>E
Contig78-snap.47	236359	G>A	nonsynonym	T>I
Contig10-snap.321	1929980	T>A	nonsynonym	R>S

Table 5.4.4: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu484* by whole-genome sequencing in the genomic region of Chromosome I showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu484				
Gene	**Position**	**Exchange**	**Kind of Mutation**	
Contig1-snap.362	2434822	C>T	nonsynonym	T>M
Contig15-snap.167	1334724	T>A	nonsynonym	M>K
Contig20-snap.55	405047	C>A	nonsynonym	P>Q
Contig37-snap.30	152574	G>A	nonsynonym	G>E
Contig37-snap.139	839027	G>A	nonsynonym	S>L
Contig56-snap.62	330160	G>A	nonsynonym	G>E

Table 5.4.5: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu487* by whole-genome sequencing in the genomic region of Chromosome III showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu487				
Gene	**Position**	**Exchange**	**Kind of Mutation**	
Contig11-snap.74	447798	G>A	nonsynonym	R>H
Contig11-snap.111	644190	G>A	nonsynonym	L>F
Contig11-snap.217	1289659	G>A	nonsynonym	V>I
Contig13-snap.22	142646	T>A	nonsynonym	I>F
Contig13-snap.42	230585	C>T	nonsynonym	L>F
Contig2-snap.311	2404760	G>A	nonsynonym	T>M
Contig2-snap.345	2577897	G>A	intronic	
Contig46-snap.9	60805	C>T	nonsynonym	T>I
Contig46-snap.24	206983	C>T	nonsynonym	P>S
Contig46-snap.85	677419	C>T	nonsynonym	A>T
Contig6-snap.59	527919	G>T	nonsynonym	M>I
Contig62-snap.64	550248	T>C	nonsynonym	V>A
Contig62-snap.65	566149	T>C	nonsynonym	V>A
Contig71-snap.74	536618	G>A	nonsynonym	R>H

Table 5.4.6: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu485* by whole-genome sequencing in the genomic region of Chromosome III showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu485				
Gene	**Position**	**Exchange**	**Kind of Mutation**	
Contig11-snap.208	1259204	C>A	intronic	
Contig13-snap.44	252933	G>A	nonsynonym	M>I
Contig2-snap.51	520437	C>T	nonsynonym	C>Y
Contig2-snap.472	3539194	T>C	nonsynonym	E>G
Contig34-snap.59	469313	G>T	nonsynonym	H>Q
Contig34-snap.80	638974	G>C	nonsynonym	W>C
Contig73-snap.95	745781	C>T	nonsynonym	V>I
Contig99-snap.4	40514	G>A	nonsynonym	P>S

Table 5.4.7: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu488* by whole-genome sequencing in the genomic region of Chromosome IV showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu488				
Gene	**Position**	**Exchange**	**Kind of mutation**	
Contig125-snap.2	3651	C>T	nonsynonym	G>E
Contig28-snap.193	1005826	G>C	nonsynonym	L>V
Contig28-snap.196	1023787	A>C	nonsynonym	S>A
Contig45-snap.165	1002334	G>A	nonsynonym	S>F
Contig48-snap.51	311295	G>T	nonsynonym	H>Q

Table 5.4.8: List of candidate genes responsible for the Euc phenotype.
The list contains all mutations found in the mutant line *tu486* by whole-genome sequencing in the genomic region of Chromosome II showing the highest significance of association between parental genotype and phenotype according to the RAD mapping results.

tu486				
Gene	**Position**	**Exchange**	**Kind of mutation**	
Contig108-snap.7	40292	C>A	nonsynonym	A>E
Contig108-snap.17	91102	G>T	nonsense	STOP
Contig31-snap.211	1168655	A>G	nonsynonym	E>G
Contig5-snap.9	187315	A>T	nonsynonym	H>Q
Contig58-snap.25	101751	C>G	nonsynonym	R>P
Contig58-snap.76	534829	C>T	nonsynonym	G>S
Contig58-snap.94	685762	G>A	nonsynonym	E>K
Contig7-snap.247	1696299	C>T	nonsynonym	G>S
Contig7-snap.363	2573726	C>G	nonsynonym	E>D

Among all mutant lines, the mutant *tu469* has shown a strong and clear association to chromosome X. The region of the highest RAD mapping score was assigned to a region close to *eud-1*, although no mutation could be found close to the *eud-1* gene. The analysis of all X-linked mutations in *tu469* revealed that only a small number of mutations could be found within or close to predicted genes. Thus, I decided to do fine mapping according to the mutations found by whole-genome sequencing.

5.4.5 Mapping of *tu469*

The *euc* mutant *tu469* showing a 100% eurystomatous phenotype was assigned by RAD sequencing to chromosome X. A list of mutations in genes and regions near to predicted genes found on chromosome X is shown above (Table 5.4.2). All of these nucleotide changes were first confirmed by Sanger sequencing. In order to find the mutation responsible for the Euc phenotype, I sequenced all of the present mapping lines (positive and negative) for these mutations (see table 5.4.9). The mutation on Contig 50 position 548426 was present in positive and negative mapping lines, which argues that this nucleotide change was already present in the strain used for mutagenesis and therefore is not affecting the mouth-from phenotype. Previous mapping of *eud* mutants has shown that false positive mapping lines are possible. Therefore, I did not exclude the mutation on Contig 50 position 283725. However, this mutation is the only one located inside a predicted gene (Contig50-snap.59) and leads to non-synonymous amino acid change in the protein (K>R).

Nevertheless, transgenic experiments showed no rescue of the Euc phenotype for Contig50-snap.59. The RSB020 wild type version of this gene was injected into *tu469*. The genomic region of the predicted gene spanned approximately 6 kb. A 2.2 kb promoter and

700 bp downstream region of the gene was included in the 8.9 kb construct. A concentration of 10 ng/µl of the construct was injected. Several independent transgenic lines showed no rescue of the all eurystomatous mouth-form phenotype. Thus, this mutation can be excluded and hence is not affecting the mouth-form phenotype. According to the mapping data, the next most likely candidate mutation is on Contig50 position 963006. This mutation is positioned in the promoter region of two genes going opposite directions. These genes are Contig50-aug14714.t1 (Augustus 2013 Prediction) and Contig50-snap.202 (SNAP Hybrid1 Predictions). The ortholog of Contig50-snap.202 in *C. elegans* is *cccp-1*, which exhibits Rab GTPase binding activity. *cccp-1* is involved in locomotion and localized at various positions (e.g. axon and cell body) in neurons. Transgenic rescue experiments have not been conducted yet and the influence of Contig50-aug14714.t1 and Contig50-snap.202 on the mouth-form phenotype awaits further analysis.

Table 5.4.9: Mapping table of candidate mutations in *tu469*.
Sequencing results of the mapping lines of *tu469* for the different candidate mutations.
1 mutation confirmed, **0** missing data, **-1** wild type sequence.

Contig Chr	base position of mutation	Ma pos 1	Ma pos 2	Ma pos 3	Ma pos 4	Ma pos 5	Ma pos 6	Ma pos 7	Ma pos 33	Ma pos 9	Ma pos 10	Ma pos 11	Ma pos 12	Ma pos 13	Ma pos 14	Ma pos 16	Ma pos 17	Ma pos 18	Ma pos 19	Ma pos 20	Ma pos 21	Ma pos 22	Ma pos 34	Ma pos 24	Ma pos 25	Ma pos 26	Ma pos 27	Ma pos 28	Ma pos 29	Ma pos 30	Ma pos31
Contig8 ChrX	1997193	1	1	-1	-1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	-1	1	1	0	1	1	1	1	0	1	1
Contig8 ChrX	1579778	1	1	-1	-1	1	1	1	H	1	1	1	H	1	1	0	1	1	1	-1	1	1	1	H	1	H	1	1	-1	1	1
Contig8 ChrX	1950207	1	1	-1	-1	1	1	1	H	1	1	1	1	1	1	1	1	1	1	-1	1	1	1	H	1	1	1	1	H	1	1
Contig50 ChrX	283725	1	1	1	1	0	H	1	1	1	1	1	1	1	1	0	1	1	1	H	1	1	1	1	1	1	1	-1	1	1	1
Contig50 ChrX	963006	1	1	0	1	0	0	1	1	1	1	0	0	1	1	0	0	0	1	H	1	0	0	0	1	1	1	0	0	0	1
Contig50 ChrX	548426	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1

Contig Chr	base position of mutation	Ma neg 2	Ma neg 3	Ma neg 4	Ma neg 5	Ma neg 33	Ma neg 1	Ma neg 7	Ma neg 8	Ma neg 9	Ma neg 10	Ma neg 11	Ma neg 12	Ma neg 13	Ma neg 14	Ma neg 15	Ma neg 16	Ma neg 17	Ma neg 18	Ma neg 19	Ma neg 20	Ma neg 21	Ma neg 22	Ma neg 23	Ma neg 24	Ma neg 25	Ma neg 26	Ma neg 27	Ma neg 28	Ma neg 29	Ma neg3 0
Contig8 ChrX	1997193	-1	-1	-1	0	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0	-1	-1	-1	-1
Contig8 ChrX	1579778	-1	-1	-1	H	H	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	H	-1	-1	-1	-1	H	-1	-1	-1	-1
Contig8 ChrX	1950207	-1	-1	-1	H	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	H	-1	-1	-1	-1
Contig50 ChrX	283725	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Contig50 ChrX	963006	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	H	-1	-1	-1	-1	-1	-1	-1	-1	-1	0	-1	-1	-1	-1	-1	-1	-1	-1	-1
Contig50 ChrX	548426	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1

5.4.6 Materials and methods

Culture conditions

All *P. pacificus* strains were kept on 6 cm Nematode Growth Medium (NGM) agar plates. Plates were spotted with 500 µl of the *Escheria coli* OP50 strain grown in L-Broth over night. For all crosses (e.g. backcrossing and generation of mapping lines) only 20 µl of OP50 was used and dried over night. All experiments were conducted at 20 °C.

Phenotypic analyses

The mouth-form type was defined by three reference characters. Briefly, were considered the width of the mouth opening, the form of the dorsal tooth, and the presence of a subventral tooth. In a stenostomatous animal the mouth opening is narrow, the dorsal tooth has a narrow shape and no subventral tooth can be found. The eurystomatous animal is defined by a broad mouth opening, a roundish dorsal tooth with a claw-like structure and the presence of a conspicuous subventral tooth (see Figure 4.1). Only adult animals were used for phenotyping. If intermediates were found (<0.1 %), they were not included in the count. In high throughput screens the SteREO DicoveryV.12 stereomicroscope from Zeiss was utilised for phenotypic analyses. The magnification for screening was always 150. A subset of animals of each mutant line was additionally screened by differential interference contrast (DIC) with a Zeiss Axioskop.

Mutagenesis

RSB020 is 2 % eurystomatous under laboratory conditions. Before mutagenesis was performed, RSB020 was phenotypically analysed on multiple plates for several generations. No phenotypic variations higher than 15 % were observed. For mutagenesis, two mixed stage plates of 2 % eurystomatous were taken. Ethyl methanesulfonate (EMS) from Sigma was used as described by Pires da Silva (Pires da Silva, 2006). The plates were screened in the F2 generation and animals showing a eurystomatous mouth-form were transferred to new plates. In the F3 a subset of 30 animals were screened for a high eurystomatous frequency. Only mutant lines of at least 50 % eurystomatous were kept. Multiple plates for screening were prepared in the F4. Only mutant lines, which consistently showed a stable eurystomatous constitutive (Euc) phenotype, were maintained.

For the preparation of mapping lines a morphological dumpy (Dpy) mutant of another highly stenostomatous strain had to be isolated. The clade C strain RSB025 shows a phenotype of 10 % eurystomatous. One mixed stage plate was used for EMS mutagenesis and

subsequently four dumpy lines were isolated. All mutants were checked for their mouth-form phenotype. The four times backcrossed dumpy mutant *tu470* was used for creating mapping lines.

Backcrossing of Euc mutants

Euc mutant J4 hermaphrodites were crossed with RSB020 males. In crossing plates containing many males indicating successful mating, 40 J4 and J3 hermaphrodites were picked in F1. The following day the mouth-form was checked and only stenostomatous animals were kept. The F2 was screened for its mouth-form frequency with plates showing mutant mouth-form frequencies excluded. In the F2, 40 animals in J3 and J4 stage from the plates showing high stenostomatous frequencies were picked onto single plates. The mouth-form was checked the next day and only eurystomatous animals were kept. In the F3, plates were screened for mutant mouth-form phenotype. Plates showing the mutant mouth-form phenotype were kept and worms of this line were used for further backcrossing. Each mutant line was at least three times backcrossed.

Whole-genome sequencing

To complement data for mapping each backcrossed mutant line was whole-genome sequenced. In preparation for sequencing five full grown plates containing only residual bacteria were washed in a 50 ml falcon tube with 0.9 % NaCl-solution. The worms were centrifuged at 1.300 rcf for 4 min at 4 °C. The supernatant was removed and the falcon tube was refilled with 0.9 % NaCl containing Ampicillin (50 µg/ml) and Chloramphenicol (25 µg/ml) and washed for 12 h at RT. The worm pellet was harvested and frozen at -20 °C.

DNA was extracted using the Epicentre MasterPure DNA purification kit. Libraries were prepared using the Illumina TruSeq Nano Library Prep Kit. Libraries were prepared following manufacturer's instruction protocol. 100 ng of DNA was used for each sample. Libraries were quantified by Qbit dsDNA BR assay kit measurements and the fragment size was verified by Bioanalyzer measurements. All libraries were diluted to 10 nM in 0.1 % EB-Tween and pooled as 10 plex. The libraries were single-read sequenced on an Illumina Genome Analyzer.

Mapping lines

In preparation of RAD sequencing for mapping, mapping lines were created. The backcrossed mutant line was crossed to RSB025 males. Two Euc J4 hermaphrodites and three to five young males of RSB025 were picked to crossing plates. Those males were killed after

two days. Males of the following generation were crossed to *tu470* Dpy J4 hermaphrodites. Non-Dpy hermaphrodites were singled out. From those plates animals were picked once again on individual plates and screened in the next generation. For mapping 32 lines showing the mutant Euc phenotype (positive mapping lines), but also 32 lines showing the highly stenostomatous phenotype (negative mapping lines) were kept for RAD sequencing.

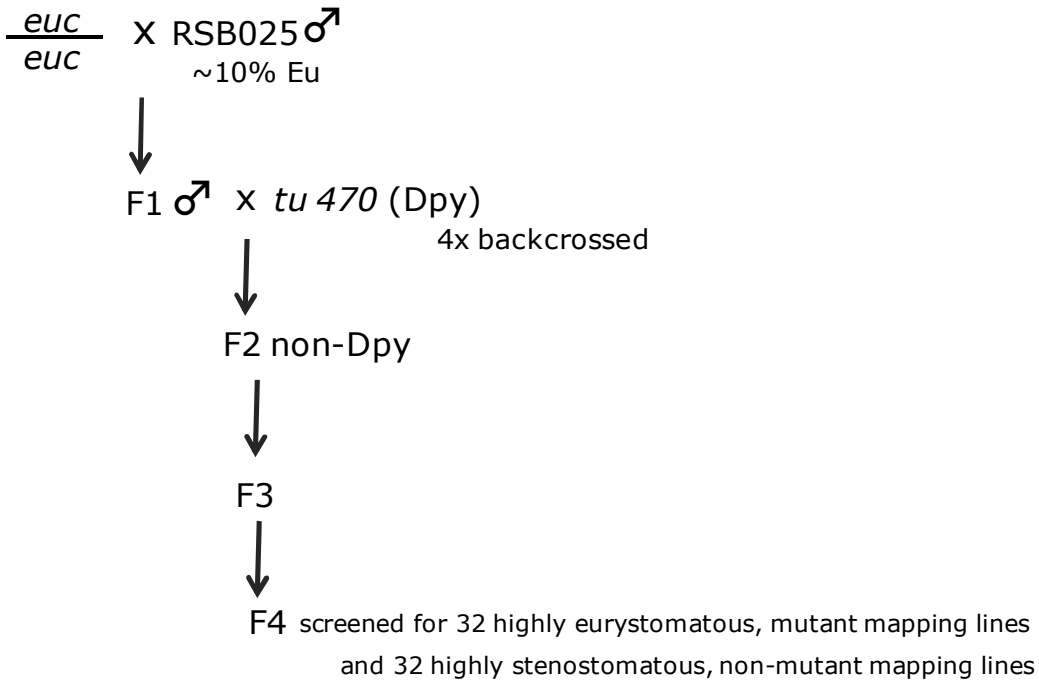

Figure 5.4.3: Crossing scheme for the creation of mapping lines used for RAD sequencing.
For each mutant line 32 positive and 32 negative mapping lines were kept.

RAD library preparation

One well-grown plate of each mapping line with limited amount of residual bacteria was washed with 0.9 % NaCl-solution in a 15 ml falcon tube. The worms were centrifuged at 1.300 rcf for 4 min at 4 °C. The supernatant was removed and the falcon tube was refilled with 0.9 % NaCl containing Ampicillin (50 µg/ml) and Chloramphenicol (25 µg/ml) and washed for 12 h at RT. The worm pellet was transferred to a 1.5 ml Eppendorf tube and stored at -20 °C. DNA of the worm pellet was extracted with the Epicentre MasterPure DNA purification kit following the manufacturer's instructions. DNA pellet was resolved with 35 µl Elution buffer instead of TE buffer. DNA was quantified by Qbit dsDNA BR assay kit. For a 96 sample library preparation the DNA of each sample was normalized to 20 ng/µl in a 96 well plate. Restriction site-associated DNA (RAD) markers were generated by using MseI and PstI restriction enzymes. 96 plex libraries were prepared by the method described by Poland (Poland et al., 2012). The libraries were quantified by Qbit dsDNA BR assay kit

measurements and the fragment size was verified by Bioanalyzer measurements. All libraries were diluted to 10 nM in 0.1 % EB-Tween. The libraries were single-read sequenced on an Illumina Genome Analyzer.

Transgenesis

Extrachromosomal arrays were created as described by Schlager (Schlager et al., 2009). Young adult hermaphrodites of the mutant line *tu469* were injected with the injection mixture. This injection mixture contained the genomic 6 kb region of the predicted gene Contig50-snap.59 including a 2.2 kb promoter region and a 700 bp 3' region of this gene (10 ng/µl), along with the transgenic marker *Ppa-egl-20*::TurboRFP (10 ng/µl) and genomic carrier DNA (60 ng/µl) from the recipient mutant. DNA extraction was done by Epicentre MasterPure DNA Extraction Kit. The transgenic marker was digested with PstI and the construct was digested with XmaI. The genomic DNA was double digested with PstI and XmaI.

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7 Appendix

Cell

A Developmental Switch Coupled to the Evolution of Plasticity Acts through a Sulfatase

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http://dx.doi.org/10.1016/j.cell.2013.09.054

SUMMARY

Developmental plasticity has been suggested to facilitate phenotypic diversity, but the molecular mechanisms underlying this relationship are little understood. We analyzed a feeding dimorphism in *Pristionchus* nematodes whereby one of two alternative adult mouth forms is executed after an irreversible developmental decision. By integrating developmental genetics with functional tests in phenotypically divergent populations and species, we identified a regulator of plasticity, *eud-1*, that acts in a developmental switch. *eud-1* mutations eliminate one mouth form, whereas overexpression of *eud-1* fixes it. EUD-1 is a sulfatase that acts dosage dependently, is necessary and sufficient to control the sexual dimorphism of feeding forms, and has a conserved function in *Pristionchus* evolution. It is epistatic to known signaling cascades and results from lineage-specific gene duplications. EUD-1 thus executes a developmental switch for morphological plasticity in the adult stage, showing that regulatory pathways can evolve by terminal addition of new genes.

INTRODUCTION

The evolution of morphological novelty is a major contributor to phenotypic diversity, particularly in adult stages. It has been suggested that developmental plasticity acts as a facilitator of phenotypic evolution (West-Eberhard, 2003, 2005), but the genetic and molecular mechanisms that regulate plasticity and specify novel traits have been largely elusive. Fundamental insight could be gained by an approach that integrates developmental biology with population genetics and ecology (Moczek et al., 2011). In particular, empirical studies of genetically identified developmental regulators in divergent populations and species are needed to test the significance of such regulators for phenotypic diversification.

Nematodes have a number of technical features, including genetic, genomic, and transgenic tools, which make such mechanistic studies practical. One model species is *Pristionchus pacificus*, which can be cultured on bacteria in the laboratory but lives in a necromenic association with scarab beetles in the wild (Herrmann et al., 2007). On the living beetle, *P. pacificus* remains in the arrested dauer stage and only resumes development with the proliferation of organisms on the dead host (Bento et al., 2010). This necromenic association has allowed the isolation of hundreds of *P. pacificus* strains and 30 *Pristionchus* species, all of which are available for population genetics and genomic studies (Herrmann et al., 2010; Morgan et al., 2012). Of particular importance is *P. exspectatus*, the presumptive sister species of *P. pacificus*, because the two species can produce sterile but viable F1 hybrids, enabling genetic studies above the species level (Kanzaki et al., 2012a). Thus, *P. pacificus* provides functional and genetic tools that can be coupled with micro- and macroevolutionary studies in a resolved phylogenetic context (Sommer and McGaughran, 2013; Figure 1).

Facilitating the necromenic lifestyle of *Pristionchus* are feeding structures that constitute an evolutionary novelty. Specifically, the mouth of *P. pacificus* and other Diplogastridae is equipped with moveable teeth that allow omnivorous feeding on bacteria, fungi, and other nematodes on beetle carcasses (Figure 1). These structures represent an ecologically important innovation because they are absent from *Caenorhabditis elegans* and other rhabditid nematodes, which are strictly microbivorous (Sudhaus and Fürst von Lieven, 2003). Moreover, the teeth exhibit developmental plasticity, whereby one of two alternative adult feeding forms is executed after an irreversible decision during larval development. The dimorphism comprises a ''narrow-mouthed'' or stenostomatous (St) form, which has a single, flint-shaped dorsal tooth, and a ''wide-mouthed'' or eurystomatous (Eu) form, which has a claw-like dorsal tooth, an opposing right subventral tooth, and more complexity in its left subventral denticles (Figure 1). The Eu form is highly derived with respect to rhabditid nematodes that lack the mouth dimorphism and is the form most associated with predation (Kiontke and Fitch, 2010; Figure 1).

Under laboratory conditions, *P. pacificus* has a ratio of Eu-to-St animals that is influenced by environmental cues such as starvation and crowding (Bento et al., 2010; Serobyan et al., 2013). An endocrine signaling module involving Δ7-dafachronic acid (DA) and the nuclear hormone receptor DAF-12 was shown to regulate the mouth dimorphism in addition to pheromone signals (Bento et al., 2010; Bose et al., 2012; Figure 1). Although

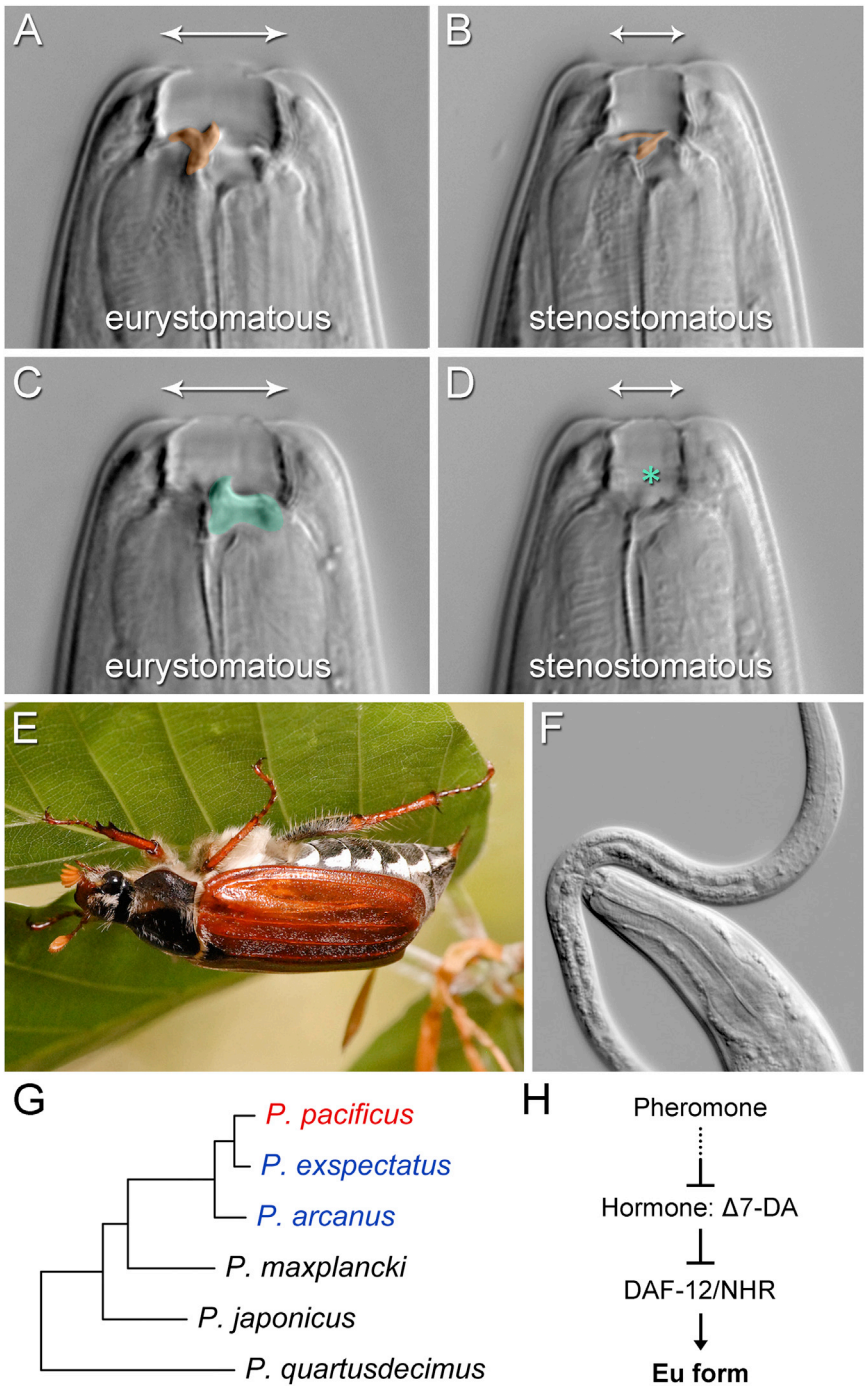

Figure 1. Developmental Plasticity in *Pristionchus* Nematodes and Its Ecological, Phylogenetic, and Mechanistic Context

(A–D) Mouth dimorphism of *P. pacificus* is presented. (A) and (C) are a single Eu hermaphrodite in two focal planes, whereas (B) and (D) are a single St hermaphrodite in the corresponding planes. (A) and (B) are sagittal, (C) and (D) right sublateral planes. The Eu form bears a claw-like dorsal tooth (A, false-colored orange) and an opposing, claw-like right subventral tooth (C, green), whereas the St form has a thin, flint-shaped dorsal tooth (B, orange) and no subventral tooth (D, asterisk). The dimorphism also includes mouth breadth (indicated by arrows) and the number of left subventral denticles (not shown).

(E) The mouth dimorphism imparts novel feeding abilities that foster a necromenic association with scarab beetles, including the European cockchafers (*Melolontha* spp.), cadavers of which are host to diverse microorganisms used as food.

(F) The complex structures of the Eu form are associated with the ability to prey on other nematodes, as pictured by a Eu *P. pacificus* hermaphrodite attacking a *Caenorhabditis elegans* larva.

(G) Phylogeny of the *pacificus* subgroup of *Pristionchus* species is presented. The ability of *P. pacificus* to form viable hybrids with *P. exspectatus* and *P. arcanus* allows functional genetic tests of macroevolutionary hypotheses. Tree is summarized from Kanzaki et al. (2013b).

(H) Regulatory model for the mouth-form decision pathway is shown.

Photographs in (E) and (F) are provided by Dan Bumbarger.

hormonal signaling and environmental cues can shift the frequencies of the two forms in a population, they are unable to act as developmental switches. Specifically, *Ppa-daf-12* mutants as well as animals treated with Δ7-DA or pheromonal small molecules have a shifted mouth-form ratio but still produce both forms. It therefore remained unknown whether any definitive switch operates in the developmental decision or whether a single form can be genetically fixed. Similarly, nothing was known about the evolution or regulation of the mouth dimorphism in other *P. pacificus* strains or among *Pristionchus* species. The capacity for genetic accommodation of a plastic trait is fundamental to the hypothesis that developmental plasticity, particularly the ability to produce an alternative morphotype, gives flexibility for responding to selection and thereby leads to the diversification of form (West-Eberhard, 2003; Moczek, 2007).

Here, we show that a developmental switch for the *P. pacificus* mouth dimorphism acts through a sulfatase encoded by the *eud-1* gene. *eud-1* mutants are completely St, whereas overexpression of *eud-1* results in completely Eu populations. Differences of the mouth-form ratio in wild isolates of *P. pacifius* correlate with *eud-1* expression, and *eud-1* overexpression transforms highly St strains to have an all-Eu phenotype. Similarly, F1 hybrids between *P. exspectatus* and *eud-1*-transgenic *P. pacificus* are completely Eu, indicating that EUD-1 is sufficient to control the mouth-form decision in different wild populations and species. We show that the *eud-1* coding region is under purifying

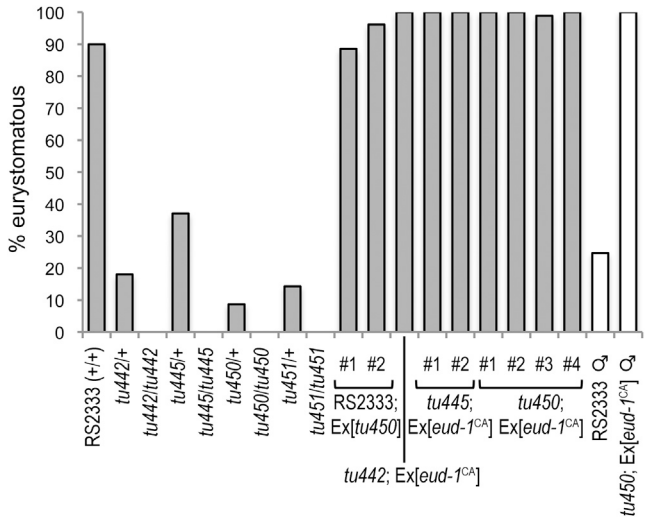

Figure 2. Phenotypes and Rescue Experiments of *P. pacificus eud-1* Mutants

Compared to the Eu-biased phenotype of wild-type hermaphrodites (RS2333), heterozygotic mutants show incomplete dominance, whereas homozygotes are completely Eud. Transformation of wild-type hermaphrodites with a mutant allele (*tu450*) did not induce a Eud phenotype. Instead, the Eu phenotype was hyperrescued in mutant hermaphrodites by an array carrying multiple copies of *eud-1*^{CA}. *tu442* was rescued by the same array as *tu450*; Ex[*eud-1*^{CA}] line #1. Males (open bars), which are highly St in wild-type animals, were also transformed to an all-Eu phenotype by the transgenic array of *tu450*; Ex[*eud-1*^{CA}] line #1, demonstrating that EUD-1 is sufficient for the sexual dimorphism of the trait. Data are represented as the total Eu frequency. See also Figures S2 and S3 and also Tables S2 and S3.

selection, and reciprocal hemizygosity experiments suggest the existence of autosomal dosage-dependent suppressors. Finally, we show that EUD-1 acts downstream of all previously known components of mouth-form development and evolved through lineage-specific gene duplications.

RESULTS

Forward Genetics Resulted in Monomorphic Mutants

To test whether regulators of developmental plasticity can act as a switch in the Eu versus St mouth-form decision, we screened for mutants that would result in the complete loss of the complex, Eu form in *P. pacificus*. We mutagenized the California strain (RS2333), which shows a high frequency (~90%) of Eu hermaphrodites (Serobyan et al., 2013), to recover such mutants. From a mutagenesis screen of 3,850 haploid gametes, we obtained 17 mutants in which hermaphrodites were strongly *eu*rystomatous form *d*efective (*eud*). Although 13 of the 17 mutants were recessive, 4 were dominant. Specifically, homozygotes of these four mutants had a completely penetrant phenotype (0% Eu), whereas heterozygotes had an incompletely penetrant phenotype (8%–37% Eu; Figure 2). Using simple-sequence length and conformation polymorphism markers, we mapped seven of the recessive mutants to six different regions on autosomes (data not shown). In contrast, the four mutants with a dominant phenotype mapped to the same genomic interval on the X chromosome (Figure 3; see Figure S1 available online). These dominant mutants showed no other phenotype or larval lethality, suggesting the existence of a single dominant locus that acts as a specific regulator of an adult phenotypic plasticity. We named this locus *eud-1*.

eud-1 Encodes a Sulfatase

The isolation of four dominant alleles of *eud-1* from a mutagenesis screen that is far from saturation suggests the importance of this gene. We obtained whole-genomic sequences for all four alleles by next-generation sequencing and searched for SNPs. A single 1 kb interval throughout the entire genome contained SNPs in all four mutants and was within the original mapping interval on the X chromosome (Figure 3A). Putative mutation sites of *eud-1* were within the boundaries of a single predicted gene, Contig8-snap.30 (http://www.pristionchus.org), and they affected predicted exons or splice acceptors (Figures 3B and S1; Table S1). Expression of Contig8-snap.30 was observed by RNA sequencing, and all four *eud-1* mutations were confirmed by Sanger sequencing.

eud-1 encodes a sulfatase putatively homologous with *C. elegans* sulfatase 2 (*sul-2*), the ortholog of human arylsulfatase A (ARSA), whose crystal structure has been solved (Lukatela et al., 1998) and active site confirmed (Waldow et al., 1999). In humans, mutations in this sulfatase lead to the lysosomal storage disorder metachromatic leukodystrophy (Schmidt et al., 1995), although no mutant phenotypes are known for *Cel-sul-2*. Supporting the function of *Ppa*-EUD-1 as a sulfatase was the location of all three coding mutations within a highly conserved active site sequence (Figure 3B). In particular, *eud-1(tu451)* causes an amino acid change, R99Q, identical to a mutation that virtually eliminates catalytic activity in a homologous sulfatase, ARSB, in Maroteaux-Lamy syndrome type VI (Litjens et al., 1996; Figure 3B). Furthermore, a presumptive null mutation, *eud-1(tu445)*, characterized the only mutant with no *eud-1* expression detected by RNA sequencing (Figures S2 and S3).

eud-1 Executes a Developmental Switch for the Mouth Dimorphism

We next tested whether the dominant *eud-1* phenotype results from gain-of-function or reduction-of-function mutations, the latter of which would indicate that *eud-1* acts as a developmental switch gene. To distinguish between these two scenarios, we performed two sets of transgenic microinjection experiments. First, we transformed the wild-type California strain with a *eud-1* mutant allele, and second, we injected a wild-type *eud-1* copy into *eud-1* mutants. If *eud-1* alleles were gain of function, transgenic animals carrying a mutant copy of *eud-1* should have an all-St phenotype. In contrast, if *eud-1* were haploinsufficient, *eud-1* alleles would be rescued with a wild-type copy of the gene.

Two independently transformed lines carrying a *eud-1(tu450)* mutant construct with an *egl-20*::*rfp* (red fluorescent protein) reporter maintained a wild-type frequency of Eu animals (Figure 2). In contrast, multiple lines of *eud-1(tu445)* and *eud-1(tu450)* mutant animals independently transformed with wild-type copies of *eud-1* were rescued to produce Eu animals (Figure 2). Interestingly, these lines were not only rescued but exceeded the wild-type frequency by forming >99% Eu hermaphrodites

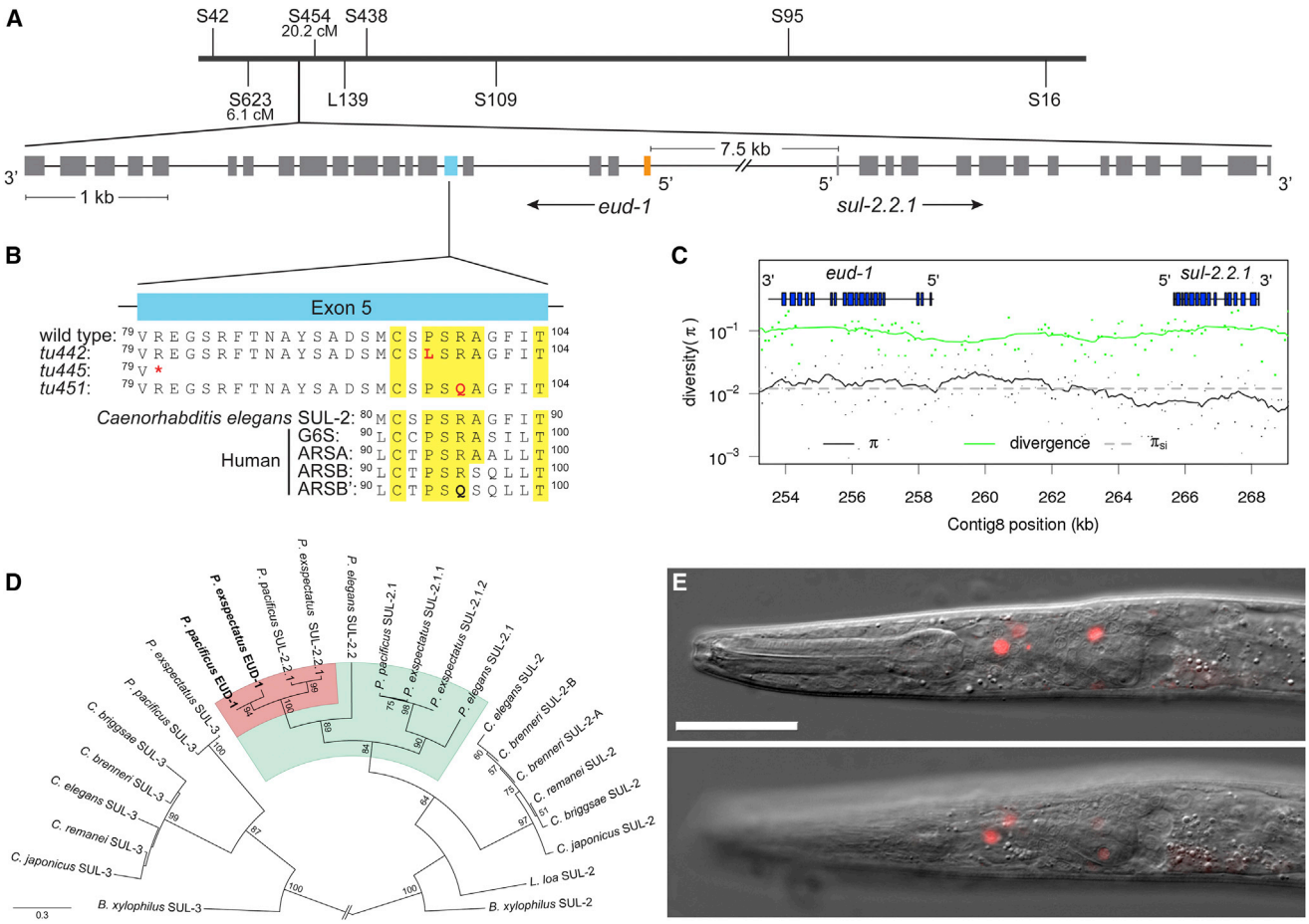

Figure 3. Mapping, Gene Structure, Mutations, Variation, and Expression of *P. pacificus eud-1*

(A) Genetic map shows a region containing the *eud-1* locus on the northern arm of chromosome X. The *eud-1* locus is next to a highly similar paralog ("*sul-2.2.1*"). Arrows indicate direction of transcription; orange box indicates first exon, encoding a predicted signaling peptide. See also Figure S1.

(B) Exon 5 of *eud-1* includes the predicted active site of the EUD-1 sulfatase. The active site is highly conserved with arylsulfatases in other animals (highlighted yellow), and two mutant lesions (red) are predicted to disrupt catalytic activity of EUD-1. The mutation in *tu451* results in an amino acid change, R99Q, identical with one causing the nearly complete loss of activity in human ARSB (ARSB′; Litjens et al., 1996). See also Table S1.

(C) Intraspecies diversity (π) and divergence with *P. exspectatus* at the *eud-1* locus are presented. Lines represent averages over 1 kb intervals as calculated from measurements in 100 bp windows (dots). The promoter of *eud-1* shows a slightly elevated π relative to the silent site diversity in the *eud-1* gene (π_{si}).

(D) Phylogenetic history of nematode arylsulfatases, including all identifiable paralogs in *Bursaphelenchus*, *Caenorhabditis*, and *Loa*, is presented. *eud-1* is the result of a recent duplication of *sul-2.2* (salmon). All three analyzed *Pristionchus* species share an additional, more ancient duplication of *sul-2* (green). The tree was inferred from amino acid sequences, invoking a WAG model, under the criterion of maximum likelihood. Bootstrap support values above 50% are shown. Branch lengths are measured in the number of substitutions per site. See also Tables S5 and S6.

(E) Spatial expression of *eud-1*, reported by a 7 kb *eud-1* promoter element driving RFP expression, is shown. Central nervous ganglia and pharynx of a single individual are shown in two longitudinal focal planes: left of pharyngeal isthmus and terminal bulb (above), and right of isthmus and through terminal bulb (below). All postembryonic stages (J3 hermaphrodite is pictured) consistently showed RFP expression in somatic and pharyngeal neurons, suggesting a role for EUD-1 in sensory transduction or neuroendocrine signaling in development. Scale bar, 20 μm.

(Figure 2). A third mutant allele, *eud-1(tu442)*, was similarly over-rescued by crossing with a line of *eud-1(tu450)*; Ex[*eud-1*^{CA}] (Figure 2). These experiments show that *eud-1* acts as a developmental switch for the *P. pacificus* mouth-form decision: zero wild-type copies of the gene in homozygous mutants resulted in all-St hermaphrodites; one wild-type copy in heterozygous mutants resulted in a low Eu frequency; two wild-type copies in the California strain resulted in a high Eu frequency; and presumed higher copy numbers in transgenic lines resulted in all-Eu hermaphrodites.

EUD-1 Regulates Sexual Dimorphism of the Mouth-Form Phenotype

The mouth-form phenotype of *P. pacificus* is sexually dimorphic, with males (XO animals) predominantly expressing the St form (~20% Eu) (Serobyan et al., 2013; Figure 2). Given that *eud-1* is on the X chromosome and that *eud-1* mutations are haploinsufficient, we investigated whether levels of *eud-1* expression control the distinct mouth-form phenotype of males. Measurements of *eud-1* expression by RNA sequencing indicated strong downregulation in males relative to mixed-stage hermaphrodites

($p < 0.05$; Figure S2), suggesting sex-specific expression of *eud-1*. We then determined whether *eud-1* overexpression by transgenes promoted the Eu mouth form in males. Indeed, transgenic males carrying extra copies of *eud-1*^{CA} were all Eu (Figure 2), demonstrating that the dosage of *eud-1* was sufficient to regulate the sexual dimorphism of the mouth-form phenotype in *P. pacificus*. Therefore, *eud-1* is necessary and sufficient for the formation of the Eu mouth form and is a dosage-dependent regulator.

The EUD-1 Switch Operates in Wild Populations of *P. pacificus*

To determine if a developmental switch gene identified by forward genetics could be linked to patterns of microevolutionary divergence, we searched for correlations between *eud-1* expression and natural variation in the mouth-form phenotype. First, we conducted a survey of 72 wild isolates of *P. pacificus* that have a known population structure as inferred from microsatellite markers and next-generation sequencing (Morgan et al., 2012; C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data). The majority of wild isolates showed a bias toward a Eu phenotype, similar to *P. pacificus* RS2333 (Figure 4A). In contrast, four of these strains had a highly St phenotype (Figure 4A). To test whether expression of *eud-1* is sufficient for the formation of the Eu mouth form in wild isolates with high St frequencies, we induced overexpression of *eud-1* in two of these strains, RS5200B (India) and RS5410 (La Réunion), using a transformation construct of *eud-1*^{CA}. RS5200B and RS5410 animals carrying copies of *eud-1*^{CA} were converted to a higher or completely Eu phenotype ($p < 10^4$ in all comparisons). Specifically, RS5200B; Ex[*eud-1*^{CA}] and RS5410; Ex[*eud-1*^{CA}] had 39%–100% and 51%–99% Eu animals, respectively (Figure 4B). In contrast, transgenic lines carrying the *eud-1(tu445)* null allele maintained a high St frequency ($\leq$6% Eu; Figure 4B), and animals that were RFP negative and thus had lost the *eud-1*^{CA} array were also highly St ($\leq$7% Eu in all but one line; Figure 4B). Similarly, transforming *P. pacificus* strains RS5200B and RS5410 with constructs of their own alleles, *eud-1*^{5200B} and *eud-1*^{5410}, respectively, resulted in higher (58%) to almost completely (98%) Eu phenotypes ($p < 10^4$; Figure 4B). Finally, using RNA sequencing, we measured the expression of *eud-1* of the two strains and found that expression of the gene was higher than in mutants, although less than half of that observed in RS2333 (Figure S2). Together, these experiments show that a switch including *eud-1* regulates the dimorphism in naturally variant populations. Moreover, the phenotype of highly St strains is not due to loss of function of *eud-1* but most likely due to the control of its expression.

Hybrid Crosses Show a Conserved Role for *eud-1* across Species

Next, we wanted to know whether the regulatory network including *eud-1* could also be linked to macroevolutionary patterns of the mouth dimorphism in *Pristionchus*. To determine the role of *eud-1* in other species, we recruited the gonochoristic sister species of *P. pacificus*, *P. exspectatus*. Specifically, we designed hybrid-cross experiments to test whether the *eud-1* switch operates above the species level. First, we identified a strain (RS5522B) of *P. exspectatus* to be completely St (<1% Eu) (Figures 4C and 4E). This strain resulted from ten generations of random inbreeding from a parental strain originally having a high Eu frequency in females (71% Eu; data not shown). Additionally, we found that *eud-1* expression in this highly St strain was lower (Figure S2). To test whether a greater number of *eud-1* copies could induce the Eu form, we crossed this strain to males of wild-type (RS2333) and *eud-1(tu450)*; Ex[*eud-1*^{CA}] lines of *P. pacificus*. Nontransgenic hybrid females had intermediate mouth-form frequencies, presumably due to genetic complementation (Figure 4C). In contrast, female hybrids carrying the *eud-1*^{CA}; *egl-20*::*rfp* array were completely (100%) Eu (Figure 4C), and transgenic hybrid males were almost completely (90%) Eu (Figure 4E). Similarly, transgenic hybrid F1 females with *P. pacificus* mothers, marked by *Ppa-unc-1*, and *P. exspectatus* fathers were 100% Eu (Figure 4C).

We next tested whether multiple copies of *Pex-eud-1* could convert the phenotype of F1 hybrids between *P. pacificus* and *P. exspectatus*. To do this, we transformed the *P. pacificus* mutant *eud-1(tu450)* with the allele from *P. exspectatus*, which resulted in complete overrescue (100% Eu; Figure 4D), and then crossed the transgenic line to *P. exspectatus*. Transgenic hybrid F1 females were completely (100%) Eu (Figure 4D). The results of hybrid crosses therefore indicate that the *eud-1* switch is evolutionarily conserved across species. However, given that overexpression studies can affect multiple developmental pathways in an uncontrollable manner, we complemented our transgenic experiments with studies of genetic variation and gene function in nontransgenic experiments.

The *eud-1* Locus Shows Strong Signs of Purifying Selection

The genetic and transgenic experiments described above indicate a role for EUD-1 in shifting the balance between the two mouth forms in different *P. pacificus* populations and in *P. exspectatus*. However, given that these experiments were based on transgenic animals with a largely uncontrolled number of gene copies in transgenic arrays, the results do not distinguish whether the activity of the EUD-1 protein differs between strains and species or whether the observed phenotypic differences were due to elements regulating *eud-1*. To discriminate between these possibilities, we analyzed the genetic variation at the *eud-1* locus among 104 resequenced *P. pacificus* strains and *P. exspectatus* (Figure 3C). We found 44 synonymous and 23 nonsynonymous substitutions among the 104 *P. pacificus* strains, although no substitutions were predicted to be deleterious. Normalizing by the number of synonymous and nonsynonymous sites within *eud-1*, these values translate into a dN/dS ratio of 0.15, indicating that 85% of nonsynonymous substitutions in *eud-1* were purged from populations quickly. Consistent with this finding was a low dN/dS ratio (0.11) between *P. pacificus* and *P. exspectatus* (Figure 3C). Thus, the coding region of *eud-1* shows signatures of purifying selection.

Similarly, we searched for signs of selection in the 7 kb upstream region of *eud-1* that is sufficient to drive *eud-1* expression. Tests for selection of noncoding sequences in 1 kb intervals failed to reject neutrality, although local peaks of up to 4%

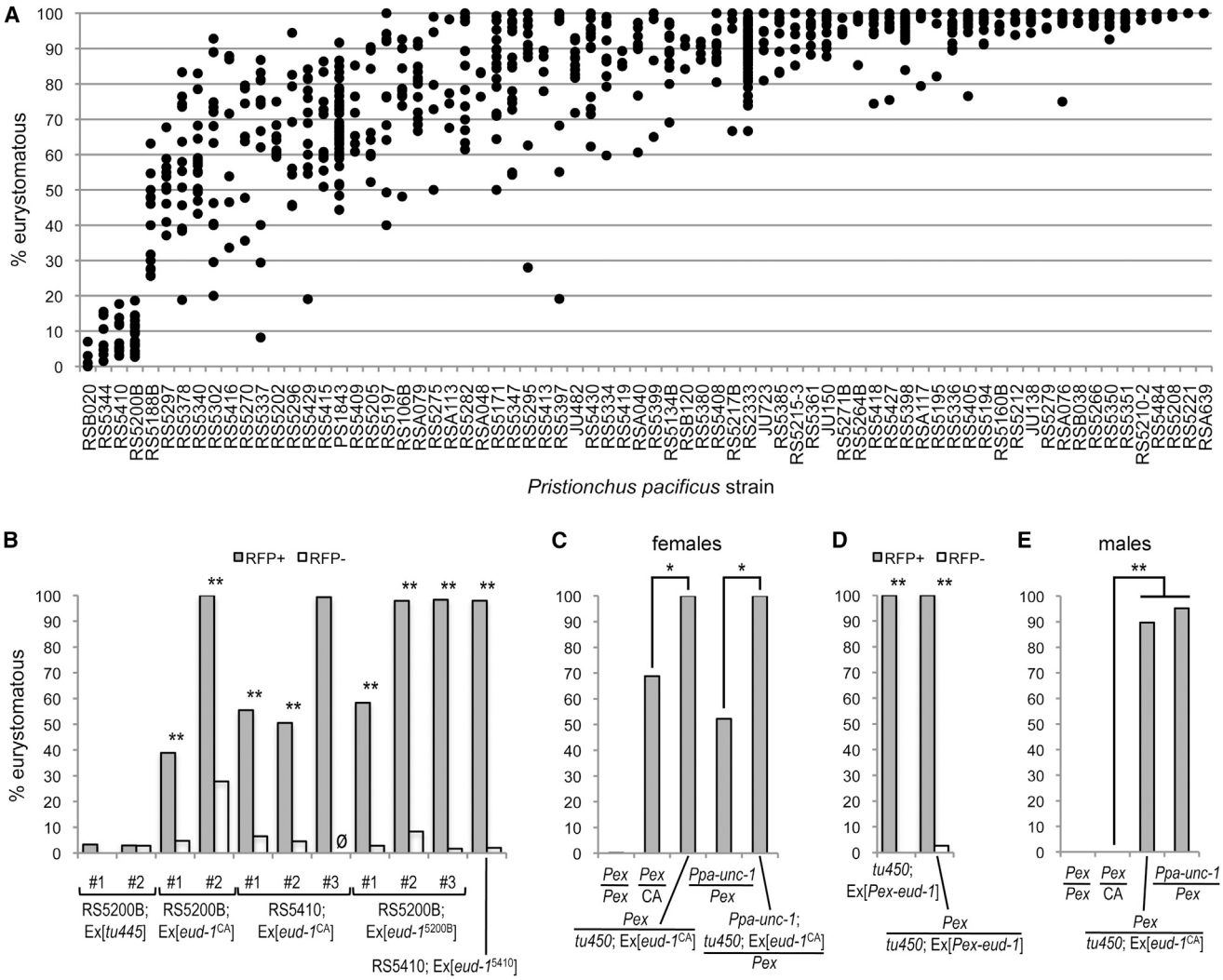

Figure 4. *eud* Phenotypes and EUD-1 Function in Microevolution and Macroevolution

Phenotypes are of hermaphrodites unless otherwise indicated.

(A) Natural variation of the mouth-form ratio in wild isolates of *P. pacificus* is shown. Out of 72 populations examined, four strains (left) have a *eud* phenotype. Each plotted point represents the Eu frequency for a single culture population. Strains are ordered from left to right by their mean Eu frequencies. Note that strains biased toward highly (>97%) Eu phenotypes were identical across multiple culture populations.

(B) Phenotypic conversion of highly St wild isolates of *P. pacificus* by overexpression of *eud-1* is presented. In addition to annotated differences, all lines transformed (RFP+) with a California (CA) or endogenous wild-type allele have a significantly higher Eu frequency than lines transformed with a presumptive null allele ($p < 10^{-4}$, Fisher's exact test). See also Table S3.

(C) Mouth-dimorphism phenotype of females of *P. exspectatus* ("*Pex*"; left column), F1 hybrids resulting from crosses of *P. exspectatus* females to *P. pacificus* males (second and third columns), and F1 hybrids from crosses of *P. pacificus* hermaphrodites to *P. exspectatus* males (two right columns) are shown. See also Table S4.

(D) Phenotypic conversion of *P. pacificus* hermaphrodites and interspecific hybrids by overexpression of a *P. exspectatus* allele (*Pex-eud-1*) is presented. See also Table S3.

(E) Phenotypic conversion of males of *P. exspectatus* ("*Pex*"; left column) and of F1 hybrids resulting from reciprocal crosses between *P. exspectatus* and *P. pacificus* is shown. See also Table S4.

All data are represented as the total Eu frequency. *$p < 0.05$ and **$p < 10^{-4}$, Fisher's exact test. See also Figures S2 and S3.

nucleotide diversity exist (Figure 3C). However, we were unable to identify bona fide transcription factor binding sites using the program ExPlain 3.1 (BIOBASE). Taken together, analyses of genetic variation at the *eud-1* locus provide strong evidence for purifying selection in the coding region of *eud-1* but do not indicate a role for specific *cis*-regulatory elements.

Reciprocal Hemizygosity Results in Contrasting Mouth-Form Frequencies in Males

Given the evidence against positive selection and functional differences of *eud-1*, we next tested for interspecific divergence of *eud-1* alleles by performing reciprocal hemizygosity experiments with *P. pacificus* and *P. exspectatus*. When we analyzed hybrid

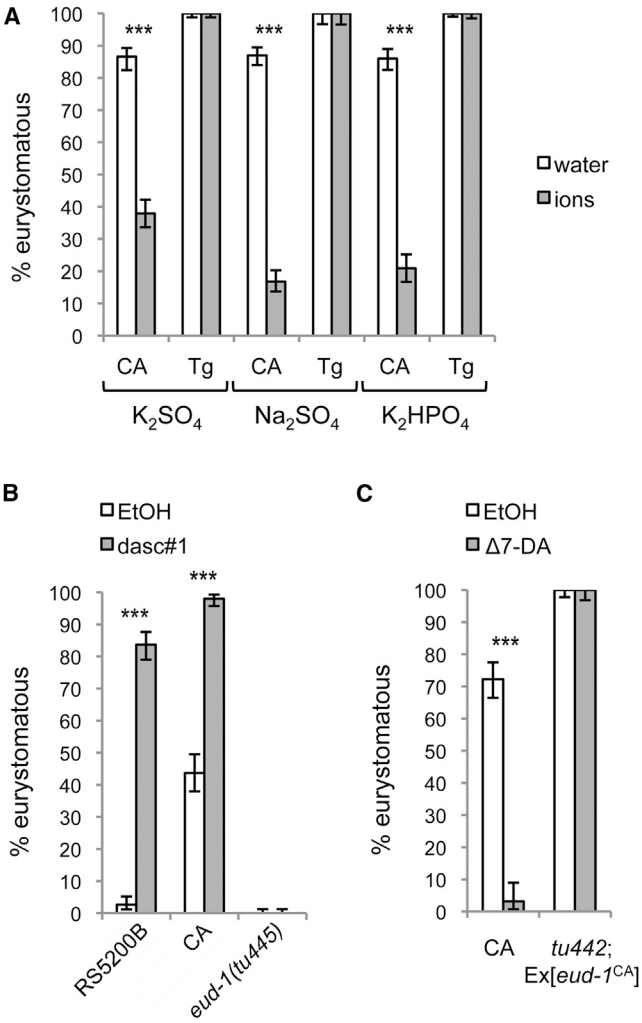

Figure 5. Activity and Epistasis of *P. pacificus* EUD-1

(A) Competitive inhibition of sulfatases by sulfate and phosphate ions is shown. Application of inhibitors at 120 mM mimics a *eud* phenotype in the wild-type California strain ("CA"), but a strain overexpressing *eud-1*, *tu442*; Ex[*eud-1*^{CA}] ("Tg") does not respond to nonlethal concentrations of salts.

(B) Epistasis of EUD-1 to pheromone signaling is shown. Whereas the pheromone dasc#1 strongly induces the Eu form in wild-type strains of *P. pacificus*, *eud-1* mutants are completely unresponsive to this signal.

(C) Epistasis of EUD-1 to the receptor of Δ7-DA is presented. A line of *P. pacificus* overexpressing EUD-1 completely inhibits induction of the St form by treatment with Δ7-DA.

Data are represented as the total Eu frequency ± a 95% confidence interval, estimated using a binomial test on the total count data pooled across replicate treatments. ***$p < 10^{-6}$, Fisher's exact test.

males carrying a single copy of *eud-1* inherited with the maternal X chromosome, we found strong differences in the expressivity of the Eu mouth form (Figure 4E). As indicated above, the female offspring from a cross of a *P. exspectatus* female to a *P. pacificus* male were 69% Eu (Figure 4C), whereas male offspring of the same cross were all St (0% Eu; Figure 4E). In strong contrast to this observation, the male offspring from the reciprocal cross, namely of a *P. pacificus* hermaphrodite to a *P. exspectatus* male, were 97% Eu (Figure 4E). Hybrid males thus expressed a high Eu frequency if the X chromosome was provided by *P. pacificus*, and not *P. exspectatus*. These results allow two major conclusions. First, the X chromosome, including the *eud-1* locus, differs between the two species. Given that hybrid females or *P. pacificus* hermaphrodites that overexpress the *eud-1* allele of *P. exspectatus* are highly Eu, the differences between the species are most likely either located in regulatory elements of *eud-1* or in other X-linked *trans*-acting factors, rather than in the coding region of *eud-1*. Second, additional, autosomal *trans*-acting factors might influence the expressivity of the Eu mouth form. Together, these findings suggest that factors in the *eud-1* regulatory network are important for evolutionary changes in mouth-form regulation. Although we cannot rule out the existence of *cis*-regulatory elements in the *eud-1* gene itself, there is no direct evidence clearly supporting their involvement.

Because reciprocal hemizygosity experiments cannot distinguish between *P. pacificus*-specific, *trans*-acting, X-linked activators and autosomal, dosage-dependent suppressors, we compared the phenotype of F1 hybrid males with *P. pacificus* mothers to males from crosses within *P. pacificus*. The predominantly St (20% Eu) phenotype of *P. pacificus* males (Figure 2), in contrast to the highly Eu phenotype of the former cross, suggests the presence of autosomal suppressors. We hypothesize that two copies of an autosomal dosage-dependent suppressor repress *eud-1* expression in *P. pacificus* males, whereas one copy of the suppressor in male interspecies hybrids is insufficient to repress *eud-1* expression. Furthermore, the haploinsufficiency of *eud-1* is consistent with the hypothesis of an autosomal dosage-dependent suppressor: whereas the Eu-biased hermaphrodites of *P. pacificus* (RS2333) would mostly escape suppression, mutant heterozygotes, which show a highly St phenotype, would not.

EUD-1 Acts as a Sulfatase and Is Expressed in Neurons

The experiments described above provide strong evidence that *eud-1* is necessary and sufficient to control the mouth-form switch and that it operates in different wild populations and species of *Pristionchus*. To assay the functional activity of *Ppa*-EUD-1, we first tested whether competitive inhibitors of arylsulfatases, sulfate ions (Dodgson and Spencer, 1953; Glössl et al., 1979), could mimic a *eud* phenotype. Indeed, application of such salts resulted in a significant reduction in the Eu frequency in the wild-type California strain, indicating that sulfates act by product inhibition (Figure 5A). Similarly, application of phosphate ions, also known to inhibit sulfatases, significantly reduced the frequency of Eu animals (Figure 5A), supporting the action of EUD-1 as a sulfatase. It is interesting to note that this inhibition was not strong enough to alter the all-Eu phenotype of the transgenic line overexpressing *eud-1*, at least at concentrations that would not result in lethality (Figure 5A). Most likely, the product inhibition seen in wild-type California animals is not reached in *eud-1* transgenic animals, which show massive overexpression of the sulfatase.

To determine the cells that express EUD-1, we generated a transcriptional reporter of a 7 kb *eud-1* promoter element, the genomic region that was sufficient for the rescue of the mutant phenotype, to drive RFP expression. This reporter was

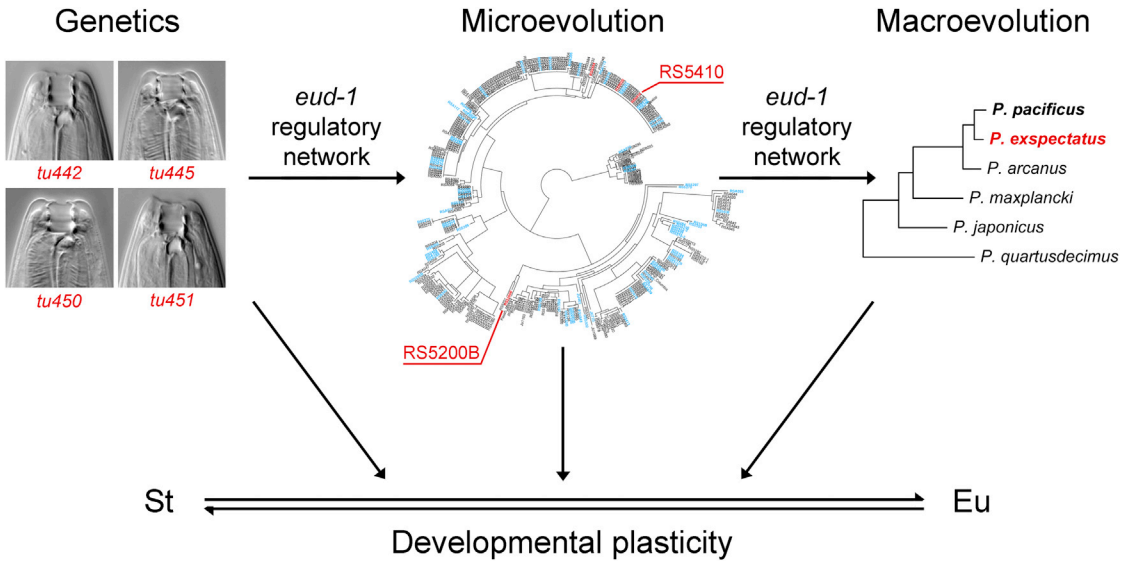

Figure 6. The Genetics of Developmental Plasticity Reflect Patterns of Microevolution and Macroevolution

Forward genetics (left) uncovered a gene, *eud-1*, as part of a switch controlling the development of an ecologically significant trait, a morphology that directly determines feeding abilities. Identifying a similar phenotype in wild populations of *P. pacificus* (center) and other species (right), all in a resolved phylogenetic context, allowed testing the relevance of this gene in natural history. Functional analysis of EUD-1 in St-biased variants (red), with respect to strains with a Eu bias or intermediate phenotype (blue), empirically demonstrates how changes in the genetic network regulating EUD-1 underlie diversification of the dimorphism. Center tree inferred by Morgan et al. (2012); tree on right, by Kanzaki et al. (2013b).

expressed in several classes of somatic and pharyngeal neurons (Figure 3E), and RFP expression was seen consistently in all postembryonic stages and all independently generated transgenic lines. The observed RFP expression suggests a role for *Ppa-eud-1* in sensory transduction or neuroendocrine signaling in development. Therefore, we next constructed tests to determine the position of EUD-1 with respect to the known cascade of mouth-form development.

Pheromone Signaling Acts through the EUD-1 Switch

Individual pheromone molecules were previously found to influence the mouth-form decision, the strongest effect resulting from the diascaroside dasc#1 (Bose et al., 2012). This molecule is only known from *P. pacificus* and, in contrast to compounds that also induce dauer formation, is specific to the mouth dimorphism. To determine whether EUD-1 acts downstream of developmental cascades initiated by pheromone, we treated *eud-1(tu445)* mutants with diascaroside #1 (dasc#1). Whereas pheromone induced significantly higher Eu frequencies ($p < 10^{-6}$) in the highly St strain RS5200B (84% Eu) and in the wild-type California strain (98%), it had no effect on the mutant (0%; Figure 5B). This finding demonstrates that the EUD-1 switch controls the response of the mouth plasticity to external, pheromonal cues.

EUD-1 Is Epistatic to Δ7-DA/DAF-12

Finally, we used epistasis experiments to determine where *eud-1* acts in the known genetic hierarchy of mouth-form regulation. Previous studies had identified an endocrine signaling module that consists of the DA derivative Δ7-DA and the nuclear hormone receptor DAF-12 to regulate the *P. pacificus* mouth dimorphism (Bento et al., 2010), but none of the tested genes or compounds acted as a complete developmental switch. In our assay, a high concentration (10 μM) of Δ7-DA resulted in a very low Eu frequency (3%) in the wild-type strain (Figure 5C). In contrast, Δ7-DA had no effect on the transgenic line *eud-1(tu450)*; Ex [*eud-1*^{CA}] (Figure 5C). These results suggest that EUD-1 operates downstream of or in parallel to the Δ7-DA/DAF-12 module. Because DAF-12 is the final common target of dauer regulatory pathways in *C. elegans* (Antebi et al., 2000), the epistasis of EUD-1 over the receptor of Δ7-DA is consistent with EUD-1 being an ultimate developmental switch for the mouth dimorphism.

DISCUSSION

This study has identified a developmental switch that controls a morphological plasticity in the nematode adult stage and that acts through the sulfatase EUD-1. Our findings result in four major conclusions. First, and most significantly, we show that a gene uncovered in an unbiased genetic screen is linked to patterns of micro- and macroevolution of an ecologically relevant trait (Figure 6). Laboratory approaches, in particular with genetic experimentation, have been powerful in many fields of biology, such as in developmental biology and neurobiology. In contrast, several aspects of evolutionary research usually escape laboratory studies, including functional analysis of genes controlling traits that mediate interaction with and directly respond to the environment. Testing the evolutionary significance of genetically identified regulatory mechanisms requires integrative approaches that link development with ecology, population genetics, and a well-resolved phylogenetic framework (Moczek et al., 2011; Sommer and McGaughran, 2013).

Here, we show that the genetically identified *eud-1* locus acts as a master regulator that is variably expressed among populations, and genetic transformation with this gene affects variation of the trait in wild isolates. The analysis of genetic variation at the *eud-1* locus shows strong signs of purifying selection in the coding region of the gene, implying that the EUD-1 protein itself does not contribute to phenotypic evolution. Rather, the genetic network regulating *eud-1*, involving potential X-linked and autosomal *trans*-acting factors, seems to be responsible for evolutionary changes in the expressivity of the mouth forms. Together, our analyses show that developmental processes inferred experimentally through mutations can reflect those that have withstood the test of natural selection. Such a link can most easily be identified for genetic networks specifying divergent postembryonic or adult traits, with the effects of BMP4 signaling on beak shape in finches (Abzhanov et al., 2004) and those of insulin signaling on horn size in scarab beetles (Emlen et al., 2012) representing prominent analogous examples.

The second conclusion is that the regulation of developmental plasticity involves a gene that controls a developmental switch. Mutations in the *eud-1* gene eliminate the Eu mouth form, whereas overexpression of this gene fixes it. Developmental switch genes are well known in signaling pathways that control multiple aspects of animal development, for example the GTPase RAS in *Drosophila* eye and *C. elegans* vulva development (Han and Sternberg, 1990; Simon et al., 1991). Most developmental switch genes have pleiotropic functions and are highly conserved throughout evolution. In contrast, the single known phenotype of *eud-1* suggests that this gene acts specifically to control the mouth dimorphism. Furthermore, *eud-1* has resulted from recent gene duplications, indicating that new genes can be recruited as master regulators of development. The phylogenetic history of *Ppa-eud-1* shows the existence of multiple paralogs of *sul-2* (Figure 3D). Duplications of the gene have occurred both since the split of *Pristionchus* from other sequenced species as well as within *Pristionchus*. This finding provides empirical evidence to complement recent discussions on the evolutionary origin and function of orphan and other novel genes (Long et al., 2003; Tautz and Domazet-Lošo, 2011). The assumption of a recently duplicated switch gene as a downstream target for mouth-form development is striking, considering that the addition of genetic elements to regulatory cascades during evolution is thought to occur primarily upstream (Wilkins, 2002). This was demonstrated in the well-characterized animal sex-determination pathway: the final target *Doublesex/mab-3* is broadly conserved (Raymond et al., 1998; Matsuda et al., 2002; Miller et al., 2003), whereas divergent components have been recruited upstream. However, in nematode mouth-form development, the epistasis of EUD-1 to pheromone signaling and the Δ7-DA/DAF-12 module shows that regulatory pathways can evolve by terminal addition of novel genes.

The third conclusion of our study is that *eud-1* encodes an arylsulfatase, the first such enzyme shown to regulate a developmental process. Two findings suggest that, in *P. pacificus*, EUD-1 acts as a sulfatase. Several mutant alleles result in amino acid substitutions that affect the active site of sulfatases, and similar mutations are known from human sulfatase-deficiency disorders (Schmidt et al., 1995; Litjens et al., 1996). Second, treatment with sulfate or phosphate ions mimics the *eud-1* mutant phenotype, indicating product inhibition, as demonstrated for other sulfatases. The target of EUD-1 cannot be deduced from its amino acid sequence and remains currently unknown. Heparan 6-*O*-endosulfatases, present in *P. pacificus* and *C. elegans* as *sul-1*, have been implicated in modifying heparan sulfate proteoglycans in growth factor signaling during embryogenesis (Dhoot et al., 2001; Kamimura et al., 2006). However, comparatively little is known about arylsulfatase function in development. An extracellular arylsulfatase was identified in sea urchin embryogenesis as a component of the extracellular matrix and was suggested to bind polysaccharides to mechanically facilitate gastrulation (Mitsunaga-Nakatsubo et al., 2009). It is thus possible that EUD-1 modifies structural molecules in *P. pacificus* mouth-form development, although the expression of EUD-1 in the nervous system does not support such a role. Instead, we speculate that a steroid hormone might be the EUD-1 target. The observed expression pattern of *Ppa-eud-1* would be consistent with the previously described role of DAF-12/NHR in mouth-form regulation and the predicted presence of sulfated sterols in nematodes (Carroll et al., 2006; Hattori et al., 2006).

Finally, this study elucidates a mechanism that generates a morphological novelty of the adult stage. Developmental plasticity has been increasingly discussed as a facilitator of phenotypic diversity and the generation of ecologically relevant traits. Several authors have argued that plasticity is essential both for the interaction between development and the environment and for driving evolution and divergence (Brakefield et al., 1998; Pigliucci, 2001; Nijhout, 2003; Schlichting, 2003; West-Eberhard, 2003). However, two major challenges result from the "facilitator" hypothesis. First, the genetic and molecular mechanisms underlying developmental plasticity need to be linked to micro- and macroevolutionary divergence. The present study provides an important example by identifying a gene that is part of a genetic network linking genetic regulatory processes to hormone signaling, a known target of developmental plasticity (Nijhout, 2003).

The second challenge concerns the long-term evolutionary success and influence of developmental plasticity. Do traits that show two or multiple morphs evolve faster than other traits, and do they contribute to phenotypic evolution in general? Although relevant studies in nematodes are still in their infancy, the *Pristionchus* mouth dimorphism supports this idea. *Pristionchus* nematodes are uniform in most morphological traits, and the majority of the 30 confirmed *Pristionchus* species were previously only diagnosed by their molecular profiles (Herrmann et al., 2006; Kanzaki et al., 2012a). In contrast, the recent description and morphological analysis of 14 new *Pristionchus* species have shown the mouth structures of these species to be highly diverse, supporting the link between plasticity and diversity (Kanzaki et al., 2012b, 2013a, 2013b, 2013c). Most interesting among these is a clade of three *Pristionchus* species that have added a novel type of plasticity to the already existing mouth dimorphism, and through phylogenetically supported intermediates, this plasticity has culminated in new mouth morphology (Ragsdale et al., 2013). Comparative analysis of *Pristionchus* mouthparts thus strongly supports the hypothesis

that developmental plasticity facilitates phenotypic diversity. The identification of EUD-1 as part of a developmental switch brings this area of evolution and ecology into the realm of genetics and molecular biology.

EXPERIMENTAL PROCEDURES

Culture Conditions

Except when used in crosses or assays, all *Pristionchus* strains were kept on 6 cm plates with nematode growth medium (NGM) agar and were fed with a lawn of *Escherichia coli* OP50 grown in 400 μl L-Broth. For crosses, plates were seeded with a lawn grown from 25 μl OP50 in L-Broth. Cultures were maintained at 20°C–25°C. Because the mouth-form ratio is sensitive to unknown environmental and putative epigenetic effects (Serobyan et al., 2013), all experiments include their own controls for the wild-type Eu frequency. Consequently, the mouth-form ratio is not comparable across all experiments, which necessarily introduce different variables. Also, to minimize the potential for laboratory evolution of the trait, a new culture of the California strain was revived annually from a frozen voucher.

Phenotype Scoring

The mouth-form phenotype was scored as previously described by Serobyan et al. (2013). In short, characters used to discriminate between Eu and St individuals, respectively, were (1) the presence versus absence of a subventral tooth, (2) a claw-like versus flint-like or triangular dorsal tooth, and (3) a wide versus narrow stoma (mouth). Characters 1 and 2 were discrete, nonoverlapping, and sufficient to distinguish the two forms. Apparent intermediates between the two forms were rare (<0.1%) and were not included in counts. In most cases, phenotypes could be scored using Zeiss Discovery V12 and V20 stereomicroscopes and were supplemented where necessary with differential interference contrast (DIC) microscopy on a Zeiss Axioskop. Transgenic lines, *P. exspectatus*, hybrids, and individuals in the pheromone and Δ7-DA assays were scored exclusively by DIC microscopy. For sample sizes of animals scored, see Tables S2, S3, and S4.

Forward Genetics

Techniques used for forward genetics in *P. pacificus* have been previously described by Pires da Silva (2006). Detailed protocols for the forward genetics screen, genetic mapping, whole-genome sequencing for mutant identification, and gene cloning are provided in the Extended Experimental Procedures.

Genetic Transformation

Extrachromosomal arrays were generated as described by Schlager et al. (2009). The germlines of adult hermaphrodites were injected with a *Ppa-eud-1* construct (15 ng/μl), the marker *Ppa-egl-20*::*TurboRFP* (10 ng/μl), and genomic carrier DNA (60 ng/μl) from the recipient strain. Transgenic lines were scored for their mouth-dimorphism phenotype over multiple generations. Sample sizes of analyzed animals are given in Table S3. All *Ppa-eud-1* constructs consisted of a 13 kb fragment containing a 7 kb promoter element, the 4.6 kb coding region including all introns, and a 1.6 kb 3′ UTR fragment. The *Pex-eud-1* locus is of similar size, and the constructs used for transformation experiments consisted of promoter fragments, the coding region including introns, and the 3′ UTR.

To rescue more than one allele with the same extrachromosomal array, a line transformed by microinjection, *eud-1(tu450)*; Ex[*eud-1*^{CA}], was crossed to the mutant line *eud-1(tu442)*. Because *Ppa-eud-1* is on the X chromosome, transgenic males were crossed to mutant hermaphrodites to eliminate the X chromosome of the transgenic line from F1 males. F1 males reporting the array were crossed back to the mutant line. F2 hermaphrodites, which carried the X chromosome from the mutant *eud-1(tu442)* as well as the array, were screened for rescue of the mouth-dimorphism phenotype.

To generate transgenic males, wild-type California males or *eud-1(tu450)* males were crossed to *eud-1(tu450)*; Ex[*eud-1*^{CA}] hermaphrodites, such that, in either type of cross, F1 males inherited both the X-linked locus and the extrachromosomal array from the transgenic line.

For transgenic experiments including other *P. pacificus* strains, control transformations of RS5200B were performed with a presumptive null *eud-1* allele, *tu445*.

Natural Variation in *P. pacificus*

To test for the presence of natural variation in the mouth-form frequency in wild populations of *P. pacificus*, phenotypes were scored for at least three culture populations spanning at least as many generations. Strains that were highly St (<20% Eu) were screened similarly over at least six culture populations.

Whole-Genomic Sequencing and Diversity Analysis

Methods for preparation and analysis of whole-genomic and transcriptomic sequences and for analysis of diversity patterns at the *eud-1* locus are provided in the Extended Experimental Procedures.

Phenotypic Conversion of Interspecific Hybrids

An extrachromosomal array carrying *eud-1*^{CA} was introduced into interspecific hybrids through reciprocal crosses. To ensure that all F1 offspring of *P. exspectatus* males and *P. pacificus* hermaphrodites were hybrids, a recessive morphological marker, *Ppa-unc-1*, was used. A transgenic double mutant, *unc-1*; *eud-1(tu450)*; Ex[*eud-1*^{CA}], was generated as a marker for transformation of hybrids. Offspring of *P. pacificus* males crossed to *P. exspectatus* females were considered hybrids. Crosses in the latter direction were performed with males of *eud-1(tu450)*; Ex[*eud-1*^{CA}], whereas control crosses in the same direction were performed with males of strain RS2333. Crosses each consisted of three to five females or hermaphrodites and of five *P. exspectatus* males or eight to ten *P. pacificus* males. Hybrid-cross experiments to introduce an array including *Pex-eud-1* were performed with *P. pacificus* RS2333 males and *P. exspectatus* females.

Reporter Constructs

The transcriptional reporter of *Ppa-eud-1*^{CA} contained the same 7 kb fragment as that of the rescue construct. The *Ppa-eud-1*^{CA} promoter element was fused with a fragment containing a nuclear localization signal, the coding region of the fluorophore TurboRFP (Evrogen), and the 3′ UTR sequence of the gene *Ppa-rpl-23*^{CA} (Schlager et al., 2009). Fragments were then fused and amplified by overlapping extension PCR. All amplified fragments were verified by sequencing. Primers for amplification of the final reporter construct contained XmaI restriction sites for subsequent digestion of *Ppa-eud-1*^{CA}::*TurboRFP*. The expression construct (15 ng/μl) was coinjected with 60 ng/μl genomic carrier DNA, cut with XmaI and PstI, and 10 ng/μl of the injection marker *Ppa-egl-20*^{CA}::*TurboRFP* cut with PstI. Three independent lines were generated. Reporting individuals were imaged using a Zeiss ApoTome wide-field microscope.

Sulfatase Inhibition and Epistasis Tests

Details of assays for sulfatase inhibition and for responses to pheromone and Δ7-DA are provided in the Extended Experimental Procedures.

Phylogenetic Analysis

Methods for the identification of *eud-1* homologs and for phylogenetic analysis are provided in the Extended Experimental Procedures. See also Tables S5 and S6.

Statistical Analyses of Phenotypic Data

In sulfatase inhibition, pheromone, and Δ7-DA assays (Figure 5), in which individuals were screened for their unique developmental responses to treatment molecules, samples were pooled across replicate treatments. In the survey for natural variation of the mouth phenotype in wild isolates of *P. pacificus* (Figure 4A), each sample was an entire culture population for which the Eu frequency was recorded. All other bar charts (Figures 2 and 4B–4E) show the Eu frequency calculated from the total individuals screened, for which sample sizes are given in Tables S2, S3, and S4. All significant differences were tested by a two-sided Fisher's exact test, as implemented in the program R. Tests were performed pairwise and with each variable including the total count data, i.e., the number of Eu individuals of all individuals scored. Tests on the phenotypic conversion of *P. pacificus* isolates RS5200B and RS5410, which

involved multiple independently transformed lines (Figures 4B and 4D), were performed as pairwise comparisons with a Bonferroni correction of α.

ACCESSION NUMBERS

The GenBank accession numbers for *P. elegans sul-2.1* and *sul-2.2* reported in this paper are KF466323–KF466325.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.09.054.

AUTHOR CONTRIBUTIONS

E.J.R. and R.J.S. designed the research, except for sulfatase inhibition experiments, which were designed by M.R.M. E.J.R. and M.R.M. performed the research, except for the analysis of whole-genomic sequence data, which was performed by C.R. E.J.R. and R.J.S. wrote the manuscript.

ACKNOWLEDGMENTS

This work was funded by the Max Planck Society and by a fellowship from the Alexander von Humboldt Foundation awarded to E.J.R. We thank Gabi Eberhardt for support in next-generation sequencing, and we thank Vahan Serobyan for assistance with RNA-seq experiments. We also thank Adrian Streit and Benjamin Schlager for conceptual discussions and helpful suggestions on the project.

Received: May 14, 2013
Revised: August 1, 2013
Accepted: September 16, 2013
Published: November 7, 2013

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EXTENDED EXPERIMENTAL PROCEDURES

Forward Genetics Screen

Mutants of *P. pacificus* were produced by previously described protocols (Pires da Silva, 2006). Mutagenized mid-J4 stage hermaphrodites (P0) were picked individually onto new plates, from which cultures F2 hermaphrodites were cloned. To isolate *eud* mutants, the F3 broods of cloned F2 were screened for a phenotype of < 5% Eu hermaphrodites. 10 St F3 individuals from each presumptive *eud* line were again cloned, after which one F4 brood with a low (<5%) Eu frequency of hermaphrodites was kept. Mutants were backcrossed twice to the wild-type strain as follows. First, mutant hermaphrodites were crossed to California males. Next, F1 males were crossed to California hermaphrodites with a recessive dumpy-like marker, *Ppa-pdl-2* (Kenning et al., 2004). Finally, the F2 were cloned to recover the mutant phenotype in the F3, cloned again in multiple as F3, and then screened in the F4 to confirm homozygosity of putatively dominant alleles.

Mapping by SSLP and SSCP Detection

For genetic mapping, mutants starting with a California background were crossed twice to the Washington strain (PS1843). In the first cross, mutant hermaphrodites were crossed to Washington males. F1 males were then crossed to Washington hermaphrodites with a recessive dumpy-like marker (*tu406*). The F2 were cloned and screened for two generations to confirm the mutant phenotype and the homozygosity of mutations. Genomic DNA of outcrossed mutant lines was extracted for genetic mapping. Simple-sequence length (SSLP) or conformation (SSCP) polymorphism markers were tested against 30-40 outcrossed mutant lines and detected as previously described (Srinivasan et al., 2002, 2003).

Whole-Genome Sequencing for Mutant Identification

To prepare samples for whole-genomic sequencing, DNA was extracted and purified using the MasterPure DNA purification kit (Epicenter). DNA was quantified and genomic libraries were prepared as described (C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data). All libraries were diluted to a concentration of 10 nM in 0,1% EB-Tween and pooled as 4-plex. The resulting libraries were sequenced as 100-bp paired ends on an Illumina Genome Analyzer II to a theoretical coverage of 9X. Raw sequencing data were processed as described in Rae et al. (2012).

Gene Cloning

Following preparation of mixed-stage RNA libraries for the California strain of *P. pacificus*, coding DNA (cDNA) was amplified by reverse transcriptase PCR and sequenced. 5′ and 3′ RACE-PCR was also performed to confirm ends of transcript sequences. Lesions in mutant alleles were confirmed by PCR and Sanger sequencing of genomic DNA. Gene-specific primers were designed according to the available genomic sequence for *Ppa-eud-1*.

RNA-Sequencing Experiments

Presence and levels of gene expression were measured by whole-transcriptome sequencing (RNA-Seq) of individual lines of mutants, wild isolates of *P. pacificus*, and *P. exspectatus*. For experiments, culture populations were allowed to grow until their food was exhausted, immediately after which the cultures were processed for sequencing. Five mixed-stage plates were washed with 40 ml M9, centrifuged immediately at 1,300 g for 4 min, rinsed with 40 ml 0.9% NaCl treated with 40 μl ampicillin and 40 μl chloramphenicol and shaken gently for 2 hr, and finally concentrated into a pellet by centrifugation and immediately frozen in liquid nitrogen. RNA-Seq libraries were sequenced as 2 × 100-bp paired-end reads on an Illumina HiSeq 2000, yielding 11-45 million paired-end reads per sample. Raw reads were aligned to the reference genomes of *P. pacificus* (Hybrid1) and *P. exspectatus*, respectively (http://www.pristionchus.org), using the software Tophat v.2.0.3 (Trapnell et al., 2012). Transcriptomes of *P. elegans*, which are also original in this study, were assembled using the Oases assembler (Schulz et al., 2012). Expression levels were estimated and compared using the programs Cufflinks and Cuffdiff v.2.0.1 (Trapnell et al., 2012).

Analysis of Diversity Patterns at the eud-1 Locus

dN/dS ratios were calculated by counting nonsynonymous and synonymous substitutions between *P. pacificus* and *P. exspectatus* orthologs with normalization by the number of nonsynonymous and synonymous sites, respectively. For intraspecies comparisons, we calculated π values as the average pairwise distance in nucleotides across 104 natural isolates with available genome sequencing data (C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data) and used the corresponding nonsynonymous and silent-site diversity measures to calculate intraspecies dN/dS ratio. For the analysis of the *eud-1* promoter sequence, we applied frequency spectrum neutrality tests based on Tajima's D and Achaz's Y in 1-kb windows at the *eud-1* locus (Achaz, 2009).

Sulfatase Inhibition

To test the enzymatic activity of EUD-1, the wild-type strain and a strain overexpressing *eud-1*, *eud-1(tu442)*; Ex[*eud-1*^{CA}], were treated with the known sulfatase inhibitors Na_2SO_4 decahydrate, K_2SO_4, and K_2HPO_4 trihydrate. Salts were dissolved in water and thereafter mixed with melted NGM agar to bring salts to a final concentration of 120 mM. NGM agar was prepared with

$MgCl_2$ as a replacement for $MgSO_4$ so that the final concentration of sulfate ions could be precisely established. Control treatments consisted of agar mixed with the corresponding volumes of water. 3.5-cm plates containing 3 ml agar were seeded with 75 μl OP50 and 120 mM of the test salt in L-Broth and were then incubated overnight at room temperature to allow bacterial growth. Two J4 hermaphrodites were picked to each plate from the same well-fed source plate. Plates were kept at 20°C. Experiments were conducted in at least four replicates for each treatment type.

Epistasis with Pheromone Signaling

To test whether EUD-1 acts downstream of pheromone signaling, dasc#1 was applied to the presumptive null mutant *eud-1(tu445)*, the highly St strain RS5200B, and the wild-type California strain. The assay was performed as described in Bose et al. (2012), with pheromone administered at 1 μM and with the following modifications: each plate was seeded with two J4 hermaphrodites, and a random sample of 100 individuals per plate was screened. Experiments were conducted in triplicate for each treatment.

Epistasis with Δ7-DA/DAF-12

An assay for the response of *eud-1(tu442)*; Ex[*eud-1*^{CA}] to Δ7-DA was used to test the epistasis of *eud-1* to the receptor of Δ7-DA. The response was assayed in parallel with the California strain. In the assay, a solution of Δ7-DA in ethanol was diluted to 10 mM and applied in 3.0-μl aliquots to 3-cm NGM plates containing 3 ml agar and previously seeded with 50 μl OP50, such that the final starting concentration of Δ7-DA was 10 μM. Control plates were treated with the corresponding volumes of ethanol. After letting plates dry for 2 hr, two J4 hermaphrodites were picked onto each plate. Plates were kept at 20°C for 7 days, with 3.0-μl aliquots of Δ7-DA or ethanol applied twice more at two-day intervals. The experiment was performed in triplicate per treatment per strain. At the end of the treatment, broods consisted completely of adults, at which time all hermaphrodite progeny ($n \geq 60$ per plate) were scored for their mouth phenotype.

Identification of eud-1 Homologs

Putative orthologs across nematodes were identified by amino-acid sequence similarity using reciprocal best BLASTp against the WormBase database. In all species where a clear homolog was found, reciprocal similarity in *P. pacificus* always identified *Ppa-eud-1* and two other predicted protein-coding genes, herein named *Ppa-sul-2.1* and *Ppa-sul-2.2.1* (Table S5). The closest paralogs of *sul-2* were, where annotated, orthologs of *C. elegans* sulfatase 3 (*sul-3*) (Table S6), except for in *Loa loa*, in which the closest paralog was the presumptive 6-*O*-endosulfatase *Llo-sul-1* and could not be aligned to *sul-2* or *sul-3*. Other sequences included in the analysis were homologs of *sul-2* and *sul-3* that could be identified in *P. elegans*.

Phylogenetic Analysis

Predicted amino-acid sequences of *sul-2* and *sul-3* were aligned automatically using MUSCLE (Edgar, 2004) and then manually in MEGA5.05 (Tamura et al., 2011), where ambiguous alignment positions were removed. The gene tree of putative *sul-2* and *sul-3* amino acid sequences was inferred under the maximum likelihood (ML) criterion, as implemented in RAxML (Stamatakis, 2006). The analysis invoked a Whelan and Goldman model with a gamma-shaped distribution of rates across sites. Forty independent runs were performed. Bootstrap support for the most likely tree among all runs was estimated by 500 pseudoreplicates.

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Figure S1. Genomic Sequence and Conceptual Translation of *Ppa-eud-1*, Related to Figure 3A

Gray bars represent exons as confirmed by RACE-PCR. Genomic sequence is available at Pristionchus.org.

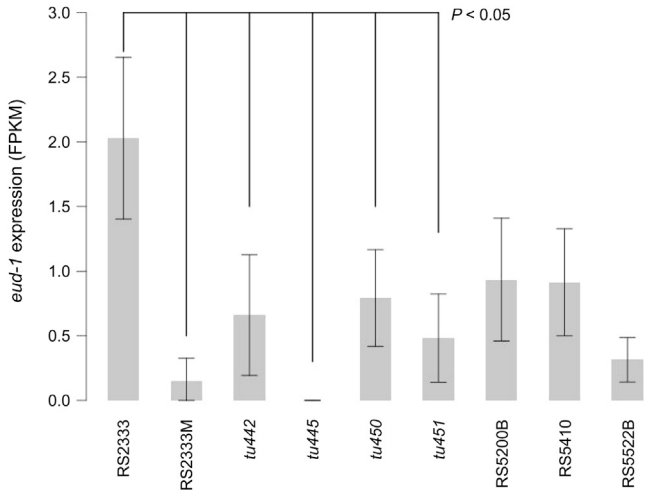

Figure S2. Expression Values for *eud-1* from RNA-Seq Experiments, Related to Figures 2 and 4

Expression values were measured as fragments per kilobase of transcript per million (FPKM) sequenced fragments estimated by a Bayesian inference method (Trapnell et al., 2012). Data are represented as FPKM +/− 95% confidence interval. Gene expression levels were quantified as relative expression with respect to all genes. Strong correlations in pairwise comparisons (Spearman's $\rho > 0.85$, Figure S3) indicated comparability of the data sets. Relative to the *P. pacificus* wild-type hermaphrodite of the California strain (RS2333), males (RS2333M), *eud-1* mutant alleles (*tu442*, *tu445*, *tu450*, *tu451*), and highly St wild isolates (RS5200B, RS5410) exhibit a pronounced downregulation of *eud-1*. The presumptive null allele of *eud-1*, *tu445*, showed zero expression. The expression level in *P. exspectatus* (RS5522B) was estimated after alignment and quantification against the *P. exspectatus* genome and annotation. Although expression levels are not directly comparable across different wild isolates and species borders, $\log_2$ FPKM values for orthologous genes (best reciprocal BLAST hits) show a strong correlation ($r = 0.81$, Pearson), suggesting very similar transcriptome profiles between the two species. In contrast, the ortholog of *eud-1* in *P. exspectatus* shows downregulation comparable to the *eud-1* mutant alleles (no test for differential expression across species borders was performed).

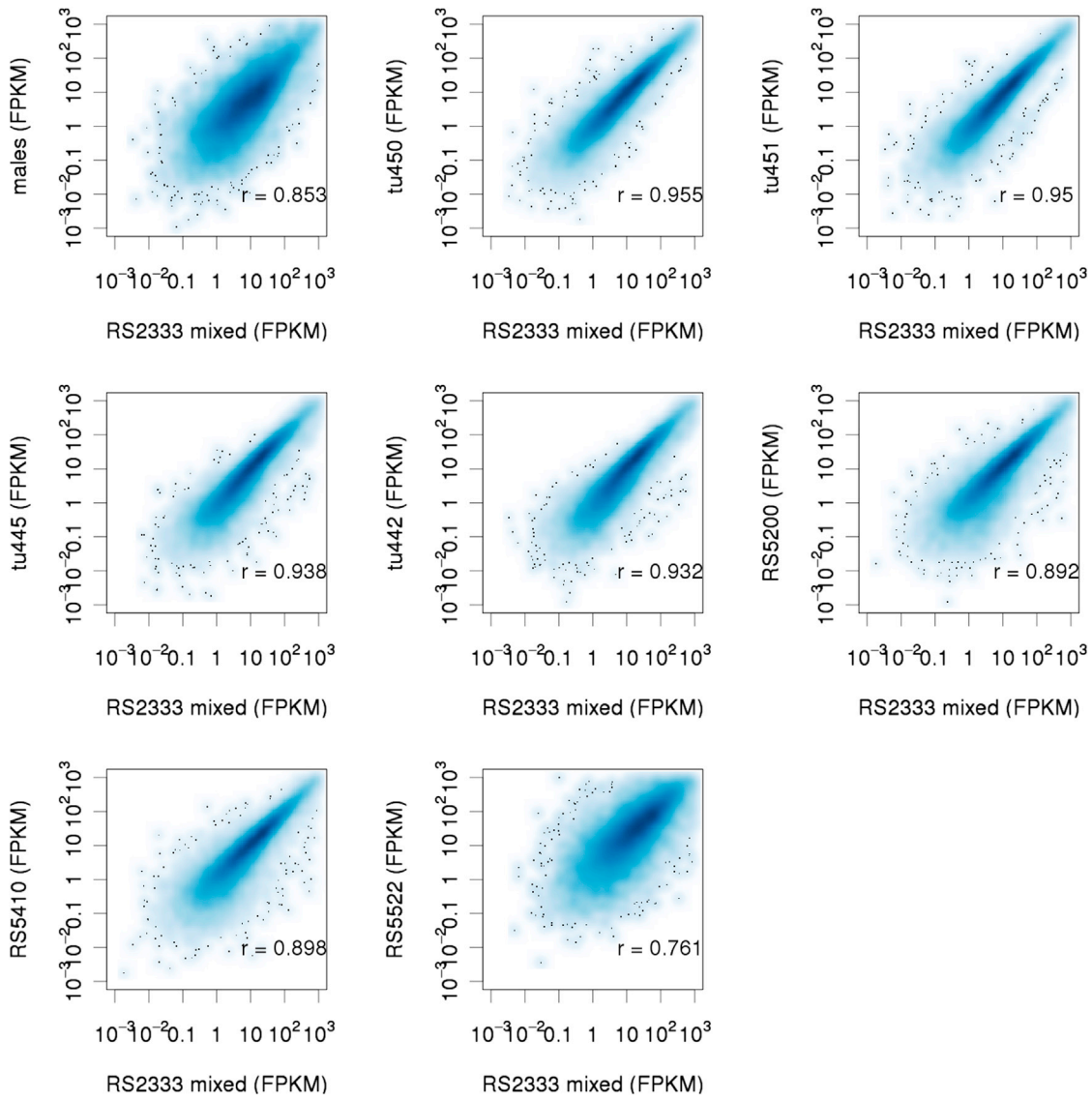

Figure S3. Correlations in Expression Values between Transcriptomes, Related to Figures 2 and 4

Expression levels for all samples were quantified independently as relative expression with respect to all genes (FPKM) using the program Cufflinks (Trapnell et al., 2012). Comparisons of *P. pacificus* mutant, male, and wild-isolate samples with the mixed-stage culture of the reference strain (RS2333) showed strong correlations (Spearman's $\rho > 0.85$). Expression levels of one-to-one orthologs across species also showed a high correlation in all comparisons (Spearman's $\rho = 0.76$). Correlations indicate that normalization with respect to all genes is valid and that the data sets are indeed comparable.

Table S1. Summary of Mutations in *eud-1* Alleles, Related to Figure 3B

Allele	**Mutation**	**Location**	**Predicted target or effect**
tu442	P97L	Exon 5	Catalytic site
tu445	R80stop	Exon 5	Null
tu450	A to T	2 bp upstream of exon 2	Splice acceptor
tu451	R99Q	Exon 5	Catalytic site

Table S2. Frequency of Eu Animals in Wild-Type and Mutant Lines of *P. pacificus*, Related to Figure 2

***P. pacificus* strain**	**% Eu**	**n**
RS2333 (+/+)	90	5894
RS2333, males	25	160
eud-1(tu442)/+	18	100
eud-1(tu442)/*eud-1(tu442)*	0	715
eud-1(tu445)/+	37	40
eud-1(tu445)/*eud-1(tu445)*	0	550
eud-1(tu450)/+	9	104
eud-1(tu450)/*eud-1(tu450)*	0	778
eud-1(tu451)/+	14	91
eud-1(tu451)/*eud-1(tu451)*	0	601

Phenotypes are of hermaphrodites/females unless otherwise indicated.

Table S3. Frequency of Eu Animals in Genetically Transformed Lines of *P. pacificus*, Related to Figures 2, 4B, and 4D

Transformed line	**RFP+**		**RFP-**	
	% Eu	**n**	**% Eu**	**n**
RS2333; Ex[*tu450*] #1	89	157	-	-
RS2333; Ex[*tu450*] #2	96	52	-	-
eud-1(tu450); Ex[*eud-1*^{CA}] #1	100	320	0	74
eud-1(tu450); Ex[*eud-1*^{CA}] #2	100	162	0	80
eud-1(tu450); Ex[*eud-1*^{CA}] #3	99	451	0	80
eud-1(tu450); Ex[*eud-1*^{CA}] #4*	100	60	-	-
eud-1(tu445); Ex[*eud-1*^{CA}] #1	100	178	0	80
eud-1(tu445); Ex[*eud-1*^{CA}] #2	100	184	0	80
eud-1(tu442); Ex[*eud-1*^{CA}], by crossing	100	178	0	90
eud-1(tu450); Ex[*eud-1*^{CA}] #1, males	100	130	0	200
RS5200B; Ex[*tu445*] #1	3	61	0	50
RS5200B; Ex[*tu445*] #2	3	101	3	105
RS5200B; Ex[*tu445*] #3	2	41	6	150
RS5200B; Ex[*tu445*] #4	6	62	3	100
RS5200B; Ex[*tu445*] #5	0	57	2	61
RS5200B; Ex[*eud-1*^{CA}] #1	39	406	5	211
RS5200B; Ex[*eud-1*^{CA}] #2	100	214	28	252
RS5410; Ex[*eud-1*^{CA}] #1	55	110	7	200
RS5410; Ex[*eud-1*^{CA}] #2	51	216	5	111
RS5410; Ex[*eud-1*^{CA}] #3*	99	469	-	-
RS5200B; Ex[*eud-1*^{5200B}] #1	58	60	3	70
RS5200B; Ex[*eud-1*^{5200B}] #2	98	98	8	60
RS5200B; Ex[*eud-1*^{5200B}] #3	98	60	2	60
RS5410; Ex[*eud-1*^{5410}]	100	75	2	75
eud-1(tu450); Ex[*Pex-eud-1*]	100	75	0	75
eud-1(tu450); Ex[*Pex-eud-1*] x RS5522B	100	31	3	44

*Only RFP+ animals observed.

Phenotypes are of hermaphrodites/females unless otherwise indicated.

Table S4. Frequency of the Eu Form in Male and Female F1 Hybrids between *P. pacificus* Strains and *P. exspectatus* (RS5522B), Related to Figures 4C and 4E

Cross type	**crosses**	**Females**		**Males**	
	(N)	**% Eu**	**n**	**% Eu**	**n**
RS5522B ♀ x RS5522B ♂	6	0	621	0	150
RS5522B ♀ x RS2333 ♂	14	69	327	0	428
RS5522B ♀ x *eud-1(tu450)*; Ex[*eud-1*^{CA}] ♂	17	100	80	90	116
Ppa-unc-1 □ x RS5522B ♂	8	52	67	97	65
Ppa-unc-1; *eud-1(tu450)*; Ex[*eud-1*^{CA}] □ x RS5522B ♂	7	100	74	98	47

Table S5. Putative Orthologs and Paralogs of *sul-2* in 10 Nematode Species, Related to Figure 3D

Species	Sequence identifier	Gene or putative gene	Transcript
Bursaphelenchus xylophilus	BUX.s01143.356	*Bxy-sul-2*	Predicted
Caenorhabditis brenneri	CBN13791	*Cbn-sul-2-A*	Predicted
	CBN06224	*Cbn-sul-2-B*	Predicted
Caenorhabditis briggsae	CBG11339	*Cbr-sul-2*	Predicted
Caenorhabditis elegans	D1014.1	*Cel-sul-2*	Confirmed by cDNA[1]
Caenorhabditis japonica	CJA12246	*Cjp-sul-2*	Predicted
Caenorhabditis remanei	CRE05490	*Cre-sul-2*	Predicted
Loa loa	LOAG_05452	*Llo-sul-2*	Predicted
Pristionchus elegans	18569	*Pel-sul-2.1*	Confirmed by cDNA[2]
	12959+21596	*Pel-sul-2.2*	Confirmed by cDNA[2]
Pristionchus exspectatus	scaffold51-snap.28	*Pex-sul-2.1-A*	Predicted
	scaffold51-snap.29	*Pex-sul-2.1-B*	Predicted
	scaffold22-snap.17	*Pex-sul-2.2.1*	Confirmed by cDNA[3]
	scaffold22-snap.18	*Pex-eud-1*	Confirmed by cDNA[3]
Pristionchus pacificus	PPA21290	*Ppa-sul-2.1*	Predicted
	PPA06135	*Ppa-sul-2.2.1*	Confirmed by cDNA[2]
	PPA06136	*Ppa-eud-1*	Confirmed by cDNA[2]

[1]Information available at WormBase.org.
[2]Present study.
[3]Rödelsperger et al. (submitted).

Table S6. Putative Orthologs of *sul-3* in Eight Nematode Species, Related to Figure 3D

Species	Sequence identifier	Gene or putative gene	Transcript
Bursaphelenchus xylophilus	BUX.s01109.101	*Bxy-sul-3*	Predicted
Caenorhabditis brenneri	CBN18180	*Cbn-sul-3*	Predicted
Caenorhabditis briggsae	CBG16830	*Cbr-sul-3*	Predicted
Caenorhabditis elegans	C54D2.4	*Cel-sul-3a*	Confirmed by cDNA[1]
Caenorhabditis japonica	CJA13201 CJA29064	*Cjp-sul-3**	Predicted
Caenorhabditis remanei	CRE00019	*Cre-sul-3*	Predicted
Pristionchus exspectatus	scaffold279-snap.12 scaffold279-snap.11	*Pex-sul-3**	Confirmed by cDNA[2]
Pristionchus pacificus	PPA23475	*Ppa-sul-3*	Confirmed by cDNA[3]

*Indexed as two sequences but are orthologous with the 5' and 3' ends, respectively, of *Cel-sul-3*.
[1]Information available at WormBase.org.
[2]Rödelsperger et al. (submitted).
[3]Present study.

EVOLUTION & DEVELOPMENT 15:3, 161–170 (2013)
DOI: 10.1111/ede.12030

Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed

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SUMMARY The increasing evidence for a role of developmental plasticity in evolution offers exciting prospects for testing interactions between ecological and developmental genetic processes. Recent advances with the model organism *Pristionchus pacificus* have provided inroads to a mechanistic understanding of a developmental plasticity. The developmental plasticity of *P. pacificus* comprises two discontinuous adult mouth-forms, a stenostomatous ("narrow mouthed") and a eurystomatous ("wide mouthed") form, the latter of which is structurally more complex and associated with predatory feeding. Both forms are consistently present in populations, but fundamental properties guiding fluctuations in their appearance have been poorly understood. Here, we provide a systematic characterization of the mouth plasticity in *P. pacificus*, quantifying a strong sexual dimorphism and revealing that, in an inbred genetic background, maternal phenotype is linked to that of male offspring. Furthermore, cues from conspecifics influenced the developmental decision in juvenile nematodes. Separating individuals from a population resulted in a lower eurystomatous frequency, which decreased incrementally with earlier isolation. Finally, the time to the reproductively mature stage was, in the presence of an abundant bacterial food supply, less for stenostomatous than for eurystomatous individuals, suggesting the potential for a fitness trade-off between developmental time and breadth of diet. This study provides a baseline understanding of the mouth dimorphism in *P. pacificus* as a necessary reference point for comparative analysis.

INTRODUCTION

The ability of a single genotype to exhibit major phenotypic differences is becoming increasingly recognized as a driver of novelty and the diversity of form (West-Eberhard 2003). The link between polyphenism and evolution is supported by numerous case studies, as highlighted in several recent reviews (Fusco and Minelli 2010; Moczek 2010; Pfennig et al. 2010; Moczek et al. 2011). It has been argued that developmental plasticity facilitates morphological innovations that result in new traits and, simultaneously, allow for novel interactions in the environment (West-Eberhard 2003). Beyond this theoretical framework developed for understanding the role of developmental plasticity in the origin of new traits, experimental evidence has revealed some specific genetic mechanisms that are involved in the accommodation of polyphenic traits (Suzuki and Nijhout 2006) or are associated with their expression (Braendle et al. 2005; Snell-Rood and Moczek 2012).

In the nematode *Pristionchus pacificus*, plasticity of feeding structures was coupled with known developmental pathways (Bento et al. 2010). The polyphenism of *P. pacificus*, as in other species of the family Diplogastridae, consists of a stenostomatous ("narrow mouthed") and a eurystomatous ("wide mouthed") form, which differ in the number and shape of teeth and in the complexity of other mouth armature (Fig. 1). The dimorphism is thought to relate to feeding differences, whereby the eurystomatous form is associated with predation of other nematodes (Kiontke and Fitch 2010). Besides its ecological significance, the genetic control of the mouth dimorphism has begun to be investigated. Specifically, it was shown that the incidence of the stenostomatous form in *P. pacificus* was higher in populations treated with Δ7-dafachronic acid (DA), a steroid hormone that inhibits the formation of a resistant, alternative juvenile ("dauer") stage by acting on the nuclear hormone receptor DAF-12 (Bento et al. 2010). Correspondingly, starvation conditions or the application of pheromone derived from high-density cultures induce both the eurystomatous form and dauer formation (Bento et al. 2010). However, mechanisms for the mouth and life-stage dimorphisms do not completely overlap, as the dauer-promoting transcription factor DAF-16/FOXO has no effect on the mouth phenotype (Ogawa et al. 2011). The unraveling of signaling pathways directly influenced by environmental parameters thus allows exciting new tests of the interaction between developmental and

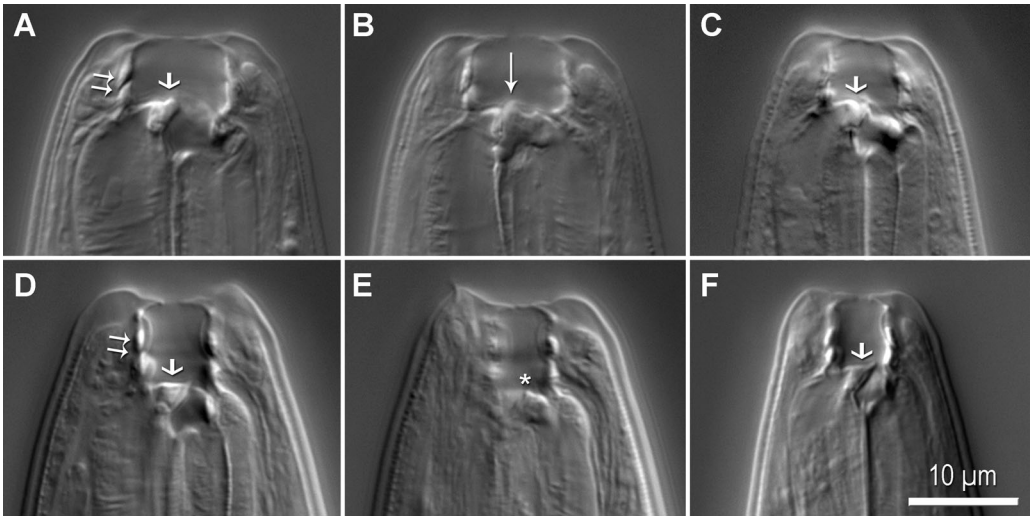

Fig. 1. DIC micrographs of the dimorphic stoma (mouth) of *Pristionchus pacificus*. All images are at same scale. Dorsal is left in all images. (A and B) Eurystomatous hermaphrodite in the sagittal and right sublateral planes, respectively. (C) Eurystomatous male in the sagittal plane. (D and E) Stenostomatous hermaphrodite in the sagittal and right sublateral planes, respectively. (F) Stenostomatous male in the sagittal plane. The eurystomatous and stenostomatous forms differ in the width of the mouth but also in several discrete characters. Short arrows indicate dorsal tooth, which is claw-like in the eurystomatous form (A–C) and thin and symmetrical, or flint-like, in the stenostomatous (D–F). Long arrow indicates opposing, claw-like subventral tooth, which is absent (asterisk) in the stenostomatous form. Stomatal walls (double arrows) are rigid and more highly sclerotized in the eurystomatous form, in contrast to their beaded appearance in the stenostomatous form. Notwithstanding a size difference in the mouth by sex, mouth-forms are qualitatively identical in the two sexes, as highlighted by the shape of the dorsal tooth in the two male forms (C and F).

ecological processes (Schlichting and Pigliucci 1998; Sommer and Ogawa 2011).

Among models for polyphenism, *P. pacificus* has a powerful set of analytical tools available to it. As a well-established satellite model to that of *Caenorhabditis elegans*, *P. pacificus* enables comparative developmental and genetic studies (Sommer 2009). Genetic analysis of dimorphism in *P. pacificus* is made feasible by androdioecious reproduction (Sommer et al. 1996), genetic and physical maps of the genome (Srinivasan et al. 2002, 2003), a sequenced and annotated genome (Dieterich et al. 2008), and the capability for forward genetics (Zheng et al. 2005; Schlager et al. 2006) and DNA-mediated transformation (Schlager et al. 2009). The recent identification of another developmental regulator, the cyclic-GMP-dependent protein kinase *egl-4*, with a mutant mouth-dimorphism phenotype has further demonstrated this genetic tractability (Kroetz et al. 2012).

Besides its amenability to genetics studies, *P. pacificus* derives power as an animal model for developmental plasticity from being rigorously quantifiable. The short generation time and large brood size of *P. pacificus* make it amenable to high-throughput screens. The phenotype can, therefore, be studied as the frequencies of forms in a population that change in statistically testable ways. Quantitative analysis of phenotypic plasticity is of significance for an ultimate understanding of the interplay of the environment and intrinsic genetic and molecular mechanisms.

Despite the inroads this system gives to understanding the precise genetic basis and evolutionary consequences of a dimorphism, the factors that guide the fluctuations in the trait are still poorly understood. The frequencies of the two forms are apparently stochastic in populations, even under consistent food and ambient conditions (Bento et al. 2010). Although starvation, dauer pheromone, or Δ7-DA can perturb these frequencies, both forms normally occur in every generation (Bento et al. 2010). What other genetic or environmental factors might influence the development of the two forms are still unknown. For example, an open question is that of sexual dimorphism of the mouth-form plasticity in *P. pacificus*. The reported absence of eurystomatous males in some other diplogastrid genera (von Lieven and Sudhaus 2000) has suggested this possibility. Hints of possible cross-generational effects and the precise influence of density-specific cues (Bento et al. 2010) are also unresolved. Here we have endeavored to thoroughly characterize the mouth-form plasticity of *P. pacificus* and thereby provide the necessary foundation for budding research on this system.

MATERIALS AND METHODS

To provide a rationale for standardization in further studies on the *P. pacificus* mouth dimorphism, we have established a method for accurately characterizing the dimorphism phenotype under a defined set of conditions.

Pristionchus pacificus

All experiments were conducted with the inbred, wild-type reference strain of *P. pacificus*, RS2333 (=PS312). Post-embryonic development of *P. pacificus* consists of four juvenile stages (J1-J4), with the first molt (J1 to J2) occurring within the egg (von Lieven 2005). Sex determination in *P. pacificus* is by an XX: X0 system, in which males occur spontaneously as a result of accidental X-chromosome non-disjunction (Sommer et al. 1996). The appearance of spontaneous males can then lead to the spread of males throughout a population by sexual reproduction. In standard laboratory culture the frequency of spontaneous males in strain RS2333 is about 0.5% (Click et al. 2009).

Nematodes were maintained on nematode growth medium agar plates seeded with a lawn grown from 400 µl (or 100 µl for crossing plates) of *Escherichia coli* strain OP50 in L-Broth. All plates were kept at 20°C. Plates showing any signs of bacterial or fungal contamination were excluded from experiments. To prevent any mechanical stresses during handling of nematodes, juveniles were picked with a buffer of viscous bacterial solution derived from OP50 lawns, such that direct physical contact with nematodes was reduced or eliminated. To avoid possible trans-generational effects of starvation or other environmental aberrations, nematodes were cultured under well-fed, non-crowded conditions for at least three generations before picking nematodes for cultures referred to as "source plates" herein. Source plates were each established from five J4 (virgin) hermaphrodite progenitors; nematodes of the ensuing generation were used to start all experiments. Thus all nematodes went through at least four generations in healthy culture, the most recent generation encountering a roughly standard population density (i.e., the progeny of five hermaphrodites), prior to experiments.

Phenotype scoring

The mouth dimorphism of *Pristionchus* spp. is discontinuous and is manifest and developmentally irreversible at the adult stage (Hirschmann 1951). Phenotypes were scored according to morphological differences detailed by von Lieven and Sudhaus (2000) and Kanzaki et al. (2012). Differences were sufficient to positively identify either of the two forms, such that neither form was scored by default. Characters used to discriminate between eurystomatous and stenostomatous individuals, respectively, were (Fig. 1): (i) the presence versus absence of a subventral tooth; (ii) a claw-like versus flint-like (i.e., dorsoventrally symmetrical) dorsal tooth; (iii) strongly versus weakly sclerotized stomatal walls; and (iv) a wide versus narrow stoma (mouth). The discrete, non-overlapping characters (i) and (ii) are sufficient to distinguish the two forms in *P. pacificus* as well as in all other examined *Pristionchus* species (E. J. R., pers. obs.). Intermediate states are possible in characters (iii) and (iv), although the polar ends of these character distributions are always correlated with the respective states for characters (i) and (ii). True intermediates, namely within or between characters (i) and (ii), are apparently rare (<0.1% of specimens examined; E. J. R., pers. obs.); they were not found in the present study and thus not included in counts. Phenotypes were authoritatively determined by differential interference contrast (DIC) microscopy on a Zeiss Axioskop. To enable higher throughput in screens, phenotypes were also scored using Zeiss Discovery V.12 and V.20 stereomicroscopes and then supplemented where necessary with DIC microscopy.

Phenotype characterization by sex, parentage, and maternal phenotype

The mouth-form phenotype of *P. pacificus* was characterized by the following measurements: (i) eurystomatous frequency of spontaneous males; (ii) eurystomatous frequency of hermaphrodites of the same cohorts as spontaneous males; (iii) eurystomatous frequency of male progeny from crosses; (iv) eurystomatous frequency of hermaphroditic progeny from crosses ("cross-hermaphrodites"); and (v) eurystomatous frequency of hermaphroditic progeny from selfing mothers ("self-hermaphrodites").

Taking these measurements in a controlled genetic and environmental background followed the occurrence of spontaneous males, due to the rarity of these males in laboratory culture. To begin, source plates, each containing cohorts born of five J4 hermaphrodites, were screened for spontaneous males after 6 days of growth. After successfully collecting and screening several ($n = 40$) spontaneous males, which were never crossed but are included in the analyzed samples, the following experimental screen was conducted for all subsequently isolated spontaneous males. The final sample of spontaneous males ($n = 125$) was obtained after screening 260 source plates. Each spontaneous male found was transferred to a crossing plate, where it was paired with a J4 (virgin) hermaphrodite randomly picked from the same source plate and then let to mate overnight. In parallel, five additional J4 (virgin) hermaphrodites were randomly picked from the same source plate onto their own individual plates. On the following day, males were recovered and screened for their mouth-form phenotype. Both the crossed hermaphrodites and the five virgin hermaphrodites picked from the same source plate were retained on culture plates overnight to lay eggs. Two days following the initial cross, crossing and virgin hermaphrodites were recovered and screened for their mouth form. Six days after crossing, mouth-form phenotypes were screened for cross-broods, which included hermaphrodites and males. Additionally, the mouth forms were screened in a self-brood of one mother of the same cohort (i.e., one of the five hermaphrodites isolated in parallel) and whose mouth-form was the same as the hermaphrodite in the cross; if such a mother was not found, then a corresponding self-brood was not included. In this manner, self- and cross-progeny of mothers of the same phenotype and source population could be directly compared.

Table 1. The eurystomatous frequency of *Pristionchus pacificus* under a laboratory culturing regime and characterized by sex, parentage, and maternal phenotype

		Self-hermaphrodites		Cross-hermaphrodites		Cross-males	
		Mean ± SE	*n* (*N*)	Mean ± SE	*n* (*N*)	Mean ± SE	*n* (*N*)
Maternal phenotype	Total	86.33 ± 2.55	19 (1352)	83.24 ± 3.06	28 (1482)	21.28 ± 3.87	28 (580)
	Eu	87.61 ± 2.13	15 (917)	85.86 ± 2.57	18 (1015)	29.65 ± 3.53	18 (416)
	St	83.15 ± 7.50	4 (435)	76.70 ± 8.53	10 (467)	0.35 ± 0.35	10 (164)

Values correspond to results in Fig. 2B. Sample size (*n*) of plates and total number (*N*) of individuals screened are given. Eu, eurystomatous; St, stenostomatous.

Entire broods resulting from 2 days of oviposition were screened and needed to comprise at least 50 individuals to be included in the experiment. To be considered a "successful" cross and thus included in the experiment, broods must have been at least 20% males. Sample sizes for all categories of individuals are given in Table 1. Morphological mutant lines were not used to distinguish hermaphroditic self- from cross-progeny in cross plates to avoid biases that could be introduced by pleiotropic effects on the mouth phenotype in those mutants (Müller and Sommer, unpublished data).

Because of the difficulty in distinguishing cross- from self-hermaphrodites, we additionally tested for differences between cross- and self-progeny by crossing males carrying a stably transmitted reporter gene to mother hermaphrodites. The reporter used was *Ppa-egl-20*::rfp (strain RS2597; Kienle and Sommer 2013), which is expressed in the tail at all life stages (Schlager et al. 2009) and which was confirmed to be transmitted with 100% penetrance ($n = 373$). Prior to experiments, reporter populations were cultured for at least four generations under a consistent population density as described above. To test the effect of paternity on the mouth-form, crosses were established between one *Ppa-egl-20*::rfp male and one young adult hermaphrodite of the reference strain. Fluorescently reporting F1 hermaphrodites were identified as cross-progeny, whereas all non-reporting hermaphrodites were considered self-progeny. As a control for the neutrality of the reporter gene toward the mouth-form phenotype, we also screened the self-progeny of each mouth-form that were produced by one young adult hermaphrodite per mating plate. Sample sizes were 19 and 15 replicates (plates) for crosses with eurystomatous and stenostomatous mothers, respectively, and were 12 and 11 for *Ppa-egl-20*::rfp selfing plates with eurystomatous and stenostomatous mothers, respectively.

Effect of population cues on the adult phenotype decision

To obtain juveniles for testing the effect of isolation on the mouth-form plasticity, five source plates were allowed to grow for 7 days (1.5 generations), such that juveniles of all stages were available in a single population. From each of these plates, 10 individuals of each juvenile stage (J2, J3, and J4) were transferred to new individual plates. After completing development in isolation, individuals were screened for their mouth form. As a control, 10 randomly picked young but already matured hermaphrodites from each of the same source plates were screened for their mouth form. The experiment was performed in triplicate to result in a sample of 150 individuals isolated per life-stage except J2, for which the sample size was 144 hermaphrodites after excluding failed developers and spontaneous males.

Developmental timing of mouth forms

To collect and synchronize juveniles for timing of their development, eggs were transferred from multiple source plates to a single new plate. J2 individuals that hatched on this plate within 2 h were transferred to their own individual plates and screened for their developmental stage once a day. J2 hatchlings were picked from the same batch of eggs at three different starting times, which were separated by 4-h intervals, to make a total of 150 individuals. After the first individuals reached the J4 stage, all animals were screened every 4 h until becoming adults, after which they were screened for their mouth form. Duration of development was calculated as the time from hatching to the adult stage. Those animals that did not molt to the J3 stage within 72 h were presumed to not have recovered from handling and were excluded from the experiment. Because of the fragility of young hatchlings, several were unable to complete the experiment: after premature deaths, failed developers, extremely late developers (see below), and one spontaneous male, the total number of samples was 141 ($n = 68$ eurystomatous, $n = 73$ stenostomatous).

Statistical analyses

Count data were obtained in two experiments: (i) phenotypes of hermaphrodites and spontaneous males individually picked for crossing experiments and (ii) isolation of individuals at different life-stages. Differences in the proportion of eurystomatous individuals from these experiments were tested using Fisher's exact test. Confidence intervals for all count data were estimated by a binomial test.

In all other experiments characterizing the mouth-form phenotype, each sample was an entire plate for which the eurystomatous frequency was recorded. Prior to statistical tests, an arcsine transformation was applied to proportional variables. Distributions of these variables after arcsine transformation did not deviate from normality (Kolmogorov-Smirnov test, $P > 0.1$ for all). To test whether (i) maternal mouth-form, (ii) cross type (self vs. cross), or (iii) sex of offspring had an effect on the mouth-form decision of offspring, we performed three-way ANOVA where these three variables were independent. Additionally, one-way ANOVA was used to separately determine whether maternal mouth-form influenced the phenotype of (i) self-hermaphrodite, (ii) cross-hermaphrodite, or (iii) male offspring. Differences in the proportions of eurystomatous animals were tested using one-way ANOVA with maternal mouth-form as the independent variable.

In a separate experiment, where differences between self- and cross-progeny were tested by crossing *Ppa-egl-20*::rfp males to wild-type hermaphrodites, one-way ANOVA was used to individually test for effects of (i) maternal mouth-form, (ii) cross type (self vs. cross), and (iii) the *Ppa-egl-20*::rfp transgene on hermaphroditic self-progeny.

In the analysis of developmental timing results, distributions of groups (times for eurystomatous vs. for stenostomatous) initially deviated from normality (Kolmogorov-Smirnov test, $P < 0.01$). Inspection for outliers identified three extremely late developers (developmental times of 88, 88, and 92 h) that matured in a second wave later than all others of both forms (non-outlier maximum = 72 h) and became stenostomatous. Extremely late developers may have been due to stress caused by trauma during handling, suggested by their comparatively small adult body size. Whether the stenostomatous program was a cause, result, or coincidence of an abnormal development rate in those individuals is unclear. After removing extreme cases, the two distributions of the developmental times no longer deviated from normality (Kolmogorov-Smirnov test, $P > 0.05$). Therefore, Student's *t*-test was used to compare the mean maturation time for independent samples (eurystomatous vs. stenostomatous).

Count data were analyzed with R; all other statistical tests were implemented in the program Statistica v. 9 (Statsoft). All figures present untransformed data. For data that were transformed for statistical analysis, whiskers represent the standard error estimated for untransformed data. All percentages given in the text are the frequency of eurystomatous nematodes. Other statistics are given in Table 1.

RESULTS

Dimorphism differs by sex and maternal phenotype

With our experimental design we sought to simultaneously test for: (i) the presence and extent of sexual dimorphism in the frequencies of the two forms; (ii) any correlation between the phenotypes of parents (i.e., mothers) and offspring; and (iii) any differences between offspring of selfing hermaphrodites and those from crosses with males. The dimorphism phenotype of *P. pacificus* was characterized for clones from the same culture conditions and, for all screens downstream of the isolation of J4 hermaphrodite and spontaneous male parents, the same parentage.

Addressing our first question, sexual dimorphism in mouth-form frequencies was evident in comparisons under all conditions. A clear difference was found for individual nematodes isolated from the same culture populations, where hermaphrodites were 71.6% ($n = 431$) and spontaneous males 11.2% ($n = 125$) eurystomatous (Fisher's exact test, $P < 10^{-6}$; Fig. 2A). Three-way ANOVA of the mean eurystomatous frequency of broods, which consisted of males and hermaphrodites under the same environmental conditions and of the same known parentage, identified a phenotypic difference between the sexes in the offspring ($F_{1,73} = 10.2$, $P < 0.0005$; a vs. bc, Fig. 2B).

An unexpected maternal effect resulted in an additional difference in the plasticity in offspring ($F_{1,73} = 5.12, P < 0.05$). Males from eurystomatous mothers showed a significantly ($F_{1,18} = 17.32$, $P < 0.001$) higher eurystomatous frequency (29.7%) than males from stenostomatous mothers (0.3%; b vs. c, Fig. 2B). No difference between hermaphrodites born of the two

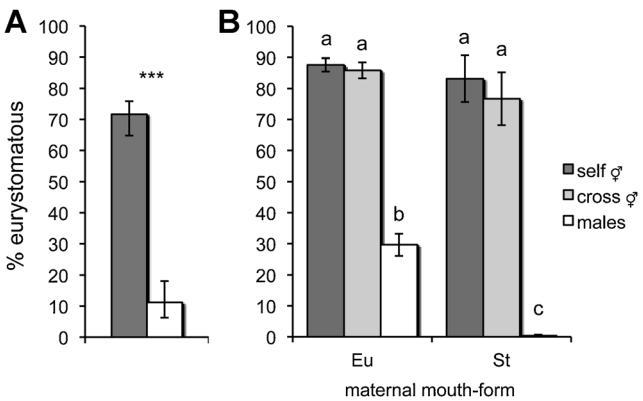

Fig. 2. The mouth-form phenotype of *P. pacificus* by sex, parentage, and maternal phenotype. (A) The total eurystomatous frequencies of spontaneous males (open bars) that is males produced by X-chromosome non-disjunction, and of hermaphrodites (dark gray) occurring in the same culture populations as spontaneous males. Difference is significant by Fisher's exact test ($^{***}P < 10^{-6}$). Whiskers represent a 95% confidence interval. (B) The mean eurystomatous frequencies of self-hermaphrodites (dark gray), cross-hermaphrodites (light gray), and cross-males (open). Cross-progeny are from spontaneous males and co-occurring hermaphrodites; self-progeny is from virgin co-occurring hermaphrodites. Each type of offspring is additionally distinguished by maternal phenotype being eurystomatous (Eu) or stenostomatous (St). Significant differences were detected by three-way ANOVA (a vs. bc, $P < 0.0005$) and one-way ANOVA (b vs. c, $P < 0.001$). Whiskers represent the standard error.

maternal forms was statistically supported ($F_{1,47} = 1.23$, $P > 0.05$), indicating that the effect in male offspring drove the difference found in the general comparison. Because hermaphrodites were crossed to clonal (spontaneous) males, and given the inability to artificially select for either mouth-form in RS2333 (PS312) by self-reproduction (Bento et al. 2010), genetic variation in this highly inbred strain is considered to be low. Therefore, such a correlation of phenotypes between mothers and sons cannot be attributed purely to genetic inheritance. Unfortunately, we were unable to test for an effect of paternal phenotype due to the inadequate number of eurystomatous males available in culture.

Finally, the phenotype of self-hermaphrodites did not differ from that of cross-hermaphrodites from crosses to spontaneous males ($F_{1,73} = 0.67$, $P > 0.05$). However, a real difference could have been underestimated by the inaccuracy built into the reproductive mode of *P. pacificus*: because hermaphroditic offspring of crossing mothers may also include self-progeny, any difference present would be partially hidden by the inclusion of unidentifiable self-offspring in counts of cross-offspring. Therefore, we performed crosses using a marker, *Ppa-egl-20*::rfp, which definitively distinguished self- from cross-progeny and which was confirmed to be neutral with respect to the mouth-form frequency ($F_{1,55} = 1.52$, $P > 0.2$; Fig. 3). This test confirmed that there was no difference in the mouth-form phenotype between self- and cross-progeny ($F_{2,31} = 0.21$, $P > 0.2$) nor any correlation of phenotype between mothers and their hermaphroditic self-progeny ($F_{1,32} = 0.22$, $P > 0.2$) or cross-progeny ($F_{1,32} = 0.09$, $P > 0.2$; Fig. 3). A similar comparison could also not be made for males, as identifying the maternal phenotype of self-cross (i.e., spontaneous) males was not feasible.

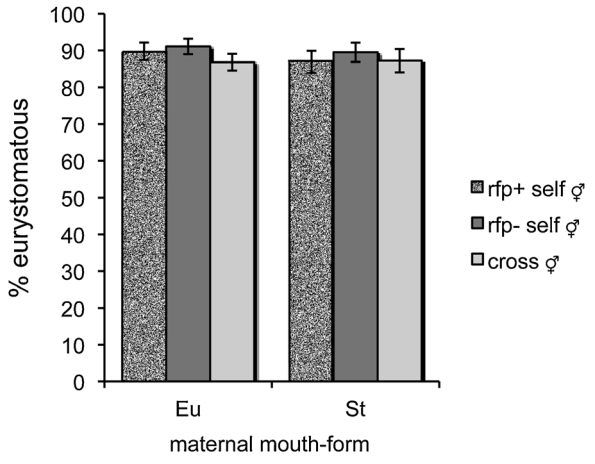

Fig. 3. The effect of paternity on hermaphroditic progeny as tested by crosses with a *Ppa-egl-20*::rfp reporter. The mean eurystomatous frequencies of *Ppa-egl-20*::rfp self-hermaphrodites (textured), wild-type self-hermaphrodites (dark gray), and *Ppa-egl-20*::rfp cross-hermaphrodites (light gray) are shown. Cross-progeny are from *Ppa-egl-20*::rfp males and wild-type hermaphrodites. Each type of offspring is additionally distinguished by maternal phenotype being eurystomatous (Eu) or stenostomatous (St). No differences by (i) maternal mouth-form, (ii) cross type (self vs. cross), and (iii) the *Ppa-egl-20*::rfp transgene on hermaphroditic self-progeny were detected by one-way ANOVA ($P > 0.2$ for all). Whiskers represent the standard error.

Isolation from conspecifics influences the developmental decision

Characterizing the mouth phenotype by sex revealed a putative discrepancy between hermaphrodites individually picked from populations with spontaneous males (71.7% eurystomatous) and their hermaphroditic self-progeny (83.2% from stenostomatous and 87.6% from eurystomatous mothers; Fig. 2B). Given the otherwise standardized genetic and environmental conditions, only one consistent difference between the two experiments was obvious: that hermaphrodites picked together with spontaneous males were always isolated as J4 juveniles, to ensure their virginity, whereas those in broods had always matured to the adult stage in a social context. Because pheromone levels are known to increase the eurystomatous frequency in culture (Bento et al. 2010), we suspected that isolation as J4 from cues given by conspecifics may have resulted in a lower likelihood of becoming eurystomatous. Therefore, we next tested whether exposure through different life-stages to signals of population density would reveal differences in sensitivity for the decision of the adult phenotype.

Isolation of each post-eclosion juvenile stage from multiple, synchronized populations of similar densities led to different phenotypes in the adult (Fig. 4). Nematodes isolated as adults,

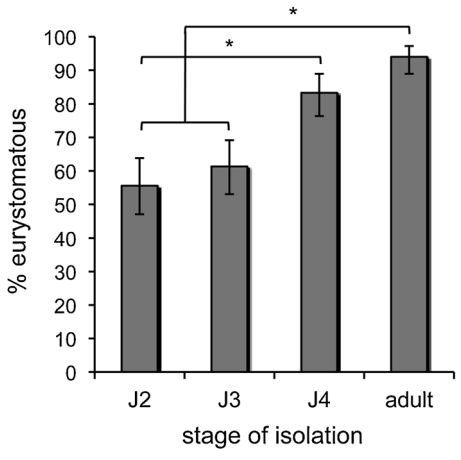

Fig. 4. The mouth-form phenotype of *P. pacificus* hermaphrodites when isolated from populations at different life-stages. Individuals were transferred at one of the three post-eclosion juvenile stages (J2–J4) or allowed to reach the adult stage together with conspecifics. Total eurystomatous frequencies are shown. Significant differences ($^{*}P < 0.05$) are according to Fisher's exact test. Whiskers represent a 95% confidence interval.

after all chances to alter the phenotype decision had passed, showed the highest eurystomatous frequency (93.3%) of any isolated stage. In contrast, those isolated as J2 or J3 juveniles showed significantly lower eurystomatous frequencies (57.3% and 61.7%, respectively) than those isolated as adults (Fisher's exact test, $P < 0.05$). Nematodes isolated during J4 showed a eurystomatous frequency (83.3%) intermediate between those isolated as J3 and as adults and which was different from that of isolated J2 (Fisher's exact test, $P < 0.05$). There was no significant difference between juveniles isolated as J2 and those as J3. Thus, sensitivity to external cues decreased gradually with successive juvenile stages and persisted at least as late as the J3 stage.

Developmental timing of the two forms

The duration of an inherent developmental program could govern the amount of exposure to external cues, and so any difference between the two forms could influence the interaction among developing nematodes. To isolate the effect of post-embryonic developmental time, we tested for differences in the absence of population cues. Tracking the developmental time of nematodes isolated as J2 hatchlings ($\leq$2 h old) revealed that individuals that became stenostomatous developed significantly more rapidly ($T_{139} = -5.67$, $P < 0.05$) than those that became eurystomatous (Fig. 5): the mean ($\pm$ SD) time to adulthood was 55 ± 3 h in stenostomatous as compared to 61 ± 2 h in eurystomatous nematodes. The time from completion of the J3–J4 molt to that of the final molt, a period of 7 ± 5 h in stenostomatous and 12 ± 5 h in eurystomatous nematodes, can account for most of this difference. Thus, the two adult phenotypes were clearly correlated with different rates of post-embryonic development, particularly at the last juvenile stage and final molt.

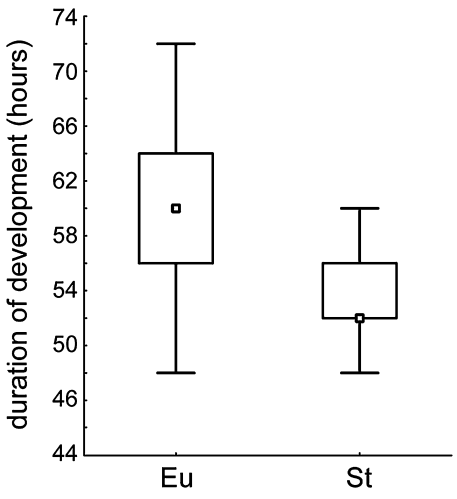

Fig. 5. The duration of post-embryonic development in the two mouth forms of *P. pacificus* at 20°C. Individuals were isolated as hatchlings ($\leq$2 h old) and their development tracked every 4 h until reaching the adult stage. Upon becoming adults, their mouth-form phenotype was recorded. Box plots show the median (center square), the lower and upper quartiles (box bounds), and the non-outlier range limits (whiskers) of the period from hatching to maturity. The difference between the eurystomatous (Eu) and stenostomatous (St) forms in duration of developmental time is significant (Student's *t*-test, $P < 0.05$).

DISCUSSION

The mouth dimorphism of *P. pacificus* is governed by a complex of sexual parameters and external cues. Although some mechanistic developmental context has been given to the phenomenon (Bento et al. 2010), the plasticity in general was not previously well described. In the present study, some of the variables governing the seemingly stochastic occurrence of its two forms were identified. A basic understanding of the dimorphism trait will be indispensible for future work on this system.

Sex plays a role in the feeding-structure plasticity

Although sexual dimorphism in the mouth plasticity in *P. pacificus* was hinted by an apparent lack of eurystomatous males in some diplogastrid taxa (von Lieven and Sudhaus 2000), the work herein is the first to systematically test and quantify such a difference. Not only is recognizing a precise difference between the sexes necessary for a complete understanding of the trait, it may narrow the search for mechanisms by warranting attention to sex-linked developmental processes. Herein we report a strong difference between hermaphrodites and males, which in populations are dominated by the eurystomatous and stenostomatous forms, respectively. It is likely that the rarity of outcrossing events shown in the laboratory (Click et al. 2009) and inferred in the wild (Morgan et al. 2012) for *P. pacificus* undermines the selection potential conferred by male-mediated differences within this species. However, sex-related effects could play a much larger role in the ecological divergence of other *Pristionchus* species, most of which are gonochoristic (Mayer et al. 2007; Kanzaki et al. 2012). Consequently, any such role would also be predicted for the evolution of hermaphroditic *Pristionchus* species from gonochoristic ancestors.

The predominance of the eurystomatous form among *P. pacificus* hermaphrodites was a surprising contrast to the findings of Bento et al. (2010), who reported hermaphrodites as being mostly stenostomatous (approximately 30% Eu) in their control experiments. Given the results obtained in this study, this discrepancy might be explained in several ways. First, the culturing regimen and thus possible cross-generational effects were controlled differently between studies. Second, a likely cause of the discrepancy is observer differences, which can never

be completely ruled out. The method used by Bento et al. to discriminate phenotypes emphasized head shape and stoma width, although these features can be variable as compared to the qualitative differences of the teeth (von Lieven and Sudhaus 2000; Fig. 1). Finally, it should be mentioned that another possibility is that of mutation accumulation in the strains used in the different studies. For the present study and in ongoing work with *P. pacificus*, strains used in experiments are freshly thawed from a frozen voucher once per year in order to minimize mutation accumulation that might affect plastic traits that are under strong environmental influence. Additionally, the number of animals used in the experimental set-up should be rigorously controlled, as the density experiments described above (Fig. 4) and the higher eurystomatous frequency induced by increased pheromone concentrations (Bose et al. 2012) both suggest that the number of progeny could influence mouth-form ratio. Taken together, we recommend the protocol used in this study as a general guideline for future studies to control for culture history and population density of source plates, mating status of mothers, the number of mothers used to start an experiment, and the stage of isolated nematodes.

A correlation between the phenotypes of mothers and sons in a genetically identical background is an intriguing result to explain. In an early study of the *Pristionchus* dimorphism, Hirschmann (1951) set up various crosses by parental mouth-form to observe, among other variables, the mouth forms of the offspring. However, because of the irregular complexity of the sampling and experimental scheme in that study, we could not interpret a similar correlation from her results. It is possible that paternal phenotype also has an additional influence on the offspring phenotype, although the scarceness of eurystomatous males prevented our testing this idea. The correlation we observed between mothers and sons could be due to hormonal cues encountered in utero or perhaps some signaling input inherited through the germline. The operation of cross-generational epigenetic effects (Greer et al. 2011; Johnson and Spence 2011; Rechavi et al. 2011; Shirayama et al. 2012) in specifying dimorphism phenotypes is an interesting possibility to test.

Conspecific cues post-embryonically influence adult morphology

Separating individuals of *P. pacificus* from their siblings showed that the presence of a population influences the developmental switch within a single generation. This is consistent with findings that "pheromone" purified from dauer-conditioned medium can influence the decision (Bento et al. 2010), but it reveals the activity of cues even when nematodes are well fed and in the absence of stress-induced dauers. Besides pheromonal cues, the introduction of mechanical cues by handling nematodes was also possible. Earlier juvenile stages are more susceptible to trauma, and so this could hypothetically translate to an influence on the developmental decision. However, the normal development to adulthood of almost all individuals, the stenostomatous of which generally develop even faster (Fig. 5), makes this effect unlikely. Furthermore, isolation of different stages showed that the decision could be altered at least as late as the J3 stage. The continuous response indicates that external information can be decreasingly incorporated into developmental regulation networks until the final morphology is executed, as known for cell-fate plasticity in nematode vulva development (Sternberg 2005).

Feeding plasticity differences in an ecological context

Variability in a feeding dimorphism has direct consequences for exploiting an ecological niche. *Pristionchus* species lead a necromenic lifestyle: they are found on beetles and other insects, and upon the death of the carrier they resume development from the dispersal (dauer) stage to proliferate on the host carcass (Herrmann et al. 2006a, b, 2007; Rae et al. 2008; D'Anna and Sommer 2011). This rapidly changing environment should in principle elicit benefits of one form over the other at different stages of change. Natural food sources include numerous types of bacteria (Rae et al. 2008; Weller et al. 2010) and presumably also fungi and other nematode colonizers (Yeates et al. 1993). If the eurystomatous form is, as assumed, a better predator than the stenostomatous form, a density-dependent switch to this form could represent a resource polyphenism in response to signals of increased competition for dwindling microbial resources (Kiontke and Fitch 2010). In this case, an opportunistic switch to a predatory form would enable predation of nematode competitors, possibly including conspecifics, as observed in anuran tadpoles (Pfennig 1990). Given form-specific feeding differences, the sexual differences in the mouth dimorphism in a population could affect the partitioning of resources among conspecifics, possibly leading to an ecological selection for the sexual dimorphism (Shine 1989). Assuming heritability of relevant loci in wild populations, any selection differentials in the dimorphism trait would, therefore, be predicted by theory to result in population divergence under the appropriate selection regime (West-Eberhard 2003). Further work to determine precise feeding differences between the two forms will be crucial for testing functional and evolutionary consequences of the dimorphism in a real ecological setting.

A developmental trade-off?

When given an abundant bacterial food supply, stenostomatous individuals of *P. pacificus* reached the stage of reproductive maturity in less time than eurystomatous individuals. This is the first evidence for a competitive advantage of the stenostomatous form. Because the eurystomatous form can access all known food sources as the stenostomatous form, and presumably more, benefits to retaining the stenostomatous form in evolution were

previously not obvious. A higher feeding efficiency of the stenostomatous form under some conditions could be supposed, although this remains to be tested. Although the stenostomatous form is less complex in its feeding morphology, differential metabolic costs of producing either form can for now only be predicted. However, if present, they could constitute a trade-off in time to maturity versus dietary breadth. Such a trade-off is supported by a difference in the duration of the J4 stage and final molt. Because the final molt is the point at which a discernibly dimorphic morphology is produced, we hypothesize that more time is needed for the organization or secretion of complex eurystomatous mouthparts. Considering the short and otherwise consistently timed life cycle of *P. pacificus*, any real difference in maturation time could theoretically be acted upon by selection. Although both forms grow well on bacteria, it is possible that a difference in developmental time would be exaggerated under more discriminating conditions. Studying the fitness consequences of a particular form on a wider array of food sources and other niche parameters will reveal whether any such trade-offs are plausible and could confer selective advantages.

A model for linking developmental plasticity to micro- and macro-evolution

Establishing a baseline understanding of the mouth dimorphism in *P. pacificus* provides a necessary reference point for comparative analysis. Anchored by a well-characterized reference strain, studies can be expanded into a population genetic context. For example, the collection of hundreds of distinct haplotypes from around the world (Herrmann et al. 2010; Morgan et al. 2012) has enabled a thorough screen for natural variation of the dimorphism, including wild strains highly biased toward either form (Ragsdale, Müller et al., unpublished data). Moreover, the laboratory availability and resolved phylogeny for some 30 new and described species of *Pristionchus* (Mayer et al. 2007, 2009), including a recently discovered cryptic species complex with *P. pacificus* (Kanzaki et al. 2012), will allow macroevolutionary studies of the plasticity. In such a framework, insight gleaned from genetic analyses in one strain of *P. pacificus* could be applied to testing genetic mechanisms at multiple tiers of evolution. An ultimate question to be addressed regard the origin of the novel morphology itself, particularly the teeth that are the hallmark of the eurystomatous form. Whether the discrete forms are the result of canalization from a continuum (Emlen and Nijhout 2000; Nijhout 2003) or the build-up of cryptic genetic variation by "developmental capacitance" (Moczek 2007) is still the subject of speculation, but the advent of *Pristionchus* and Diplogastridae as a model for plasticity and evolution promises exciting opportunities to put theory to the test.

ACKNOWLEDGMENTS

We thank Dr. Cameron Weadick and Vladislav Susoy for helpful criticism and suggestions. We gratefully acknowledge the Alexander von Humboldt Foundation for the support of a fellowship to E. J. R.

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