

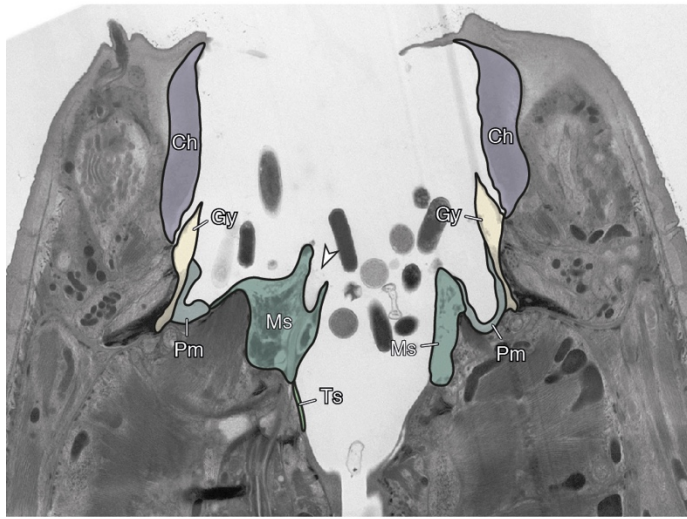
In the format provided by the authors and unedited.

Geometric morphometrics of microscopic animals as exemplified by model nematodes

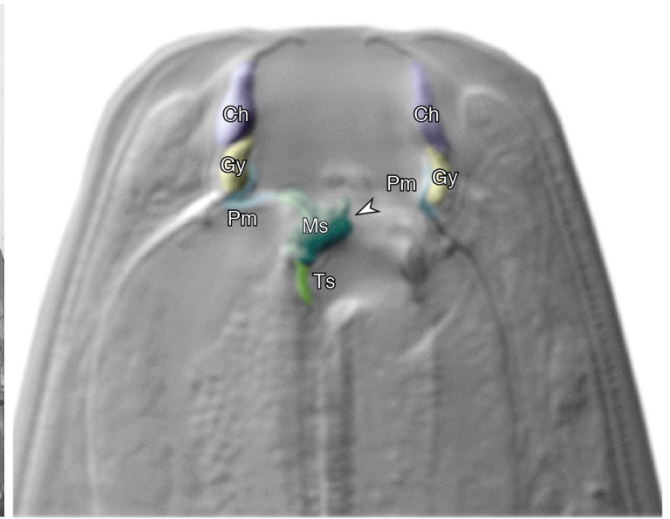
Tobias Theska¹ , Bogdan Sieriebriennikov^{1,2} , Sara S. Wighard¹ , Michael S. Werner¹   and Ralf J. Sommer¹  

¹Department for Integrative Evolutionary Biology, Max Planck Institute for Developmental Biology, Tübingen, Germany. ²Present address: Department of Biology, New York University, New York, NY, USA. e-mail: michael.werner@tuebingen.mpg.de; ralf.sommer@tuebingen.mpg.de

a TEM section (sagittal) of eurystomatous morph

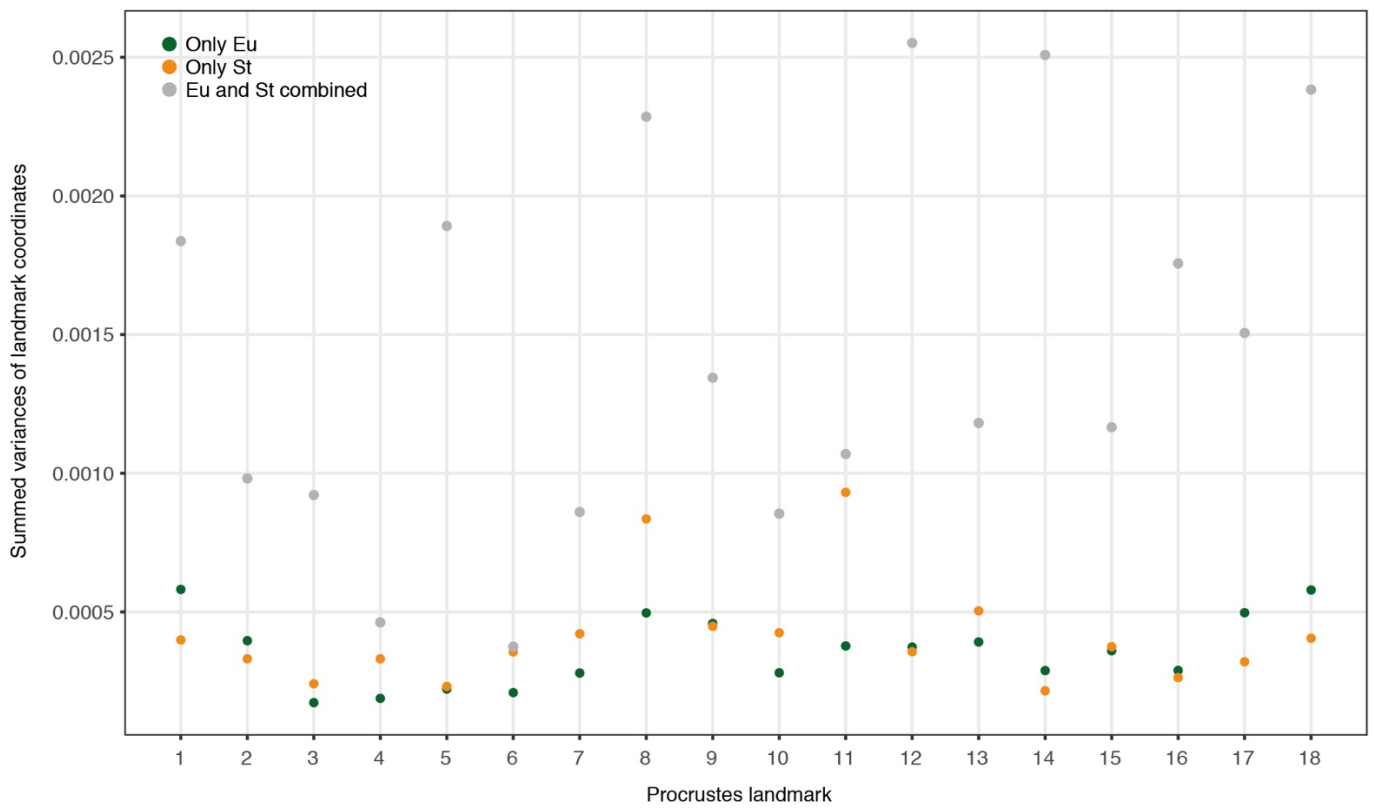


b Microscopic image (DIC) of eurystomatous morph

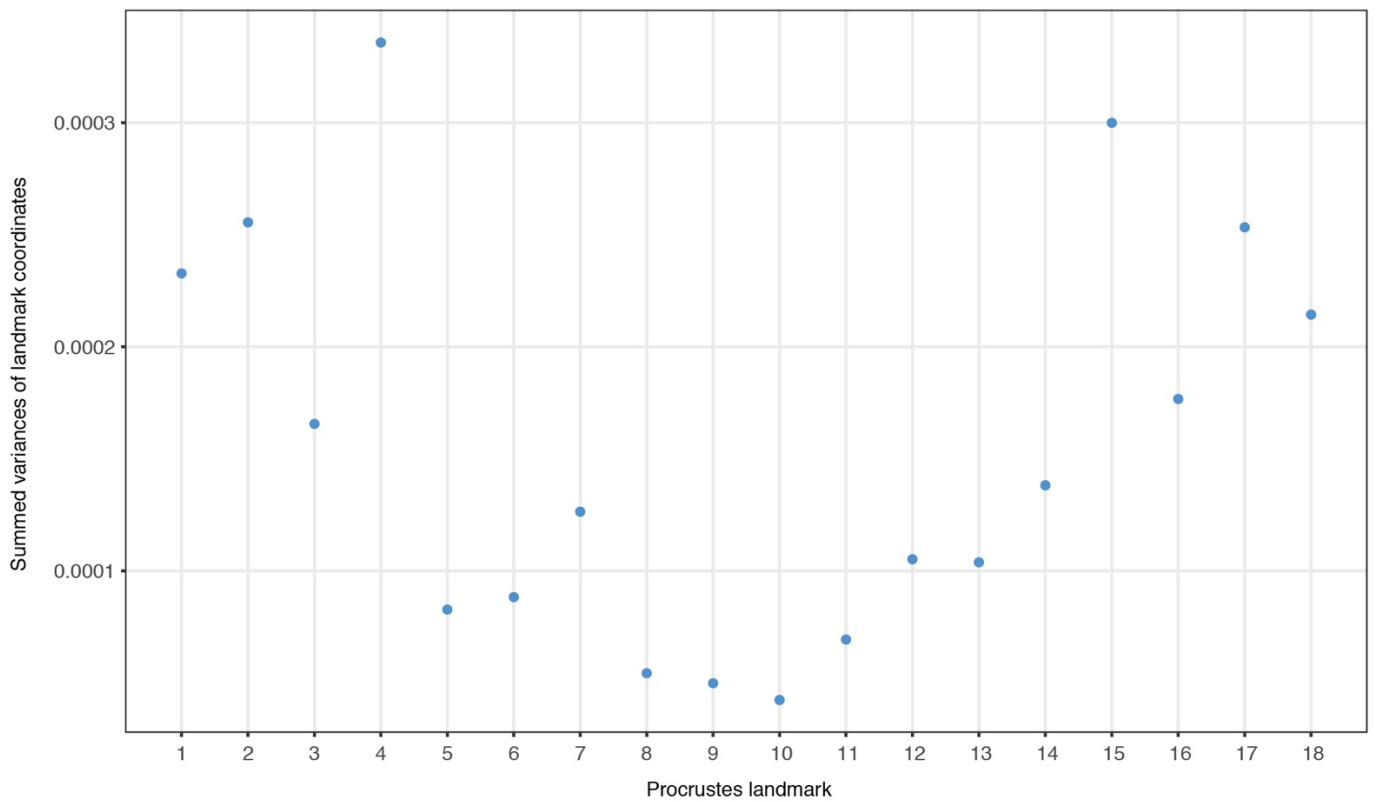


Supplementary figure 1. Comparison of the eurystomatous morph of *P. pacificus* under TEM and DIC microscope. **a**, TEM image of the stoma. **b**, Light microscope (DIC) image of the stoma (100x). Both images show the sagittal plane in right lateral view. Arrowheads indicate the position of the dorsal gland opening. ch, cheilostom; gy, gymnostom; ms, metastegostom; pm, promesostegostom; ts, telostegostom.

a Summed variances for each landmark in *P. pacificus*

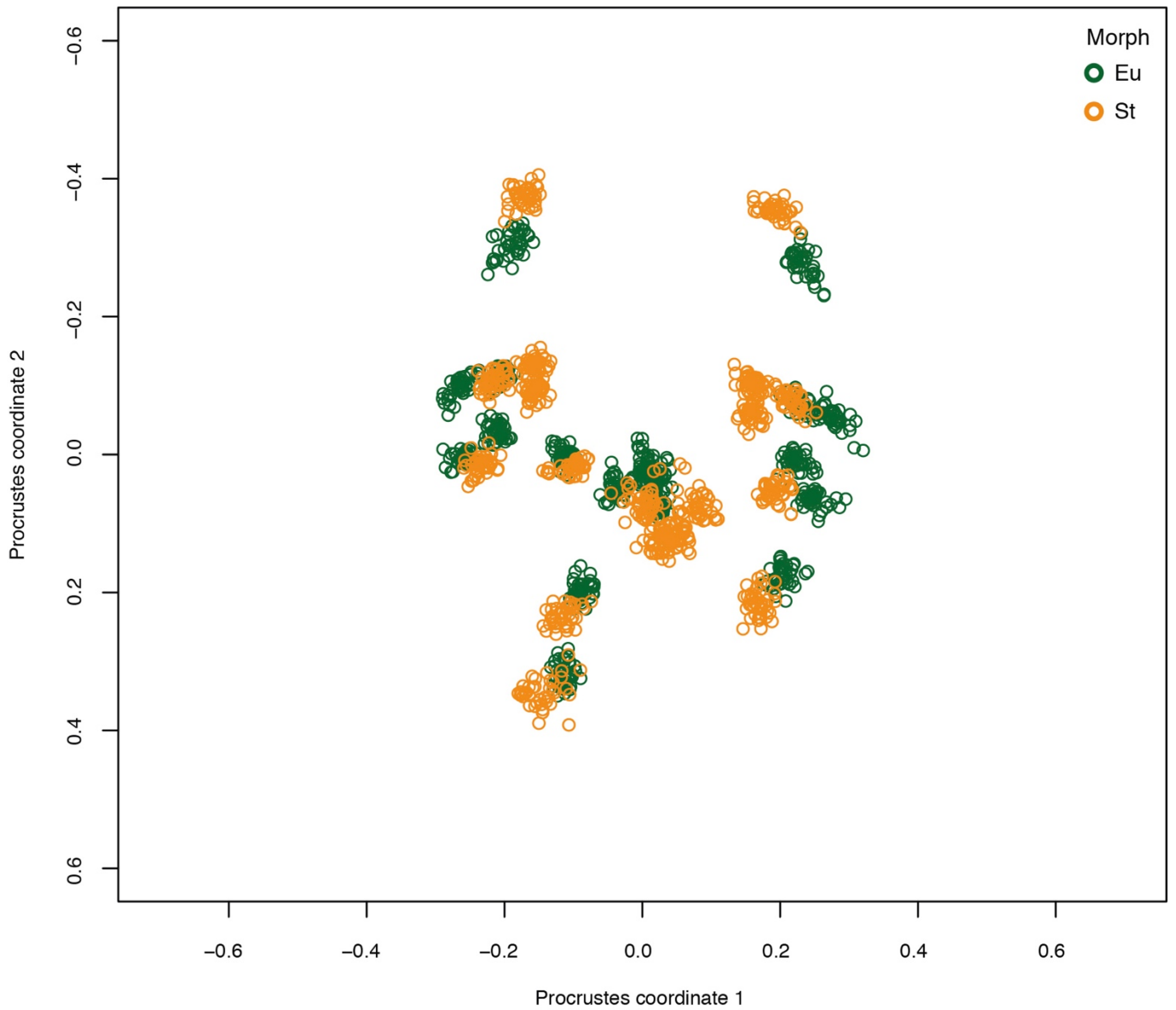


b Summed variances for each landmark in *C. elegans*



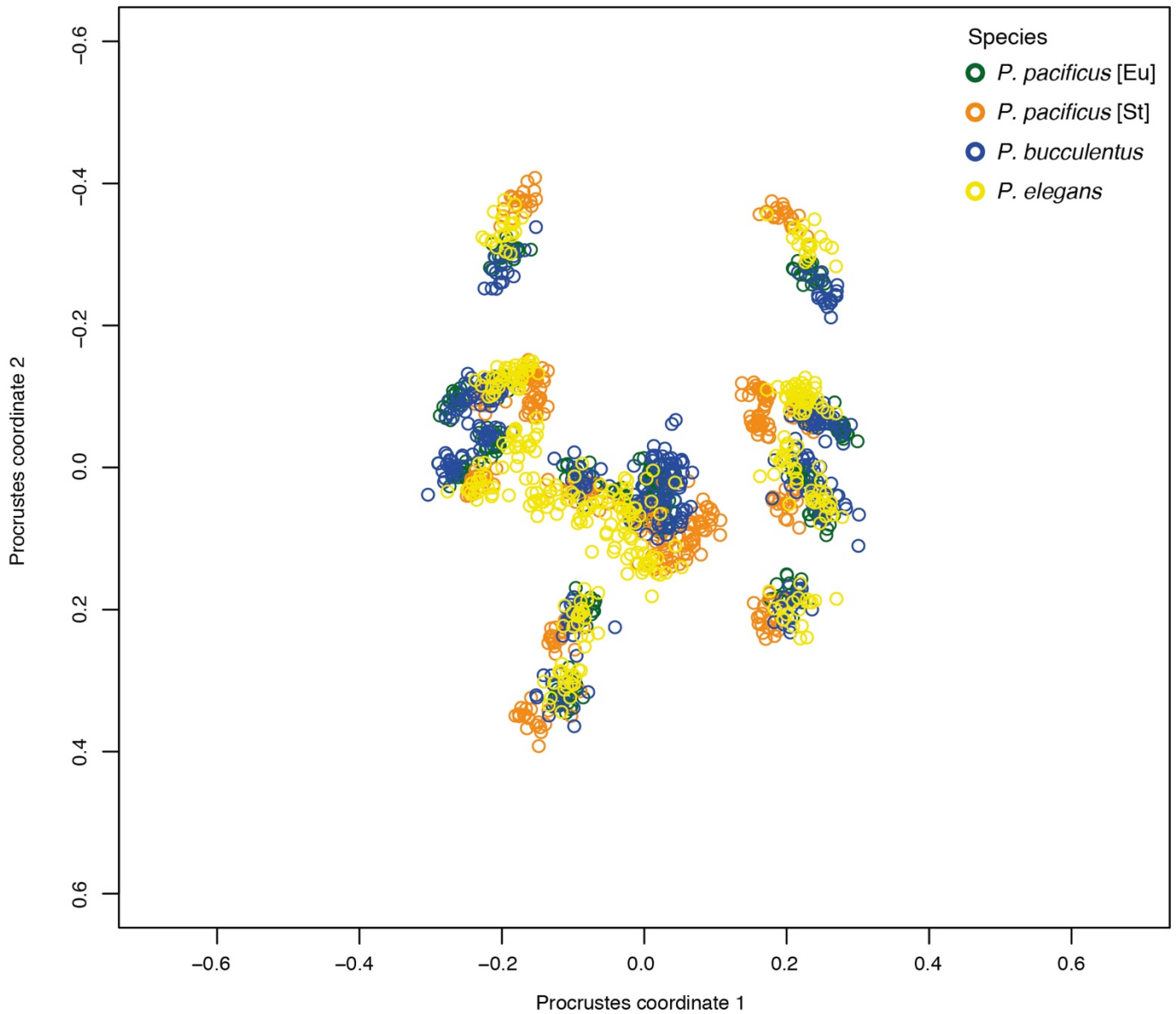
Supplementary figure 2. Variances of single landmarks for *P. pacificus* and *C. elegans*. Total variances for each landmark were calculated by summing up the variance in both coordinates of a respective landmark. Single coordinate variances were calculated using the **var()** function of the R statistics package. Note that the *Procrustes coordinates*, and thus also their variances, are dimensionless. **a**, Total variance of single landmarks for each morph (Eu and St) of *P. pacificus*, as well as total variance of single landmarks across all specimens regardless of the morph. **b**, Total variance of single landmarks for all *C. elegans* specimens. **a, b**, Smaller variances indicate a robust landmark positioning. Larger variances can indicate either inherently noisy landmarks (i.e. a high degree of naturally occurring variation in the structure), or biologically meaningful differences between groups. For example: Landmark “5” shows very small variances within each of the two morphs of *P. pacificus*, which indicates that it can be found reliably within each morph. However, the variance in landmark position is very high if all individuals (i.e. Eu and St combined) are used for estimation. This indicates that the position of landmark “5”, relative to all other landmarks of the configuration, is very different between the morphs and thus potentially relevant in explaining biological shape differences. On the other hand, the high variance of landmark “11” across all *P. pacificus* specimens is due to the slightly increased variance of this landmark in stenostomatous animals (St) as compared to the low variance observed for eurytostomatous animals (Eu). Thus, this landmark might be less reliable for the identification of shape differences between morphs. Note that landmarks which are jointly placed on movable elements (e.g. landmark 6, 7, 8, 9 & 10 in *P. pacificus*) do not show systematically increased variances as opposed to landmarks which are placed on non-movable elements (e.g. 1-4 and 15-18 in *P. pacificus*). This indicates that landmarks on movable and non-movable elements can be treated equally for downstream analysis.

Aligned Procrustes landmarks of all individuals of *P. pacificus* (stomatal polyphenism)



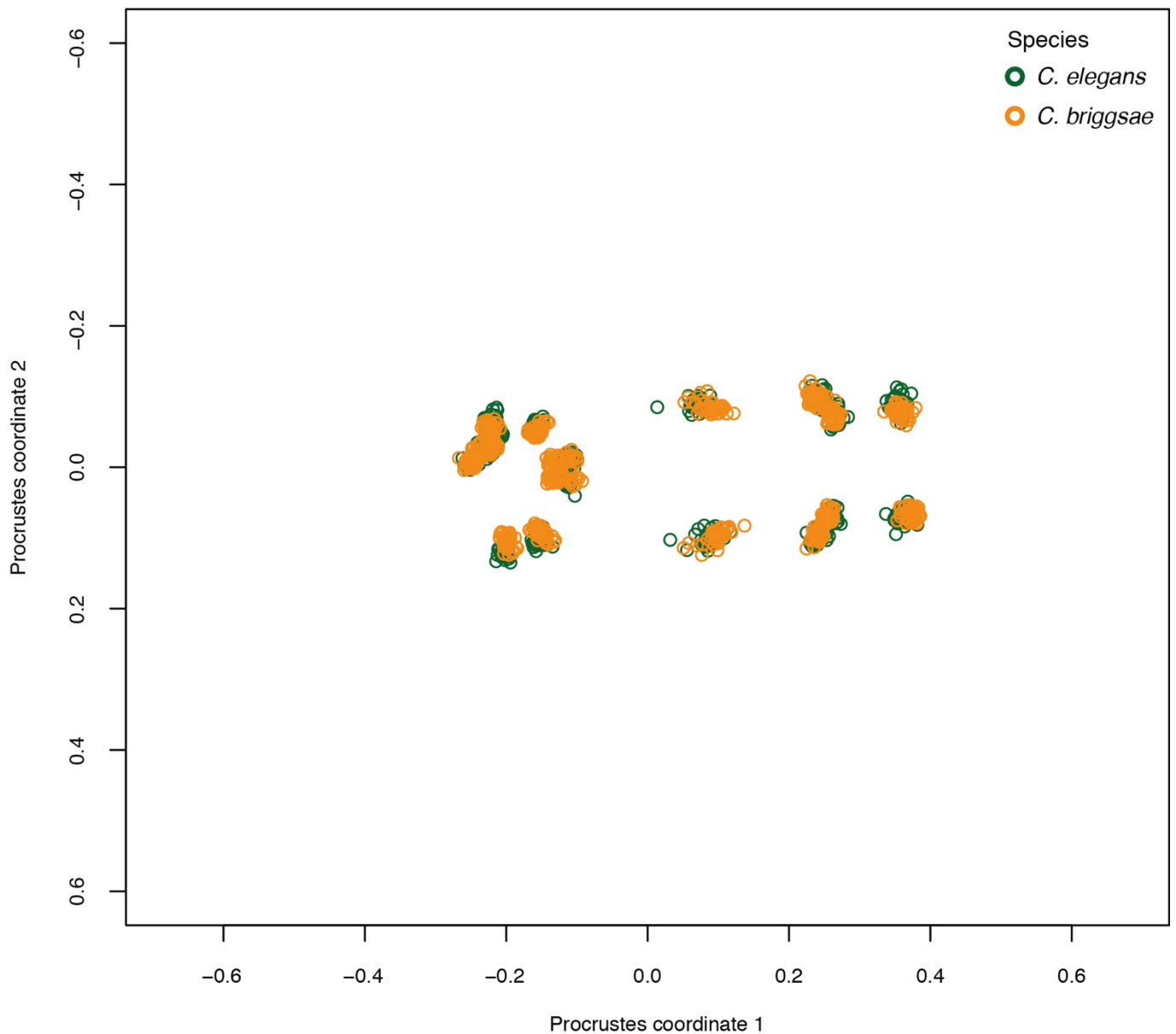
Supplementary figure 3. Scatterplot of Procrustes aligned coordinates obtained for the *P. pacificus* dataset (including both morphs). Generalized Procrustes analysis (GPA) was carried out using the **gpagen()** function of the *geomorph* package (see step 20 of the Procedure). *Procrustes coordinates* are projected into a linear (euclidean) tangent space. Semilandmarks were slid by minimizing bending energy. Output image was flipped horizontally, to depict aligned shapes in the original (right lateral) orientation.

Aligned Procrustes landmarks of all individuals in the interspecific comparison (*Pristionchus* sp.)

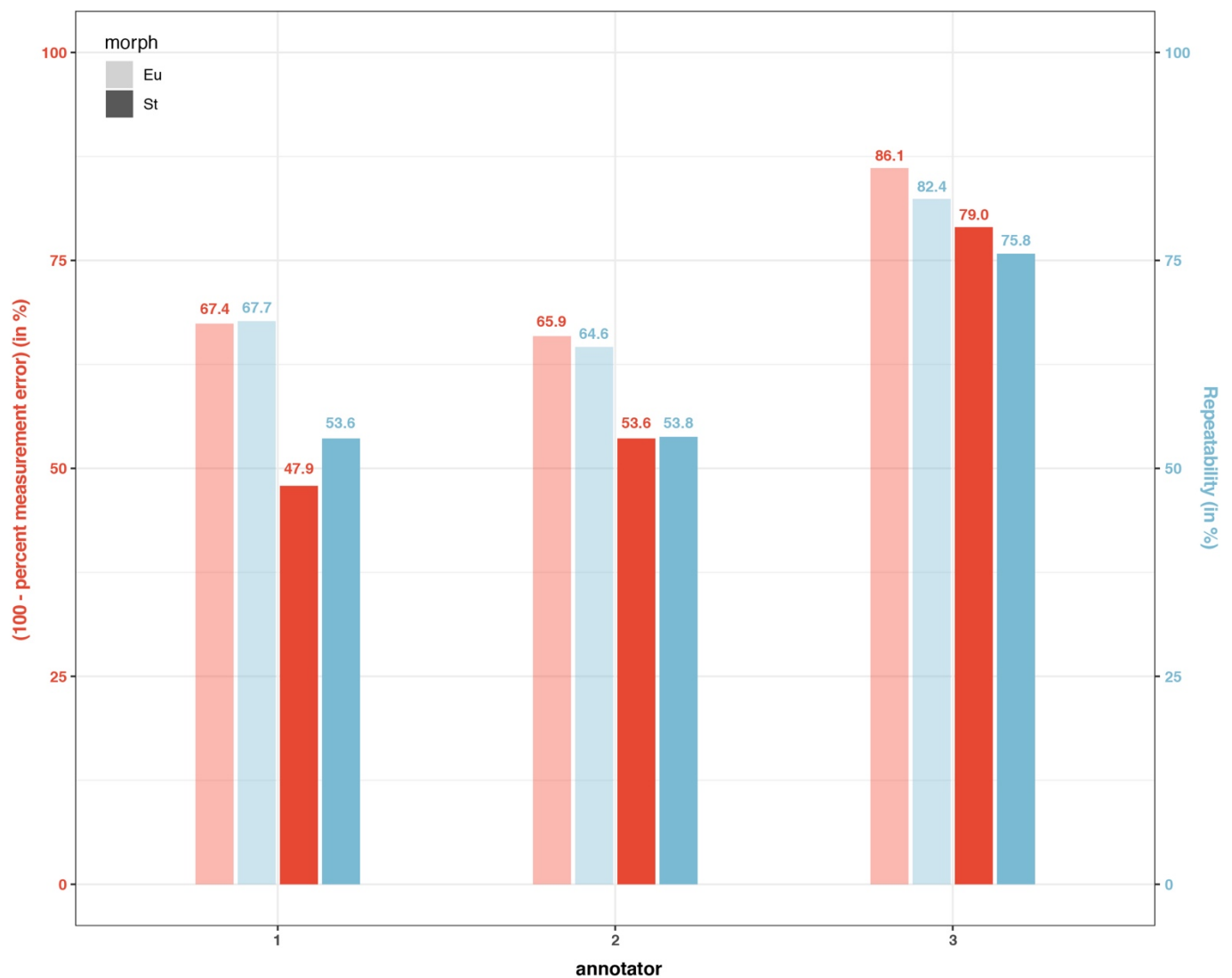


Supplementary figure 4. Scatterplot of Procrustes aligned coordinates obtained for the *Pristionchus* dataset (including *P. pacificus*, *P. bucculentus* and *P. elegans*). Generalized Procrustes analysis (GPA) was carried out using the **gpagen()** function of the *geomorph* package (see step 20 of the Procedure). *Procrustes* coordinates are projected into a linear (euclidean) tangent space. Semilandmarks were slid by minimizing bending energy. Output image was flipped horizontally, to depict aligned shapes in the original (right lateral) orientation.

Aligned Procrustes landmarks of all individuals of *C. elegans* and *C. briggsae*



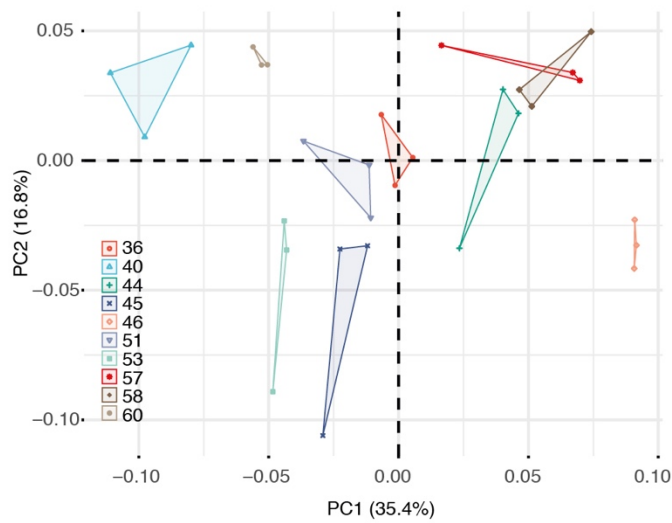
Supplementary figure 5. Scatterplot of Procrustes aligned coordinates obtained for the *Caenorhabditis* dataset (including *C. elegans* and *C. briggsae*). Generalized Procrustes analysis (GPA) was carried out using the **gpagen()** function of the *geomorph* package (see step 20 of the Procedure). *Procrustes coordinates* are projected into a linear (euclidean) tangent space. Output image was flipped horizontally, to depict aligned shapes in the original (right lateral) orientation.



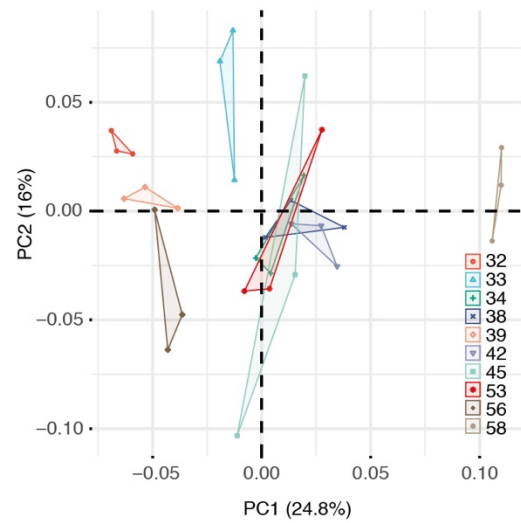
Supplementary figure 6. Replicability performance of annotators with different levels of training. Repeatability (i.e. the amount of variation between individuals) and percent measurement error (i.e. the amount of variation within individuals/among replicates) were calculated for shapes using the code in Box 2 of the main text. Annotators 1 and 2 are less trained in the procedure of landmark placement, while annotator 3 is familiar with the procedure. Color coding in y-axis corresponds to color coding of bars. Red, percent measurement error (%ME); blue, repeatability (RPT). Light bars indicate the values obtained for Eu, and dark bars indicate those obtained for St animals. Abbreviations: Eu, eurytomatous and St, stenostomatous morph of *P. pacificus*. Superimposition was performed using the **gpagen()** function of *geomorph* (see step 20 of the Procedure). Semilandmarks were slid by minimizing bending energy. For repeatability estimation, the number of parametric bootstraps and permutations was set to 1,000. In all cases (across morphs and annotator), when estimating the %ME of shapes, the specimen factor was significant while the replicate factor was not. This means that the variation between worms was always

stronger than the variation across technical replicates of the same individual. Statistics (PERMANOVA on shape) for annotator-1: ME[Eu] = 32.6%, ME[St] = 52.1%. Specimen factor [Eu], $F = 7.2156$, effect size (Z) = 8.7559, $P < 0.001$. Replicate factor [Eu], $F = 0.4828$, effect size (Z) = -1.6918, $P = 0.96$. Specimen factor [St], $F = 3.7629$, effect size (Z) = 7.5127, $P < 0.001$. Replicate factor [St], $F = 0.7633$, effect size (Z) = -0.7179, $P = 0.76$. Statistics (PERMANOVA on shape) for annotator-2: ME[Eu] = 34.1%, ME[St] = 46.4%. Specimen factor [Eu], $F = 6.8098$, effect size (Z) = 7.4885, $P < 0.001$. Replicate factor [Eu], $F = 0.7233$, effect size (Z) = -0.63636, $P = 0.72$. Specimen factor [St], $F = 4.465$, effect size (Z) = 7.4693, $P < 0.001$; Replicate factor [St], $F = 0.6583$, effect size (Z) = -1.0927, $P = 0.88$. Statistics (PERMANOVA on shape) for annotator-3: ME[Eu] = 13.9%, ME[St] = 21.0%. Specimen factor [Eu], $F = 19.647$, effect size (Z) = 9.2233, $P < 0.001$; Replicate factor [Eu], $F = 0.1969$, effect size (Z) = -2.9198, $P = 0.999$. Specimen factor [St], $F = 12.286$, effect size (Z) = 11.612, $P < 0.001$; Replicate factor [St], $F = 0.3595$, effect size (Z) = -2.5964, $P = 0.995$.

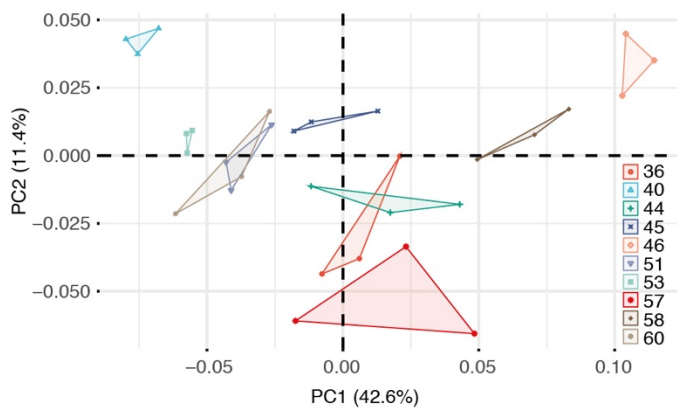
a PCA of *P. pacificus* [Eu] replicates - annotator 1



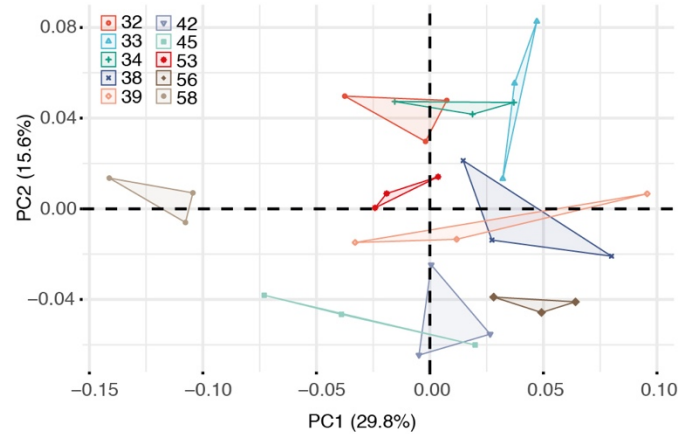
d PCA of *P. pacificus* [St] replicates - annotator 1



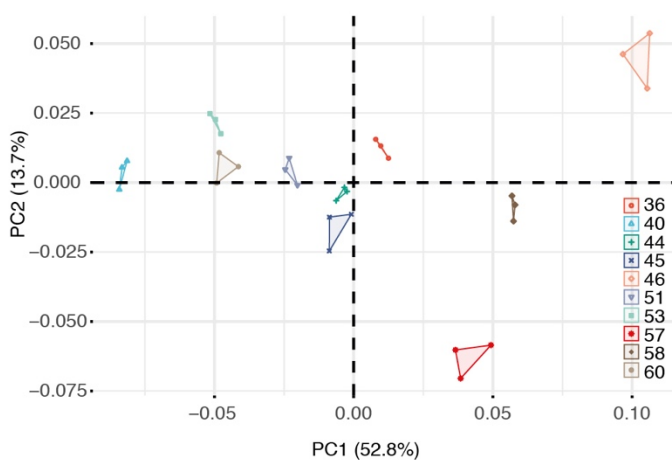
b PCA of *P. pacificus* [Eu] replicates - annotator 2



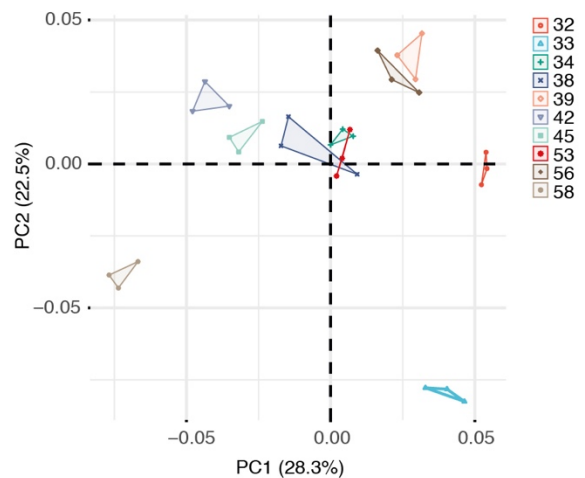
e PCA of *P. pacificus* [St] replicates - annotator 2



c PCA of *P. pacificus* [Eu] replicates - annotator 3



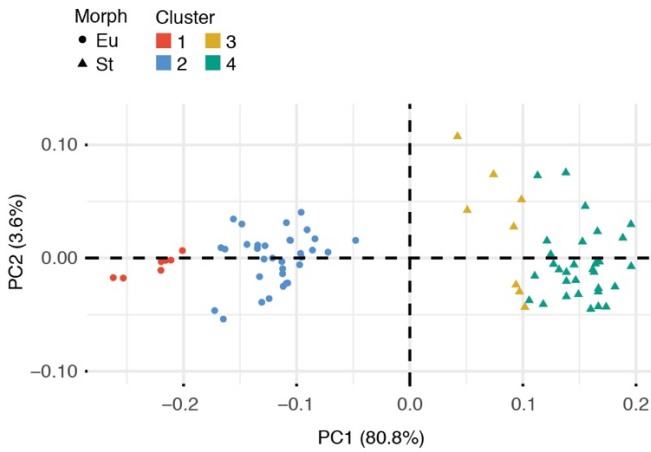
f PCA of *P. pacificus* [St] replicates - annotator 3



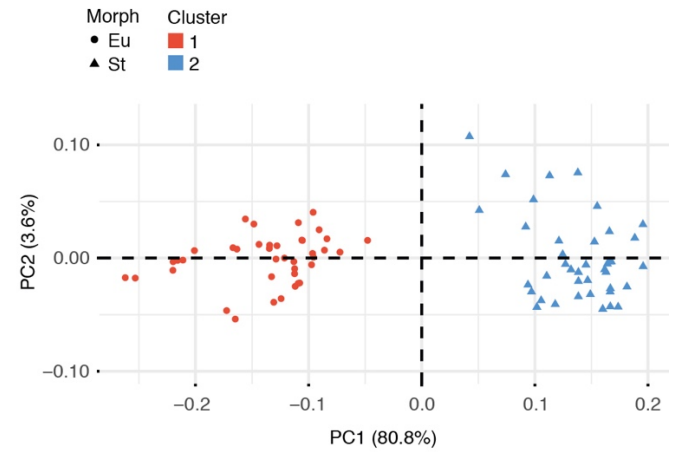
Supplementary figure 7. Annotator-specific distribution of technical replicates (stomatal shapes) in the shape tangent space. Each individual was labeled three times independently by three annotators of

different levels of training in landmark placement. Numbers in the legends refer to identifiers of the ten eury stomatous (a-c) or ten stenostomatous (d-f) specimens (i.e. the biological replicates of each morph). Generalized Procrustes analysis (GPA) was carried out using the **gpagen()** function of the *geomorph* package (see step 20 of the Procedure). *Procrustes coordinates* are projected into a linear (euclidean) tangent space. Semilandmarks were slid by minimizing bending energy. PCA was performed according to step 21 of the Procedure. Note that specimen ID numbers are *not* identical between eury stomatous (a-c) and stenostomatous (d-f) individuals (e.g. '58' refers to two different animals in a-c versus d-f).

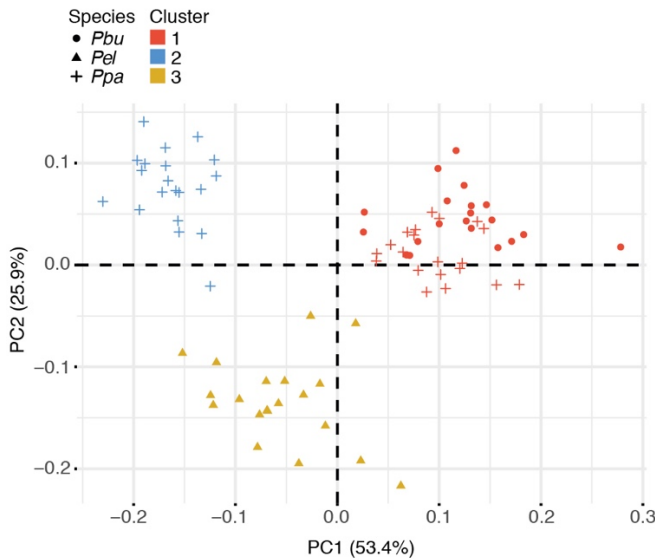
a Stomatal polyphenism in *P. pacificus* (mPCs)



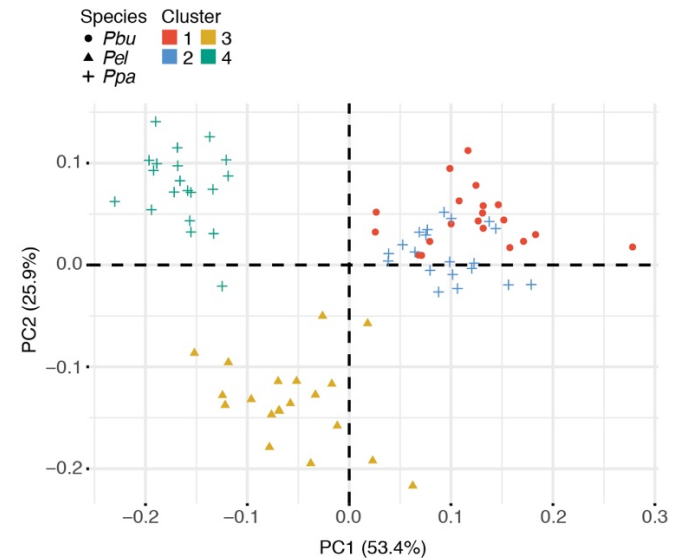
b Stomatal polyphenism in *P. pacificus* (PCs > 1%)



c Stomata of different *Pristionchus* species (mPCs)

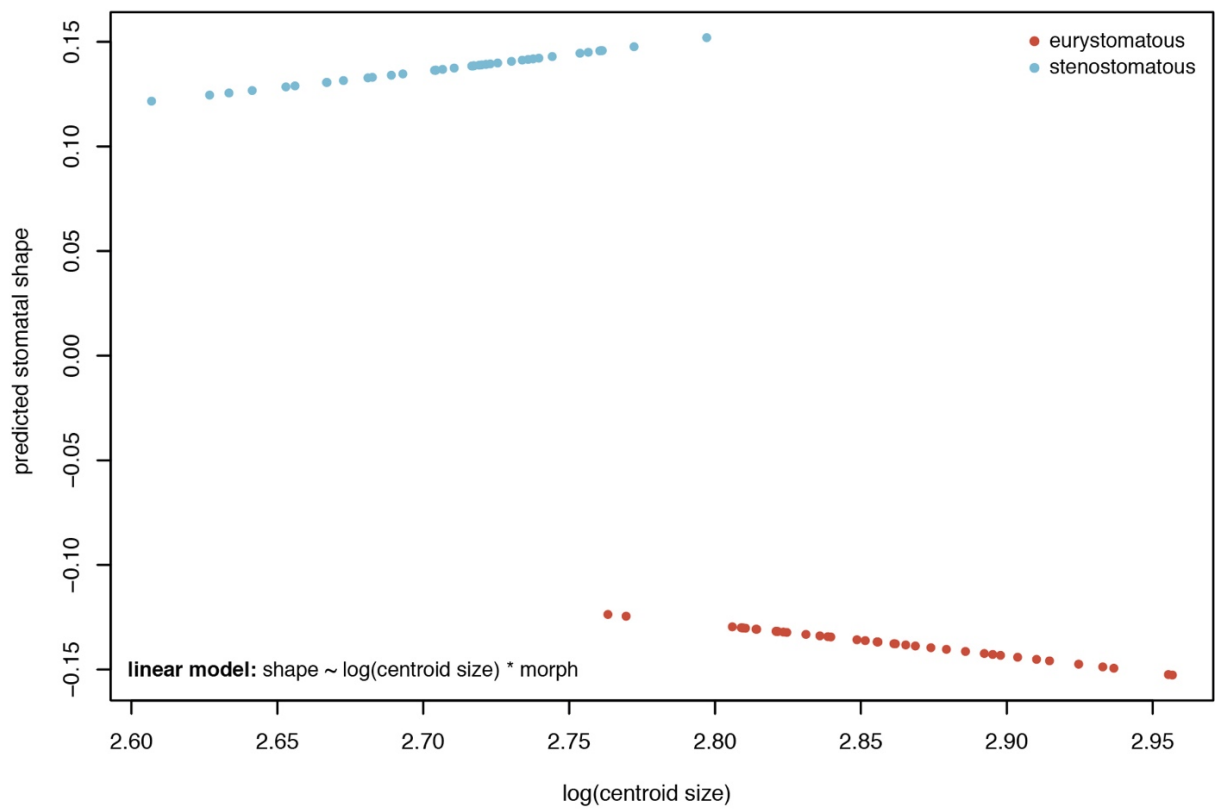


d Stomata of different *Pristionchus* species (PCs > 1%)

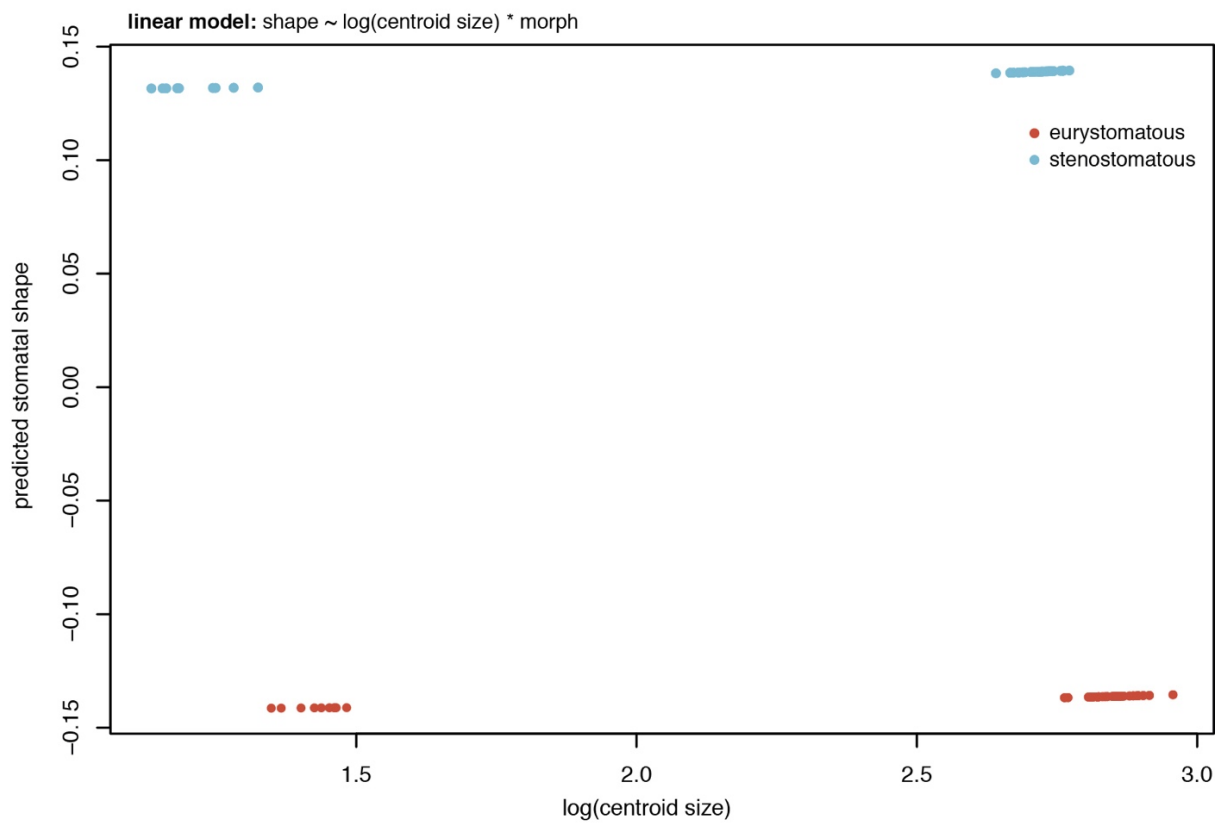


Supplementary figure 8. model-based clustering performed on the two *Pristionchus* datasets using two different approaches for PC inclusion. **a**, Clusters identified for the *P. pacificus* dataset by using only meaningful PCs (mPCs) as an input. **b**, Clusters identified for the *P. pacificus* dataset by including all PCs which describe at least 1% variation (PCs>1%) as an input. **c**, Clusters identified for the *Pristionchus* species dataset by using only meaningful PCs (mPCs) as an input. **d**, Clusters identified for the *Pristionchus* species dataset by including all PCs which describe at least 1% variation (PCs>1%) as an input. Abbreviations: Eu, eury stomatous morph; St, stenostomatous morph; *Pbu*, *P. bucculentus*; *Pel*, *P. elegans*; *Ppa*, *P. pacificus*. Meaningful PCs are estimated by the `getMeaningfulPCs()` function of the *Morpho* package.

a Allometric trajectories of the two different stomatal morphs in *P. pacificus*

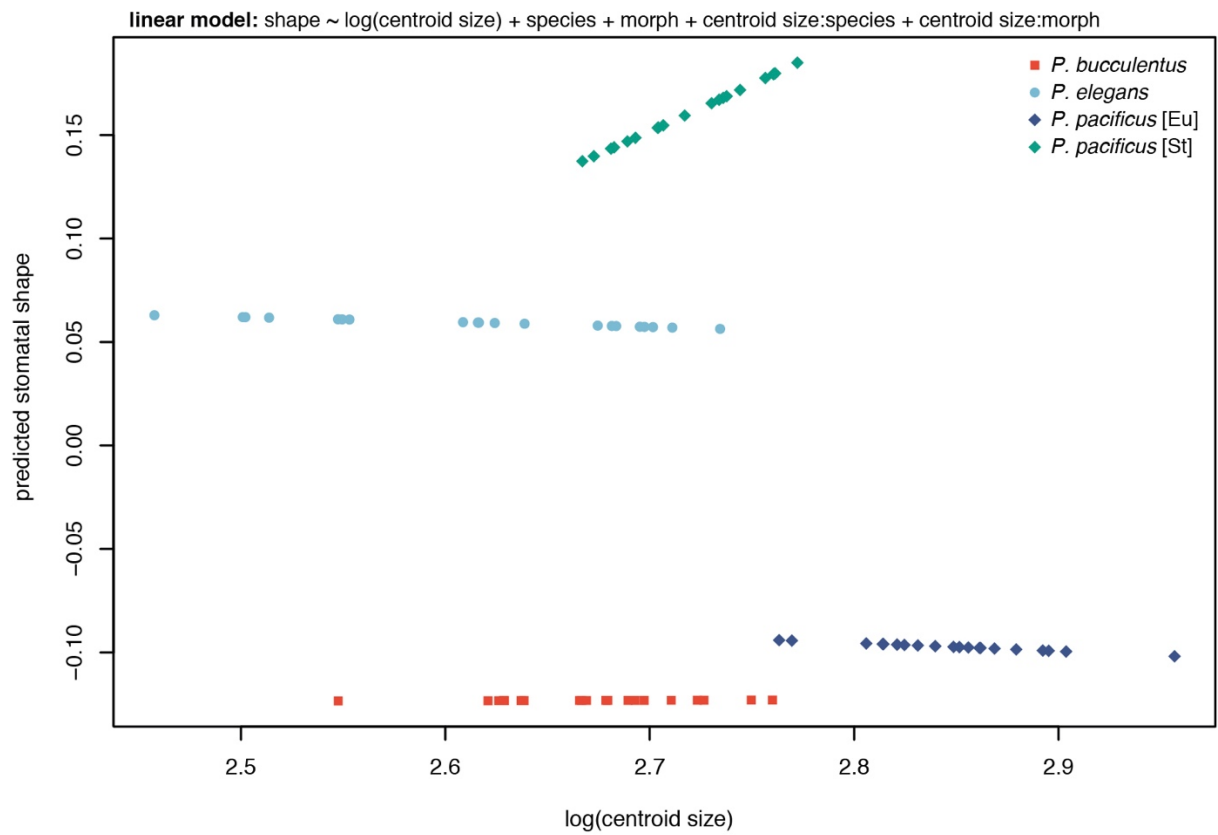


b Allometric trajectories for the two morphs of *P. pacificus* based on uncorrected centroid sizes

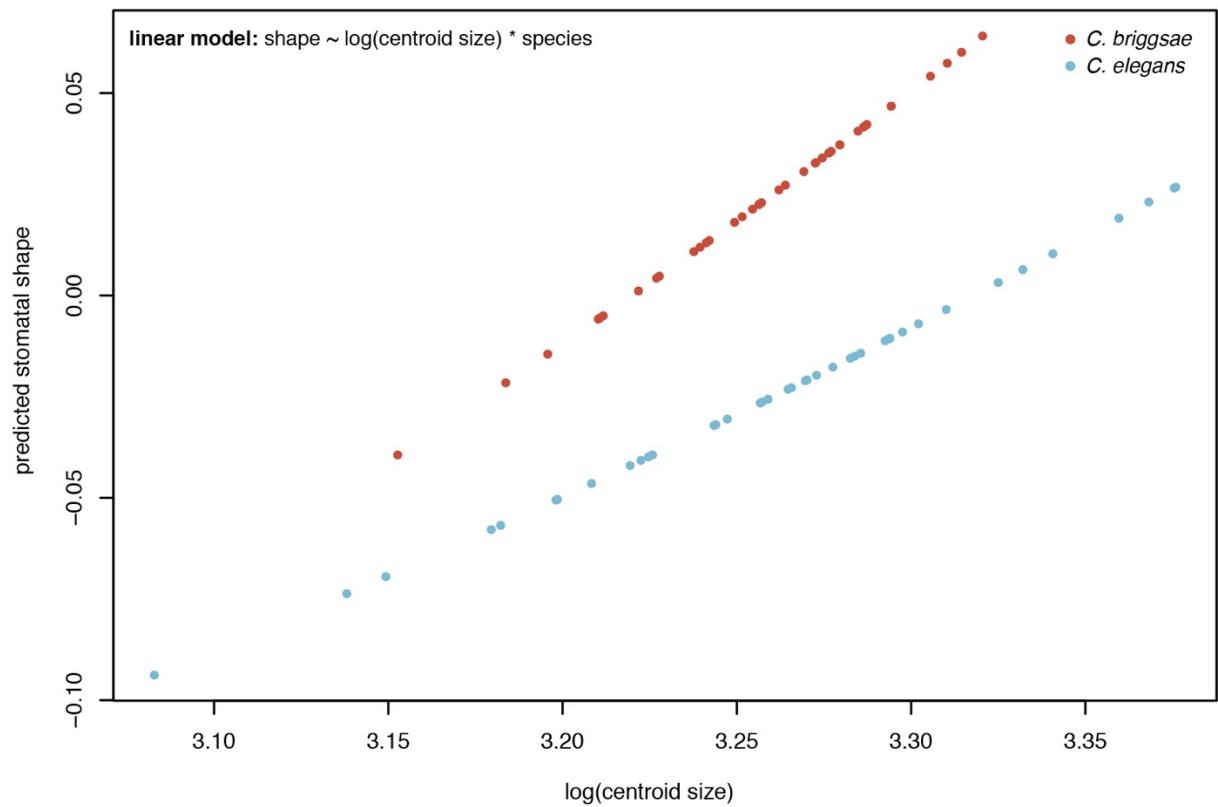


Supplementary figure 9. Allometry plots for young adults of the two *P. pacificus* morphs. **a**, Morph-specific (static) allometric trajectories after troubleshooting (see main text) differences in centroid size due to inconsistent image stack scaling (inches versus microns). **b**, Morph-specific (static) allometric trajectories before troubleshooting centroid sizes (note that individuals whose centroid size was in inches separated from their kin towards the left of the x-axis). Predicted stomatal shapes on the y-axis represent the PC1 scores of the PCA that was performed on the predicted values from multivariate regression on shape versus log(centroid size). Superimposition was performed using the **gpagen()** function of *geomorph* (see step 20 of the Procedure). Note that, while each morph shows static allometry, the differences between morphs are not manifestations of allometric scaling as they do not share a common trajectory. Linear models are indicated in the panels. Statistics on shape (PERMANCOVA): size, $F = 165.8679$, effect size (Z) = 5.7801, $P < 0.0001$. morph, $F = 59.6372$, effect size (Z) = 6.1827, $P < 0.0001$. size x morph, $F = 1.7444$, effect size (Z) = 1.356, $P = 0.097$.

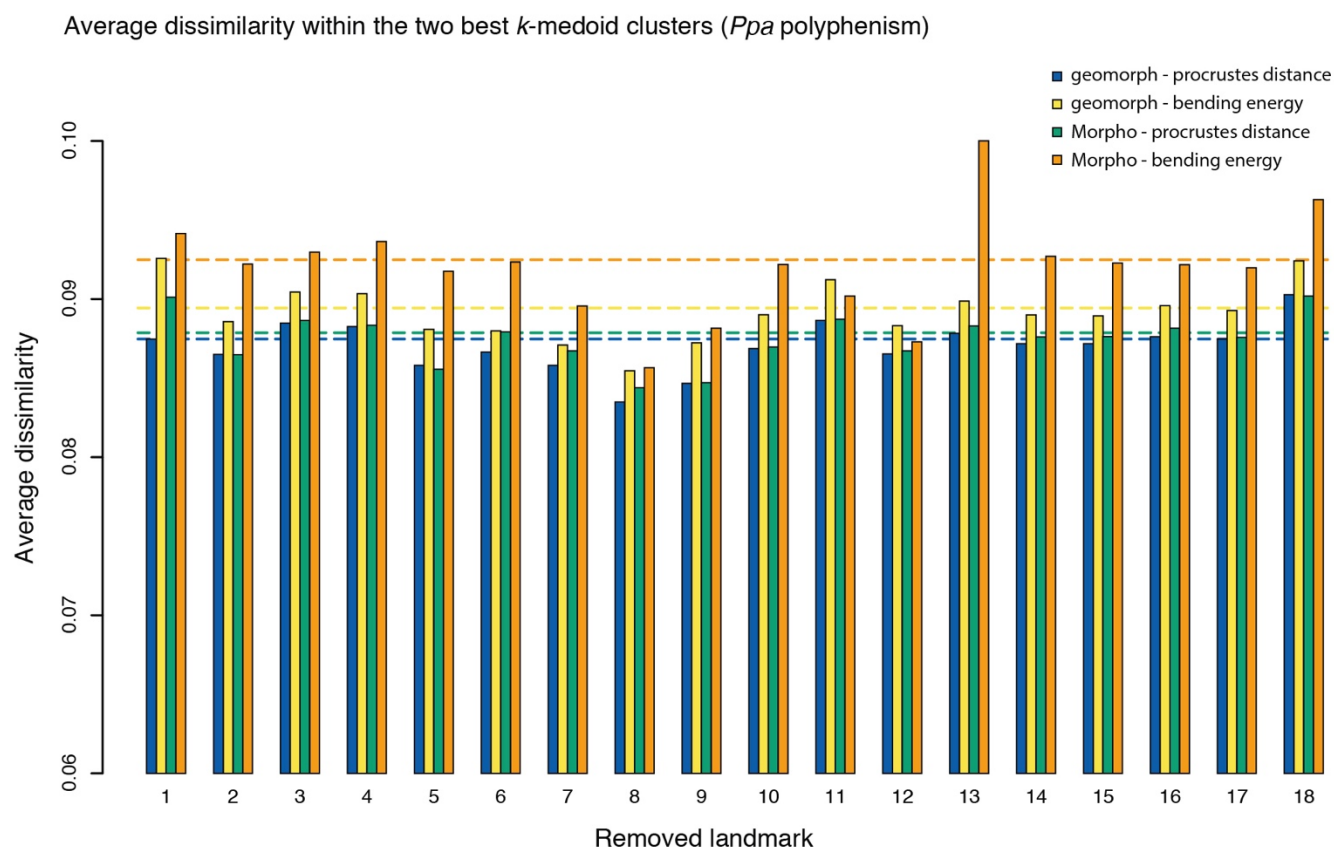
a Allometric trajectories of the stomata in various *Pristionchus* species



b Allometric trajectories of the stomata in *C. briggsae* and *C. elegans*

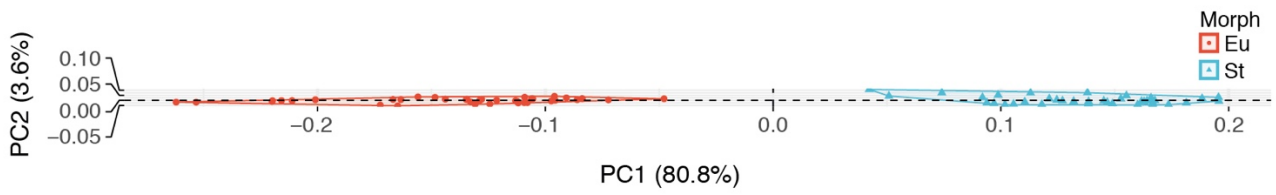


Supplementary figure 10. Static allometry trajectories estimated for young adults of **(a)** three *Pristionchus* species and **(b)** two *Caenorhabditis* species. Note that differences in centroid size due to inconsistent image stack scaling (inches versus microns) were corrected before plotting panel **a** (see ‘troubleshooting’ in the main text). Predicted stomatal shapes on the y-axis represent the PC1 scores of the PCA that was performed on the predicted values from multivariate regression on shape versus log(centroid size). Superimposition was performed using the **gpagen()** function of *geomorph* (see step 20 of the Procedure). Note that *P. elegans*, eury stomatous *P. pacificus* and *P. bucculentus* (**a**) show essentially isometric trendlines (i.e. shapes do not change with increasing body size), while the stenostomatous morph of *P. pacificus* shows a specific allometric trajectory. Both *Caenorhabditis* species (**b**) show strong allometric relationships in stomatal shape, indicative of increasing morphological divergence between species with growth. Abbreviations: Eu, eury stomatous and St, stenostomatous morph. Linear models are indicated in the panels. Statistics on shape (PERMANCOVA) for the *Pristionchus* data set **(a)**: size, $F = 20.2303$, effect size (Z) = 4.3164, $P < 0.0001$. species, $F = 62.7531$, effect size (Z) = 7.4549, $P < 0.0001$. morph, $F = 48.0781$, effect size (Z) = 6.2528, $P < 0.0001$. size x species, $F = 1.2129$, effect size (Z) = 0.7009, $P = 0.23831$. size x morph, $F = 2.1124$, effect size (Z) = 1.758, $P < 0.05$. Statistics on shape (PERMANCOVA) for the *Caenorhabditis* data set **(b)**: size, $F = 27.1836$, effect size (Z) = 5.1015, $P < 0.0001$. species, $F = 30.6839$, effect size (Z) = 6.4582, $P < 0.0001$. size x species, $F = 4.3322$, effect size (Z) = 3.7579, $P < 0.001$.

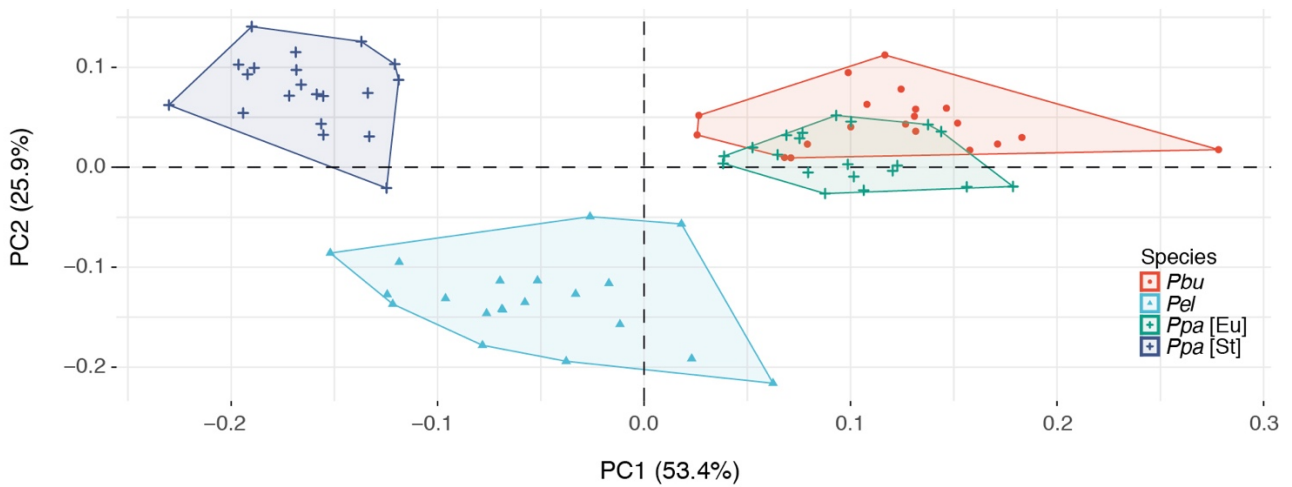


Supplementary figure 11. Change of average dissimilarity within the two-best k -medoid clusters upon single landmark removal in the *P. pacificus* dataset (including both morphs). Dotted lines represent the average dissimilarity of the best two k -medoid clusters in the data set, when no landmarks are removed (colors correspond to the package and sliding approach used to perform GPA). Note that the detected change for each landmark removal shows a similar direction, regardless of the package which was used for GPA (*geomorph* and *Morpho*) and regardless of the sliding approach that was chosen for GPA (minimization of Procrustes distances or bending energy). Changes of dissimilarity are generally subtle, indicating that the proposed landmark configuration is robustly able to quantify shape differences, even after single landmarks are removed.

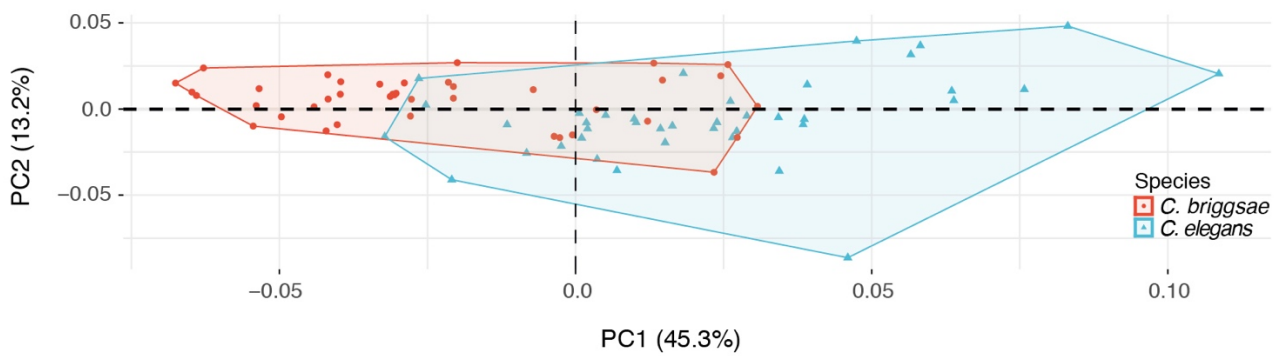
a Variation-weighted PCA plot showing the two different mouth-forms of *P. pacificus*



b Variation-weighted PCA plot showing stomatal shapes of various *Pristionchus* species



c Variation-weighted PCA plot showing stomatal shapes of two *Caenorhabditis* species



Supplementary figure 12. Variation-weighted PCA plots. Length of the y-axis is scaled relative to the x-axis, based on the variation described by PC1 and PC2. **a**, Comparison of two stomatal morphs in *Pristionchus pacificus*; **b**, Comparison of stomatal shapes across various *Pristionchus* species; **c**, Comparison of two *Caenorhabditis* species. Abbreviations: Eu, eurystomatous; St, stenostomatous; *Pbu*, *Pristionchus bucculentus*; *Pel*, *Pristionchus elegans*; *Ppa*, *Pristionchus pacificus*.

landmark	type	description
1	fixed	anteriormost point of cheilostom on the dorsal side
2	fixed	posteriormost point of cheilostom on the dorsal side
3	fixed	anteriormost point of gymnostom on the dorsal side
4	fixed	posteriormost point of gymnostom on the dorsal side
5	fixed	anteriormost point of promesostegostom on the dorsal side
6	sliding	most convex point on the dorsal outline of the dorsal tooth
7	sliding	most concave point on the dorsal outline of the dorsal tooth
8	fixed	anterior tip of the dorsal tooth
9	fixed	opening of the dorsal pharyngeal gland
10	fixed	posteriormost point of the metastegostom on dorsal side (i.e. base of dorsal tooth)
11	fixed	lateral base point of the right ventrosublateral tooth or ridge
12	sliding	anterior tip of the right ventrosublateral tooth or ridge
13	fixed	ventral base point of the right ventrosublateral tooth or ridge
14	fixed	anteriormost point of promesostegostom on the ventral side
15	fixed	posteriormost point of cheilostom on the ventral side
16	fixed	anteriormost point of gymnostom on the ventral side
17	fixed	posteriormost point of cheilostom on the ventral side
18	fixed	anteriormost point of cheilostom on the ventral side

Supplementary table 1. Description of landmarks for *Pristionchus*.

landmark	type	description
1	fixed	anteriormost point of cheilostom on the dorsal side
2	fixed	posteriormost point of cheilostom on the dorsal side (deepest point within the cheilostomal ridge)
3	fixed	anteriormost point of gymnostom on the dorsal side
4	fixed	border point of cheilo- and promesostegostom on the dorsal side (at the level of the pharyngeal sleeve tip)
5	fixed	posteriormost point of promesostegostom on the dorsal side
6	fixed	posterior base point of the dorsal flap
7	fixed	anterior tip of the dorsal flap
8	fixed	anteriormost point of the telostegostom on the dorsal side
9	fixed	opening of the dorsal pharyngeal gland
10	fixed	posteriormost point of the telostegostom on the dorsal side
11	fixed	lateral base point of the right ventrosublateral flap
12	fixed	anterior tip of the right ventrosublateral flap
13	fixed	ventral base point of the right ventrosublateral flap
14	fixed	posteriormost point of promesostegostom on the ventral side
15	fixed	border point of cheilo- and promesostegostom on the ventral side (at the level of the pharyngeal sleeve tip)
16	fixed	anteriormost point of gymnostom on the ventral side
17	fixed	posteriormost point of cheilostom on the ventral side (deepest point within the cheilostomal ridge)
18	fixed	anteriormost point of cheilostom on the ventral side

Supplementary table 2. Description of landmarks for *Caenorhabditis*.