

Computational Analysis of The Effects of Nitrogen Source and Sin1 Knockout on Biosilica Morphology in The Model Diatom *Thalassiosira Pseudonana*

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ABSTRACT

Morphogenesis of the silica based cell walls of diatoms, a large group of microalgae, is a paradigm for the self-assembly of complex 3D nano- and microscale patterned inorganic materials. In recent years, loss-of-function studies using genetic manipulation were successfully applied for the identification of genes that guide silica morphogenesis in diatoms. These studies revealed that the loss of one gene can affect multiple morphological parameters, and the morphological changes can be rather subtle being blurred by natural variations in morphology even within the same clone. Both factors severely hamper the identification of morphological mutants using subjective by-eye inspection of electron micrographs. Here we have developed automated image analysis for objectively quantifying the morphology of ridge networks and pore densities from numerous electron micrographs of diatom biosilica. This study demonstrated differences in ridge network morphology and pore density in diatoms growing on ammonium rather than nitrate as sole nitrogen source. Furthermore, it revealed shortcomings in previous by-eye evaluation of the biosilica phenotype of the silicanin-1 knockout mutant. We anticipate that the computational methods established in the present work, will be invaluable for unraveling genotype-phenotype correlations in diatom biosilica morphogenesis.

INTRODUCTION

Diatoms represent a large group of unicellular, eukaryotic microalgae that biosynthesize silica based cell walls. The 3D nano- to microscale architecture of the biosilica is a species specific trait indicating that its morphology is genetically controlled [1]. The intricate and highly regular, hierarchical porous patterns of diatom biosilica are regarded as paradigms for the bottom-up synthesis of mineral based materials under environmentally benign [2-4]. Furthermore, diatom biosilica microparticles have interesting materials properties that can be exploited for a wide range of applications in photonics, chemical sensing, catalysis and drug delivery [5-8]. The diatom cell wall consists of two halves of identical structures that are assembled in a petri-dish like arrangement. The slightly larger half (epitheca) overlaps the other half (hypotheca). Each theca is composed of a plate- or dome-shaped valve, exhibiting a species specific silica pattern, and several ring-shaped girdle bands. The biosilica is perforated with numerous, usually regularly arranged pores enabling a light-weight yet mechanically robust architecture and the exchange of ions and molecules with the environment [9, 10]. The formation of valves and girdle bands takes place inside diatom cells in specialized compartments called silica deposition vesicles (SDVs) [11]. Apart from amorphous SiO₂ as the main component, biosilica contains proteins, long-chain polyamines (LCPA), and polysaccharides [12-14].

In recent years, genetic manipulation studies have identified genes involved in biosilica morphogenesis in the radial centric model diatom *Thalassiosira pseudonana*. Individual

knockdowns of each the genes *sap1*, *sap2* and *sap3* resulted in various aberrations in biosilica morphology [15]. Knockdown of the gene *Thaps3_21880* caused a reduction of the pore density while increasing the mean area of the same [16]. Knockout of the silicanin-1 gene (*sin1*) had affected valve morphology and caused substantially reduced strength and stiffness of the cell wall [17]. Besides such targeted genetic modifications, the effect of many external parameters on biosilica morphology has been investigated. These include, among others, drug-based inhibition of cytoskeleton function [18-22] and of a V-type H⁺-ATPase [23], as well as controlled changes in (i) salinity and pH of the growth medium [24, 25], (ii) growth temperature [26], and (iii) light wavelength as well as intensity [27, 28]. In most of these studies a multitude of structural changes was observed upon alteration of only one gene or one environmental parameter. Furthermore, some of the morphological changes were rather subtle and somewhat heterogeneous among individual cells even from the same clone. As a consequence, biosilica morphology changes were prone to biased interpretation or might even have been overlooked. To eliminate such problems, unbiased, automated, and quantitative computational analysis tools are required that enable thorough and impartial evaluation of the effects on biosilica structure by gene disruptions and external perturbations.

Previous work has applied supervised learning for the analysis of scanning electron microscopy (SEM) images of the biosilica ridge pattern of the valves of *T. pseudonana* [26]. This led to the identification of a phenotype switch from a mesh-like to a tree-like ridge pattern in cells that were depleted of silicic acid, the precursor for silica [26]. Artificial neural networks (NNs) were used to identify in SEM images slight structural differences between the valves from *T. pseudonana* wild type and the *Thaps3_21880* gene knockdown mutant [16]. However, although NNs can be used to determine if any morphological differences exist between multiple groups of images, they do not reveal what the specific morphological differences are. Therefore, NNs offer little aid in reaching a mechanistic understanding of the biosilica morphogenesis. Therefore, it is desirable to develop computational methods that not only enable rapid and unbiased in-depth analysis of diatom biosilica phenotypes, but also point to an understanding of the mechanisms that create them.

A key goal of diatom based materials synthesis is the development of cell lines that produce biosilica with structures and properties tailored for specific applications. Recently, a genetic engineering based method called live diatom silica incorporation (LiDSI) has been established that allows for generating diatom strains, which contain desired fluorescent proteins, receptor proteins, or enzymes into the biosilica [29]. While biosilica functionalization using LiDSI is well developed and even enables regioselective incorporation of proteins [30], the genetic engineering of biosilica structure is still in its infancy. Only quite recently, the first genetically induced morphological mutants were generated in diatoms through knockdown and knockout of specific genes [15, 17]. One of the mutants (*Sin1KO*), which was generated by knocking out the *sin1* gene in *T. pseudonana*, exhibited a pleiotropic phenotype. Tedious manual inspection

of transmission electron microscopy (TEM) and atomic force microscopy images revealed differences in the height and number of cross-connections between the ridges in the valves [17]. The analysis of other structural parameters was not pursued.

Here we describe the development of two complementary computational approaches for impartial and quantitative in-depth image analysis of valve biosilica morphology. These tools were then applied to quantify and compare the influence of growth conditions and genetic modifications on biosilica morphology of *T. pseudonana*.

RESULTS

As test cases for developing computational tools for the analysis biosilica phenotypes we chose wild type cells and three previously described *sin1* knockout mutants of *T. pseudonana* (KO1, KO2, KO3), which exhibited subtle changes in biosilica morphology [17]. The four strains were cultivated under two growth conditions, in which nitrate (nit) or ammonium (am) served as the sole nitrogen source. The reason for varying the nitrogen source is the fact that the nitrate reductase promoter/terminator cassette, Pnr/Tnr, is the only well-established inducible promoter in diatoms [31]. When an introduced gene is placed under control of Pnr/Tnr, it is repressed when the cells are grown in medium containing ammonium as the sole nitrogen source [32]. Expression of the transgene is induced when the cells are transferred into nitrate containing medium that lacks ammonium [32]. It has previously been demonstrated that the type nitrogen source exerts large scale effects on metabolism [33], yet it is unknown whether this has an effect on silica morphogenesis. We envision that use of the inducible Pnr/Tnr cassette may become useful for evaluating the effect of candidate genes suspected to be involved in silica morphogenesis. Therefore, it needs to be clarified whether or not biosilica morphology is influenced by the metabolic changes that are elicited solely through changing the nitrogen source in the growth medium.

Imaging of valves

Our structural analyses in the present work were entirely focused on the valve part of the biosilica, which is the structurally most elaborate part of a diatom cell wall. To prepare valves for wild type (WT) cells and *sin1* knockout strains for transmission electron microscopy (TEM), cell walls were isolated, valves were severed from the cell walls by mild ultrasound treatment, and the samples air dried on a TEM grids. The valves remained largely intact during the preparation procedure but frequently contained small cracks. *T. pseudonana* valves exhibit five main structural features: fultoportulae, costae, cross-connections, areola pores and cribrum pores. A fultoportula is a tubular structure surrounded by three or four satellite pores. Fultoportulae are regularly spaced near the valve rim (Fig. 1a, yellow circles), and in about ~ 50 % of the valves one or two fultoportulae are present slightly off the valve center (Fig. 1a,

yellow circle). The valve surface contains numerous silica ridges that radiate from the valve center, branch out towards the rim of the valve and span in-between. A radially oriented ridge

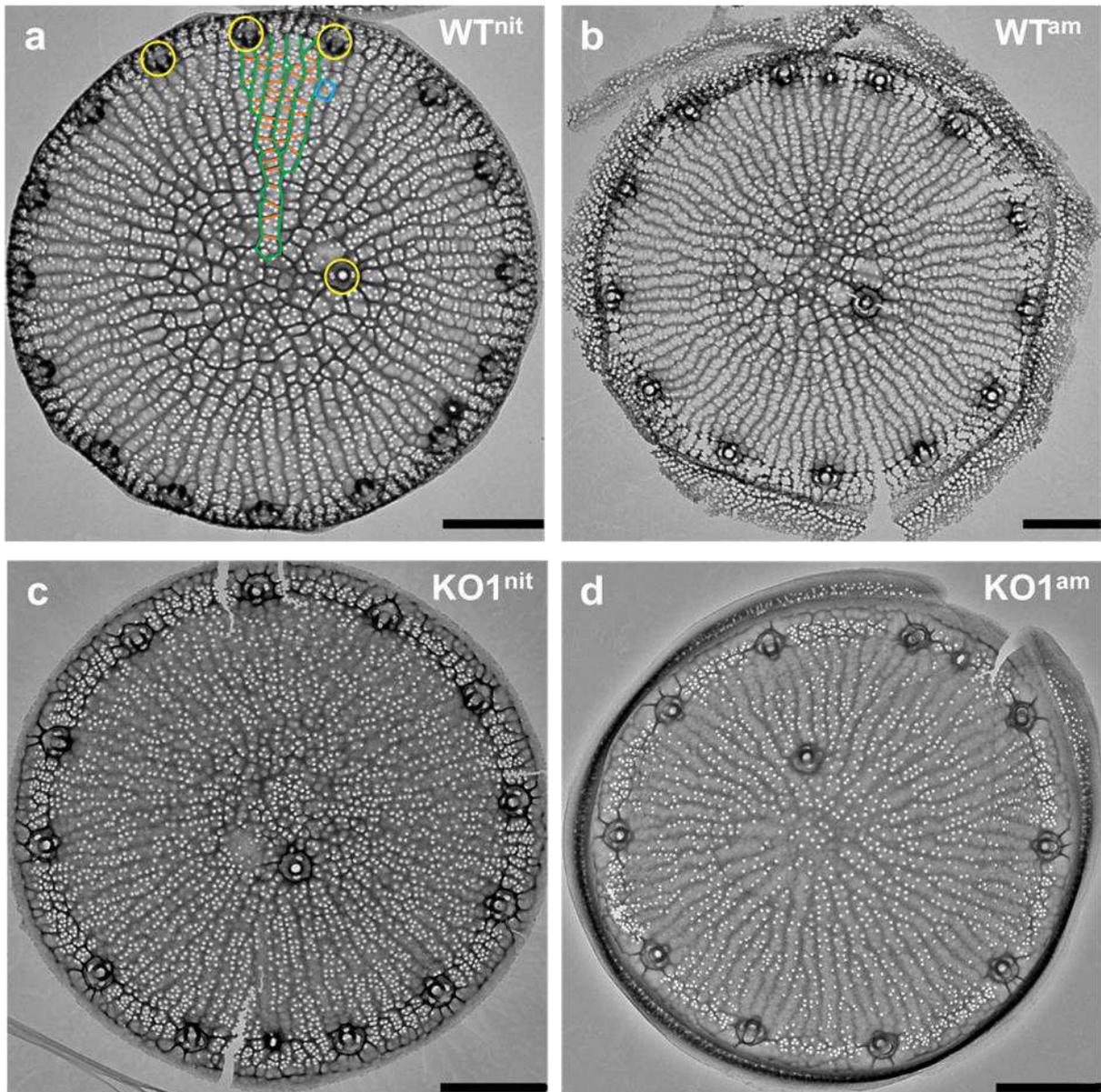


Fig. 1. TEM images of valve biosilica from *T. pseudonana* wild type (WT) and a representative *sin1* knockout mutant (KO1) grown in media containing different nitrogen sources. Representative images of individual valves are shown for the indicated strains (see top right corner). The indices nit (for nitrate) or am (for ammonium) refer to the sole nitrogen source in the growth medium. (a) WT grown in nitrate. Characteristic structural features of the biosilica are highlighted in green = costa, orange = cross-connection, cyan = areola pore, yellow and dark blue = fultoportula. (b) WT grown in ammonium, (c) KO1 grown in nitrate, (d) KO1 grown in ammonium. Scale bars: 1 μ m.

is called costa (Fig. 1a, green) and an azimuthally oriented one is called a cross-connections (Fig. 1a, orange). A region bounded by adjacent cross-connections and costa segments is called an areola pore, which typically exposes a trapezoid shape (Fig. 1a, cyan). Inside each areola pore are several circular openings termed cribrum pores (Fig. 1a). Visual inspection of transmission electron microscopy (TEM) images revealed that the *sin1* knockout mutants

exhibited a strongly reduced number of cross-connections compared to the valve of wild type cells, as expected (Fig. 1 and Fig. S1). The reduction in cross-connections was apparent for valves from both nitrate and ammonium grown cells (Fig. 1c, b and Fig. S1). When comparing WT valves from cells grown on different nitrogen sources, the cross-connections seem to be less pronounced in the valves from ammonium growing cells (Fig. 1a, b). In *sin1* knockout strains, the cribrum pore densities seemed to be lower in valves from ammonium grown cells compared to valves from nitrate grown cells (Fig. 1c, d and Fig. S1). However, the differences in the expression of cross-connections and in cribrum pore densities are not equally obvious from each individual valve. As these features are difficult to assess manually, we aimed to develop computational methods for their automated quantitative analysis.

Automated analysis of the ridge morphology of valves

To quantify valve morphology, we focused on the pattern of silica ridges (costae and cross-connections), which are thickenings of the cell wall that appear as a network of dark lines in TEM images (Fig. 1). We aimed to extract the ridge network as a pixel-thin skeleton image representation. However, extraction of the skeleton is challenging as the contrast of the ridge network varies widely between images, indicating differences in the thickness of the ridges in

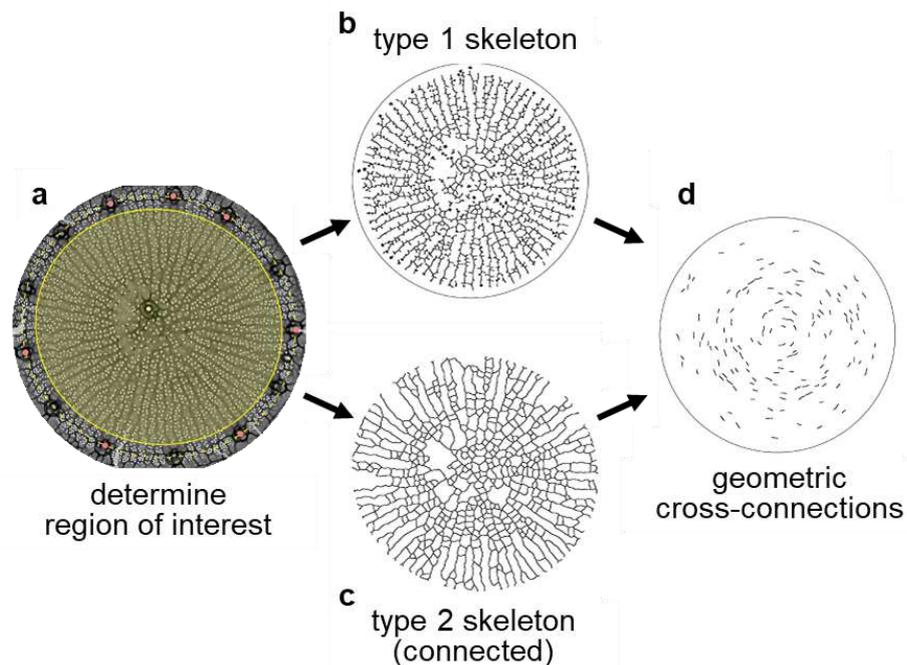


Fig. 2. Analysis pipelines for the characterization the ridge pattern of a valve. (a) The region of interest is identified as a circle that is interior to the circular pattern of the fultoportulae at the rim. The ridge pattern is extracted as (b) type 1 skeleton (maintaining high-contrast features) and (c) type 2 skeleton (maintaining connectedness). (d) Cross-connections are identified based on their orientation and length in both skeletons.

the valves from different strains (Fig. 1). Indeed, it was previously demonstrated that *Sin1KO* strains had reduced silica content and less elevated costae [17]. To deal with the challenging

contrast issue, we developed two different approaches to extract ridge networks. In the first approach, we implemented an adaptive thresholding method, which uses local contrast differences to extract a binary image from the TEM data. This method mainly detects ridges with high local contrast, which is likely indicative of high silicification. In the following, we refer to the skeletons generated with this method as “type 1 skeletons” (Fig. 2b). Type 1 skeletons extracted from low-contrast images are typically fragmented. However, it is known that during valve formation, the ridges develop as a continuous branched network, with cross-connections between branches appearing at later stages of development [34, 35]. To recover this continuous branching structure, we developed a second approach, designed to extract a connected (i.e. non-fragmented) skeleton even from low-contrast images (Fig. 2c). This approach relies on a so-called “ridge detection image filter”, which can identify dark lines of a chosen thickness by estimating the main principal curvature at each point in the image. As the pixel values are assumed to represent elevations in the landscape, the ridge detection filter identifies geographical ridges of the chosen broadness, regardless of gradual changes in elevation along the ridgelines. In contrast, thresholding would identify places of high elevation. We refer to the skeletons it generates as “type 2 skeletons”. The two skeleton extraction approaches produce similar results from high-contrast images, but differ when used for low-contrast images (Fig. 3).

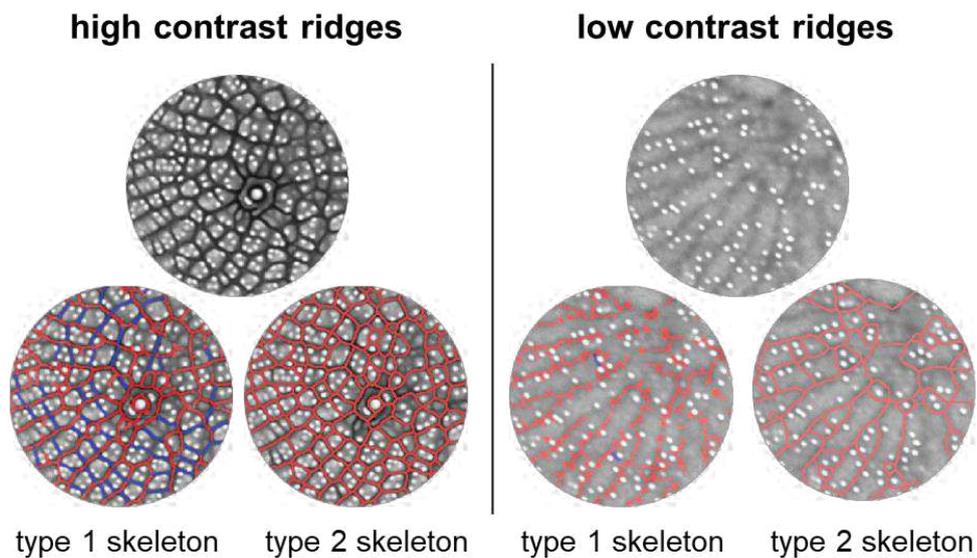


Fig. 3. Comparison of type 1 and type 2 ridge skeletons generated from TEM images with high and low contrast ridges. Type 1 and type 2 skeletons are indicated with a red overlay. Additionally, geometric cross-connections are highlighted in blue in the type 1 skeletons.

Once the ridge network is extracted, several of its features can be measured. In earlier work, valve morphology was quantified by manually counting the number of cross-connections per valve [17]. Therefore, the first feature we analyzed in the extracted skeletons was the number of “geometric cross-connections”, defined as valve ridges oriented in an azimuthal direction

(Fig. 2d). We developed an algorithm to classify parts of the network as belonging to costae or cross-connections. Each segment of the ridge network was classified based on a geometric criterion: the angle it forms with a line connecting it to the valve's centerpoint. Segments oriented at an angle smaller than a threshold (here: 45°) were considered to be parts of costae, while those forming an angle greater than the threshold were classified as cross-connections. Additionally, azimuthal segments shorter than 70 nm were discarded. This size was chosen by comparing the azimuthal segments for a range of lengths and choosing a value around which the cross-connection count varied the least. Geometric cross-connections per unit area (i.e. cross-connections densities) were determined in both, type 1 skeletons (representing highly silicified features) and type 2 skeletons (representing the fully connected ridge network) (Fig. 4). The cross-connection densities in type 1 skeletons of the valves from wild type cells (WT) grown on the different nitrogen sources were very similar (Fig. 4a). However, the cross-connection density was, surprisingly, higher in the valves from nitrate grown *sin1* knockout strains (KO1-3) compared to the valves from the same strains grown in ammonium (Fig. 4a). These data suggested that in the absence of the *sin1* gene, the cross-connection density is highly dependent on the nitrogen source. To further investigate this unexpected result, the geometric cross-connection density was analyzed in the type 2 skeletons. Here, the densities of cross-connections between the valves from cells grown in nitrate and ammonium were very similar for all strains irrespective of the nitrogen source (Fig. 3b).

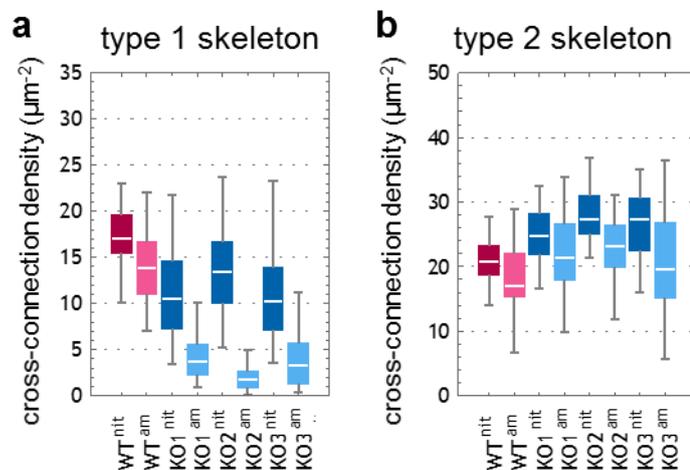


Fig.4. Geometric cross-connection counts per unit area. (a) Type-1 skeletons representing high-contrast features, and (b) type-2 skeletons representing a connected ridge network.

The discrepancy between the cross-connection densities in the two types of skeletons was caused by the fact that the two methods for skeletonization have different capabilities for identifying cross-connection. The method for extracting type 2 skeletons is geared towards identifying costae and cross-connections also in low contrast regions, where the other skeleton extraction method (type 1 skeletons) yielded fragmented networks and thus a lower number of cross-connections. The ridge networks in valves from ammonium grown cells exhibited

consistently lower contrast of the ridge network in TEM images compared to valves from nitrate grown cells (Fig. S2). Therefore, the discrepancy between the cross-connection counts by the two skeletonization methods is more drastic in valves from ammonium grown cells (Fig. 3).

Automated analysis of the density of cribrum pores

A characteristic feature of *T. pseudonana* are the ~20 nm sized cribrum pores, which are seemingly randomly distributed in the areas between the costae. Each areola usually contains two or more cribrum pores (Fig. 5a). The center of each pore was located in the TEM images by first accentuating pores using the “difference of Gaussians” method followed by identification for tall local maxima. For all strains the cribrum pore density was consistently higher in valves from nitrate grown cells than in valve from cells grown in ammonia (Fig. 5b). Furthermore, in nitrate medium the *sin1* knockout mutant strains exhibited higher cribrum pore densities than the wild type (Fig. 5b). The same trend applied for the ammonium grown strains, except for the KO3 strain, which had the same cribrum pore density as the wild type (Fig. 5b).

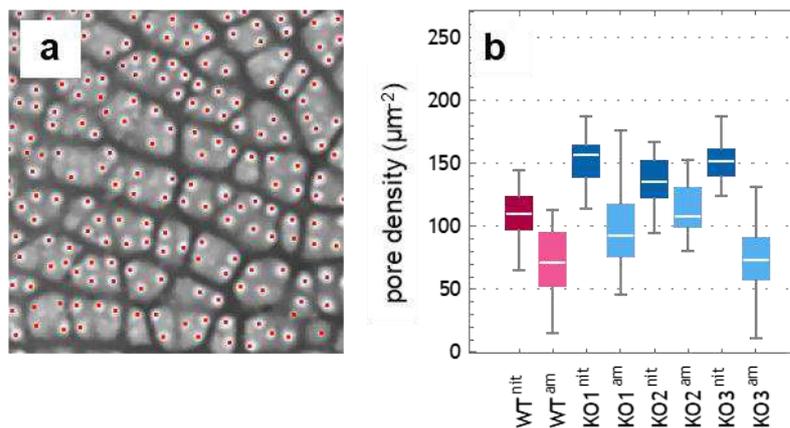


Fig. 5. Analysis of cribrum pore density. (a) Pores are highlighted in red in part of an image of a valve. (b) Box plots of the distribution of the number of pores per unit area for each dataset.

DISCUSSION

In the present work we have developed automated image analysis for evaluating the ridge patterns and pore densities from large numbers of TEM images of diatom biosilica. The new methods for morphological analysis replaced previously used manual methods [17], enabling significantly faster evaluation of much larger data sets. To analyze valve morphologies, we established two different approaches for extracting the characteristic networks of ridges (costae and cross-connections), which we termed type 1 and type 2 skeletons. Both approaches are suitable for determining the number of cross-connection from images in which the gray-level contrast between the ridge network and the surrounding areas is high. However, when the contrast is low only the method extracting type 2 skeletons is able to identify the

connected ridge network, because it is based on a “ridge detection image filter” which identifies continuous lines comparable to geographic ridgelines.

It is reasonable to assume that gray-level contrast in TEM images of biosilica reflects differences in silica content. Diatom biosilica is composed of ~90% hydrated silica and ~10% organic material, and thus electron contrast in TEM images of biosilica is dominated by silicon, which is the element with by far the highest electron density in this material. Consistent with this assumption is the observation that the TEM contrast between the ridges and the surrounding areas is lower in valves of *sin1* knockout mutants (see Fig. 1a, c), which have a ~40% reduced z-height of costae compared to wild type [17]. For reasons described above, skeletonization method 1 is more susceptible than method 2 to differences in TEM contrast. Therefore, strongly reduced silicification of a cross-connection ridge is often interpreted as a missing cross connection in type 1 skeletons (see Fig. 3). This is consistent with the interpretation gained from by-eye inspection of TEM micrographs (see Fig. 1 and reference [17]). In contrast, skeletonization method 2 is capable of detecting weakly silicified cross-connections (i.e. low TEM contrast), and thus revealed that the ridge network of *T. pseudonana* wild type is still fully intact in the *sin1* knockout mutants (see Fig. 4b). The previous interpretation of missing cross-connection in the *sin1* knockout mutants [17], was thus a misinterpretation of a phenotype that exhibits weakly silicified cross-links. This result demonstrated that by applying both skeletonization methods, a more realistic description of the valve phenotypes could be obtained.

Another unexpected result of this study was the observation that the type of nitrogen source had an effect on the density of cribrum pores and the cross-connection density of the ridge network in type 1 skeletons. The effect on cross-connection density was very mild in wild type cells but much more pronounced in the *sin1* knockout mutants, with valves from cells grown on ammonium exhibiting lower cross-connection densities than valves from nitrate grown cells (see Fig. 4a). Analysis of the type 2 skeletons demonstrated for each strain that the cross-connection densities were independent of the nitrogen source (see Fig. 4a), thus indicating that reduced silicification is responsible for the diminished cross-connection densities in the type 1 skeletons from ammonium grown cells. This remarkable finding raises a new question: How can the nitrogen source influence silica morphogenesis in diatoms? We believe that part of the answer lies in the observation that the nitrogen source effect on silicification is stronger in the *sin1* knockout mutants than in wild type. There is evidence that the Sin1 protein enhances the silica forming activity of long-chain polyamines (LCPA) [36], which are general components of the biosilica forming machinery in diatoms [12]. We hypothesize that LCPA biosynthesis might be downregulated in diatoms grown on ammonium medium compared to nitrate grown diatoms, which would lead to reduced silica formation. In *sin1* knockout mutants, the silica formation capability may be further reduced due to the lack of the Sin1 protein. Despite these shortcomings there is still sufficient silica forming activity

present to biosynthesis a complete valve including the ridge network. However, the thickening of the ridges (called z-expansion), which is the final stage of silica biosynthesis [34] may be hampered by reduced levels of LCPA and the lack of Sin1. The reduced silica content of valves compromises their mechanical performance [17], which is problematic even for laboratory cultures as the valve needs to counteract the turgor pressure exerted by the protoplast. One counter measure of the cell for increasing the stability of valves with reduced silica content, could be to change the pattern of cribrum pores. Whether a reduction of cribrum pore density, as we observed here (Fig. 5), generally leads to an increase in mechanical performance of the valve, remains to be determined.

The results from the present study clearly demonstrate that automated computational analysis is highly useful for unraveling the influences of external and internal factors on diatom biosilica morphology. We anticipate that the computational geometric methods established here will be instrumental in future work for characterizing the function of biomineralization proteins in silica morphogenesis in *T. pseudonana* and possibly many other diatoms species.

METHODS

Culture conditions

T. pseudonana (clone CCMP 1335) was grown at 18 °C with a 16 h light/8 h dark cycle at 5000–10,000 lux, in artificial seawater (ASW) [35] lacking the nitrogen source. Sterile filtered NH_4Cl or KNO_3 was added to a final concentration of 550 μM .

Segmenting the high-contrast valve ridges (type 1 skeleton)

Each grayscale TEM image was converted into a binary image by applying adaptive thresholding to differentiate the network structure from the background. From the network structure, a single pixel thick skeleton was extracted using the Ridge Detection Plugin of ImageJ [37]. Examples of skeletons are shown in Fig. 2b and Fig. 3.

Segmenting the connected valve ridge network (type 2 skeleton)

First, we corrected any uneven brightness in the electron micrograph: The local brightness was estimated by removing (inpainting) the pores, removing the valve ridges using a closing filter, and finally applying Gaussian blur. The brightness field obtained this way was subtracted from the image. Then the location of valve ridges (costae and cross-connections) was identified with a ridge detection image filter that computes the main principal curvature at each point, using the typical costae width as the scale parameter. Finally, the result was thresholded and skeletonized to create a binary one-pixel thin representation of the ridge network (Fig. 2c). The extraction of the ridge network requires care, so that we may obtain a network with a large connected component while making sure that the parts of the valve plate that separate

neighboring pores can be reliably distinguished from true ridges, even in low-contrast images. We manually selected a single set of parameters which produced good results across all image datasets. Examples of final skeletons are shown in Fig. 2c and Fig. 3. These examples have one level of open branches pruned away to aid comparison with the original image. We noted that such pruning has no effect on the topological ridge network analysis.

Classifying cross-connections using geometric criteria

We designed an algorithm to identify geometric cross-connections from the electron micrograph of a diatom valve based on their orientation. Since *T. pseudonana* is a centric diatom with radial symmetry for the valves, we manually choose a center point (i_c, j_c) for the each valve. Cross-connections (green lines) would appear nearly perpendicular to radial lines, while costae are approximately parallel to radial lines. In a first step, raster scans of every pixel (i, j) belonging to the skeletonized valve network were generated and classified belonging to either the cross-connections or the costae with the following selection rules: A pixel is part of a cross-connection if its line segment forms an angle $\theta_{i,j}$ greater than a threshold θ_{thresh} (which we choose as 45°) and as part of a costa otherwise. A second criterion for defining a geometrical cross connection is a minimum length of > 70 nm. The resulting pattern (**Error! Reference source not found.**d) consists of only cross-connections, which we count by connected component labeling [38].

Pore detection

The pores were located using the difference of Gaussians method. This method will accentuate features at a certain chosen length scale in the image. The center of pores was detected by choosing the pore size as the length scale and looking for maxima of sufficient height in the image. In order to find an appropriate length scale for each image, an initial pore detection pass was run with an approximate, assumed pore size. The mean size of the initially detected pores was then measured, and used in subsequent passes to refine the result. This method usually converges in at most 2-3 iterations.

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DECLARATIONS

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Conflicts of interest/Competing interests

The authors have no conflict of interest and no competing interests.

Data availability

The data are available from the corresponding authors upon request.

Code availability

The codes are available from the corresponding authors upon request.

Authors' contributions

N.K., C.D.M. and S.H. conceived the study. All authors planned and interpreted experiments and computational analyses. S.G. conducted experiments, S.H. and A.F. developed analysis algorithms and performed computational analyses. All authors wrote the paper.

SUPPLEMENTARY INFORMATION

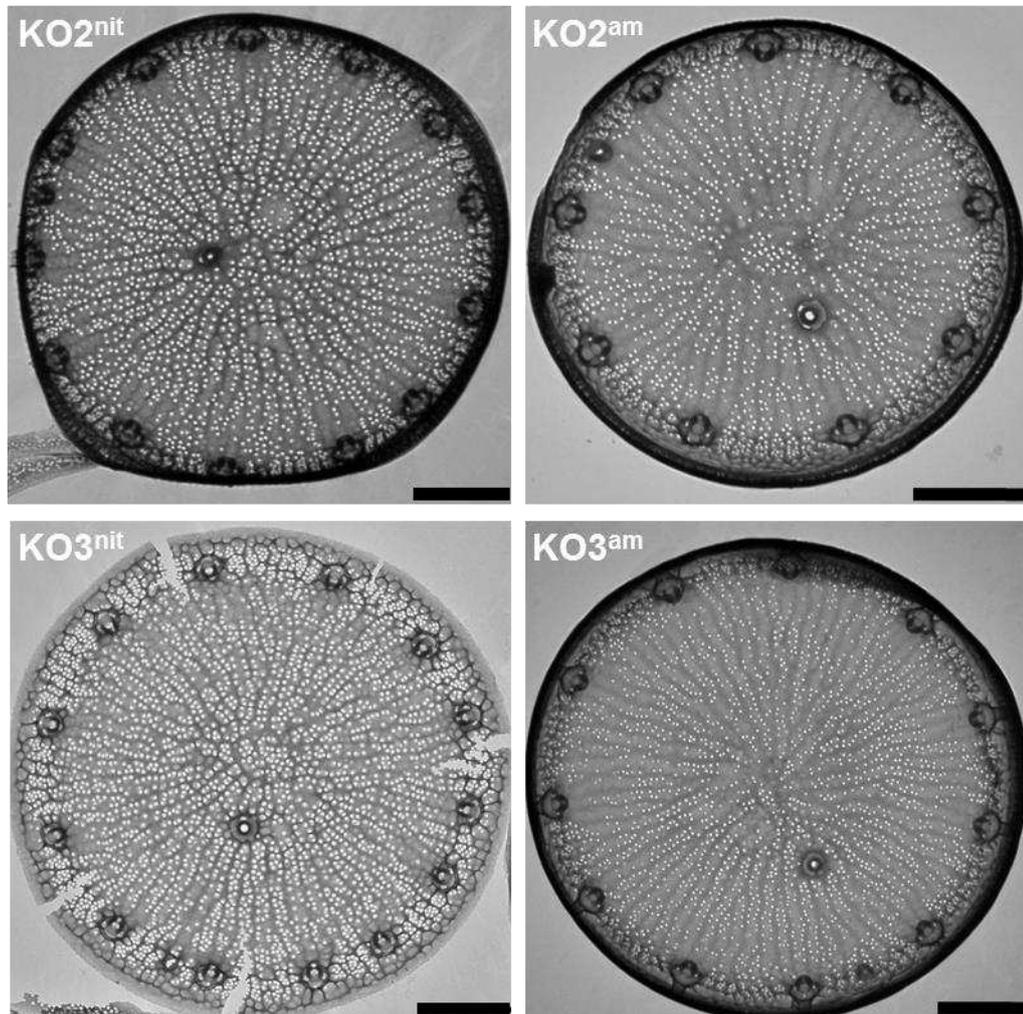


Fig. S1. TEM images of valve biosilica from the *sin1* knockout mutants KO2 and KO3 grown in medium containing nitrate or ammonium. Representative images of individual valves are shown for the indicated strains (see top right corner). The indices nit (for nitrate) or am (for ammonium) refer to the sole nitrogen source in the growth medium. Scale bars: 1 μm.

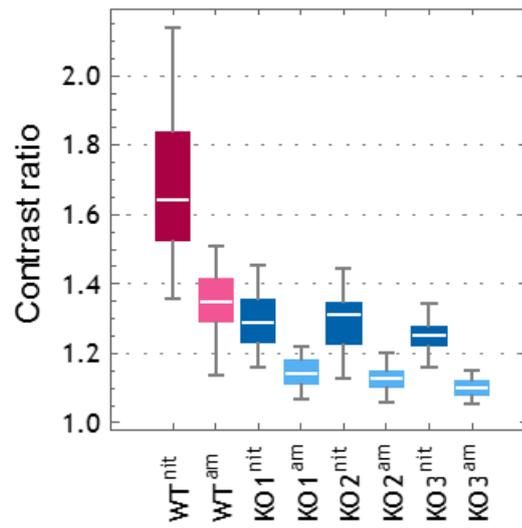


Fig. S2. Relative contrasts of ridge networks in TEM images. The ratios between the gray levels of valve ridges and the areas between the ridges (excluding the pores) are shown. The ridge networks were based on type 2 skeletons.

Figures

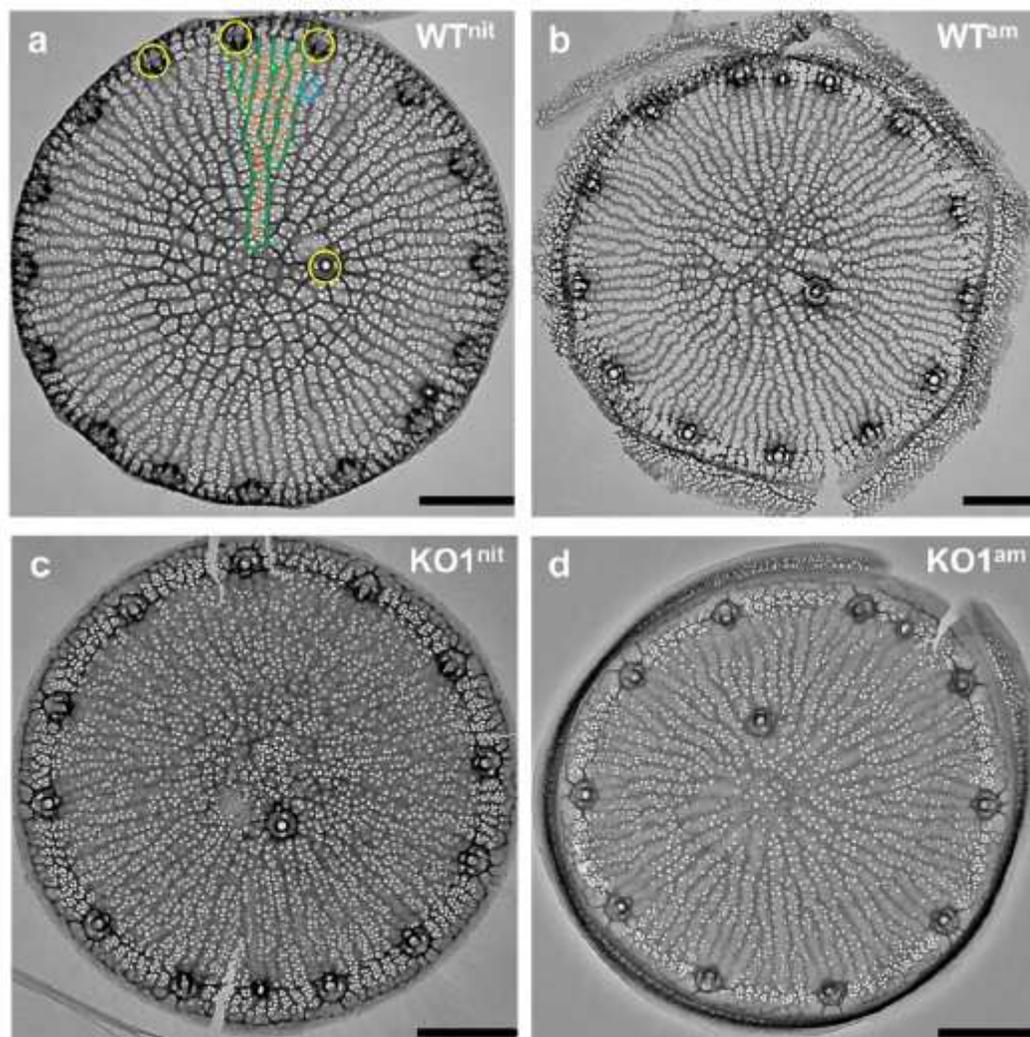


Figure 1

TEM images of valve biosilica from *T. pseudonana* wild type (WT) and a representative *sin1* knockout mutant (KO1) grown in media containing different nitrogen sources. Representative images of individual valves are shown for the indicated strains (see top right corner). The indices nit (for nitrate) or am (for ammonium) refer to the sole nitrogen source in the growth medium. (a) WT grown in nitrate. Characteristic structural features of the biosilica are highlighted in green = costa, orange = cross-connection, cyan = areola pore, yellow and dark blue = fultoportula. (b) WT grown in ammonium, (c) KO1 grown in nitrate, (d) KO1 grown in ammonium. Scale bars: 1 μm .

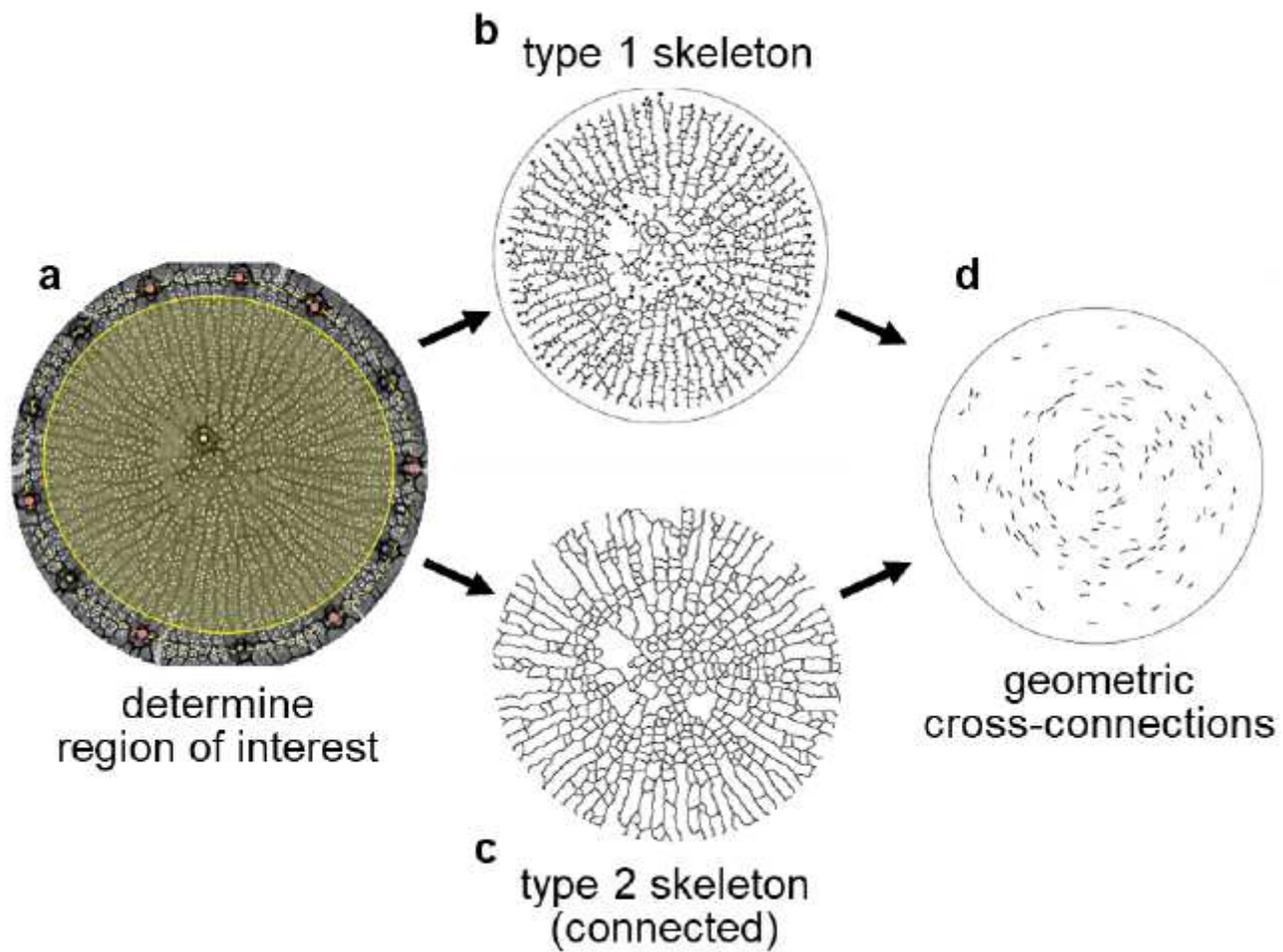


Figure 2

Analysis pipelines for the characterization the ridge pattern of a valve. (a) The region of interest is identified as a circle that is interior to the circular pattern of the fuloportulae at the rim. The ridge pattern is extracted as (b) type 1 skeleton (maintaining high-contrast features) and (c) type 2 skeleton (maintaining connectedness). (d) Cross-connections are identified based on their orientation and length in both skeletons.

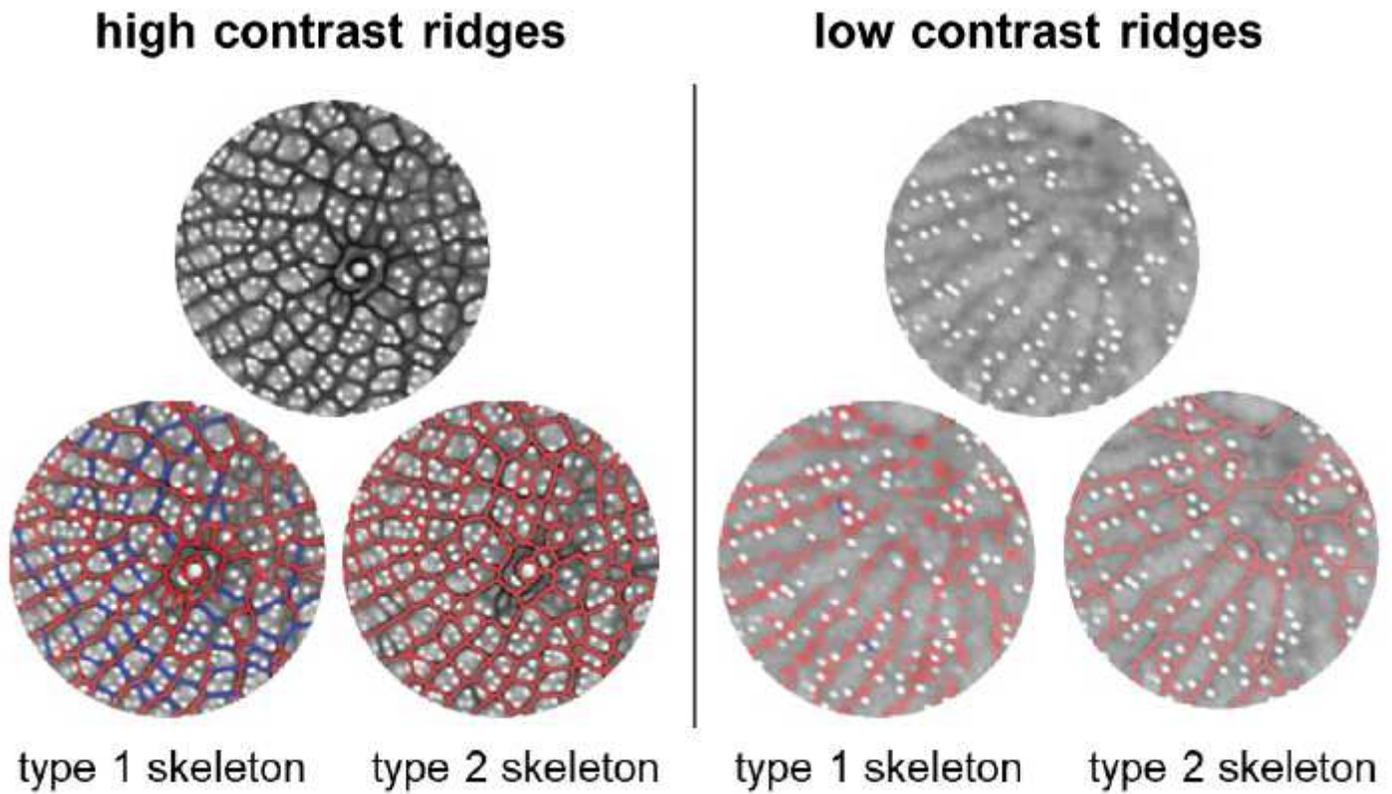


Figure 3

Comparison of type 1 and type 2 ridge skeletons generated from TEM images with high and low contrast ridges. Type 1 and type 2 skeletons are indicated with a red overlay. Additionally, geometric cross-connections are highlighted in blue in the type 1 skeletons.

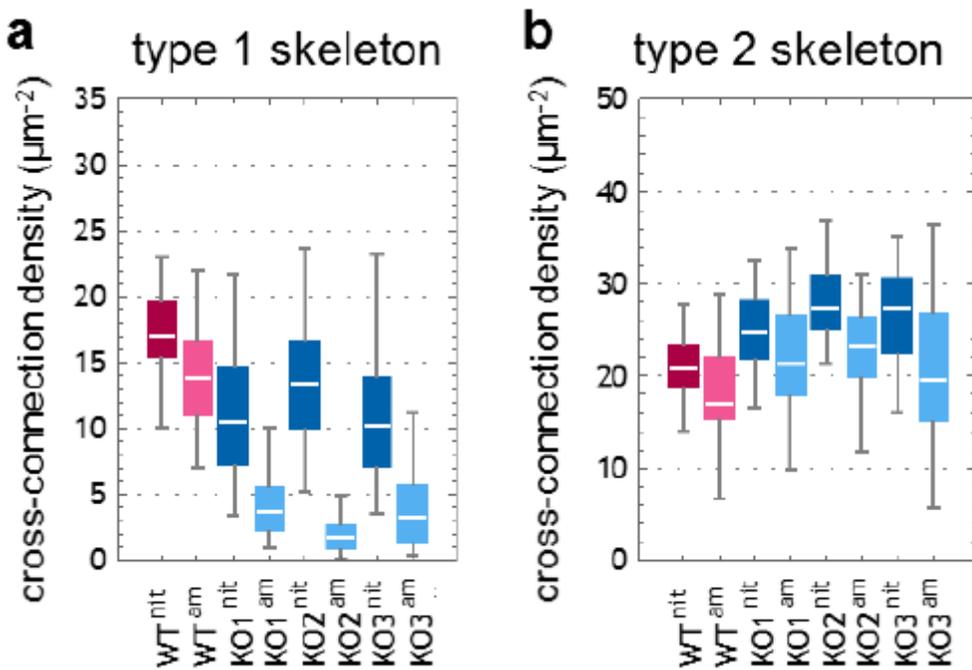


Figure 4

Geometric cross-connection counts per unit area. (a) Type-1 skeletons representing high-contrast features, and (b) type-2 skeletons representing a connected ridge network.

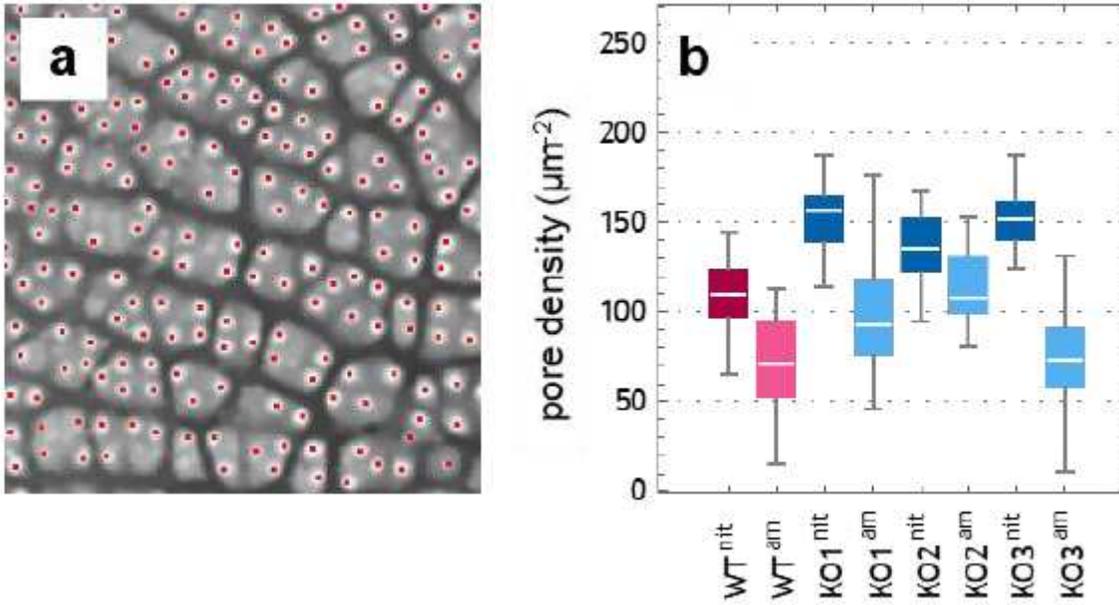


Figure 5

Analysis of cribrum pore density. (a) Pores are highlighted in red in part of an image of a valve. (b) Box plots of the distribution of the number of pores per unit area for each dataset.