

Guidelines for the MorphoLeaf application.

(Version 1.0, December 2015)

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Presentation of MorphoLeaf

The MorphoLeaf application allows you to extract the contour of multiple leaf images and identify their biologically-relevant landmarks. These landmarks are then used to quantify morphological parameters of individual leaves and to reconstruct average leaf shapes.

The MorphoLeaf plug-in is subdivided into 7 modules, each performing a specific task within a global process. You may either go through the whole process step by step, or stop the process at a given step and restart later.

Installation of MorphoLeaf

Go to the MorphoLeaf web site at <http://morpholeaf.versailles.inra.fr/> to get the latest version of MorphoLeaf and installation instructions.

Some advice to start with optimal leaf images

- **Place a single leaf horizontally in each image**, centering the blade in the middle and leaving a **clear homogenous background** around it (i.e. free of other leaves parts and/or debris).
- **Orient all leaves in the same project similarly**. For example, all leaves oriented with their petiole on the left and tip on the right.
- **Maximize as much as possible the contrast** between the leaf and the background. Try different backgrounds if possible.
- **Optimize the resolution** of your picture. For most leaves, 1000 pixels along the main leaf axis are enough. You may lower the resolution for leaves with a simple structure and increase it for leaves that are highly dissected.
- Keep a record of the calibration in size of your pictures. If possible, keep the same magnification for all your pictures. This will simplify the treatment of your pictures but is not compulsory as MorphoLeaf can handle pictures with different magnifications in the same stack.
- Treat different genotype, growth conditions, or species as different projects assembling them into different stacks (see section 2).

1. Importing leaf images into Free-D

Here you will import your pictures into a Free-D stack that will contain as many slices as the number of pictures you have imported.

A comprehensive guide for the use of the Free-D software can be downloaded from <http://free-d.versailles.inra.fr/>.

- 1.1. Before taking your leaf pictures consult the section “[Some advice to start with optimal leaf images](#)”. Make sure that your pictures are in .tif. If not, you can convert them using softwares like ImageJ. Free-D and MorphoLeaf accept either Grayscale (8 bits) or RGB (24 bits) images.
- 1.2. Start Free-D by double clicking the **Free-D** icon.
- 1.3. In the **File** menu, click **New stack** to open the *New stack* dialogue window.
- 1.4. In the *New stack* window, fill-in the **Stack name**, the **Length unit** and the **Pixel width** fields. If your pictures have different calibrations you will have the opportunity to add this information later on ([1.9](#)). You don't need to modify the slice spacing and aspect ratio fields and you can leave the store absolute path to images unchecked.
- 1.5. Click the **Add** button to open the *Select image files* window from which you can **choose the images** you want to analyze. Validate your choice by clicking **Open** in the *Select image files* window and clicking **Done** in the *New stack* window.
- 1.6. The pictures will appear as different slices within a single stack. You can switch between single slice view and panorama view by clicking the  and  icons at the top of the *Free-D* window.
- 1.7. You can navigate through the stack to check the different pictures by clicking on their name in the **Slices** part of the *Free-D* window or using the     navigation icons at the top of the *Free-D* window
- 1.8. You can zoom in and out by clicking the  and  icons and resize to the full window using the  icon. Once you have zoomed-into your picture you can change the local view using the *Overview* window active *via* the  icon, using the scroll icons    , or by

- maintaining the space bar pressed and moving the mouse while clicking.
- 1.9. You can switch between views of only the leaf picture, only the segmented items you will create or both by clicking on   or .
 - 1.10. To change the calibration of a single picture, **select the picture** by clicking on its name in the **Slices** part of the *Free-D* window, select **Edit > Slice properties** and in the *Slice properties* window change the **Pixel width** value and validate your choice by clicking on **Apply**.
 - 1.11. Save your stack by clicking the  icon. This will create a STACK DESCRIPTION FILE (.sdf) that contains the data related to your picture stack.
 - 1.12. You can close your stack at anytime clicking on  or in the **File** menu, clicking **Close stack**.

2. Create a new MorphoLeaf project or load an existing MorphoLeaf project

Here you will create a new MORPHOLEAF FILE (.mlf) or open an existing MORPHOLEAF FILE (.mlf). The MORPHOLEAF FILE (.mlf) contains the metadata associated with the leaf pictures.

- 2.1. You can continue directly from step **1.9** with an already open .sdf file (skip to 2.3).
- 2.2. Alternatively, you can launch a new session of Free-D and open an existing .sdf file containing your pictures by clicking the  icon or by clicking **File > Open stack**, selecting your file of interest in the *Open stack* window.
- 2.3. In the **Plugins** menu start the **MorphoLeaf** plugin. This will open a *MorphoLeaf* window in the **Project Manager** module.
- 2.4. To start a new project, click **New Project** and fill the fields in the *New project* dialogue window (no field is compulsory).
Select the **Venation Type** of the leaves: choose  for pinnate venation or  for palmate venation. If you choose palmate, the part of the leaf surrounding its tip will be included in the analysis while if you choose pinnate it will be excluded.
Click the  icon and in the *Find directory* window set the location where the .mlf file containing the metadata of the project will be saved and then click **Choose**. Validate by clicking **Done**.
- 2.5. To work on an existing project, click **Open Project** to open the *Open project* window in which you can select the .mlf file you want. Validate your choice by clicking **Open**.

3. Extraction of the leaf contour and positioning of the petiole/blade junction

Here you will automatically extract the contours of the leaves in all the slices of the stack and have the opportunity to manually edit them if necessary. You will also have to set manually two landmarks corresponding to the petiole/blade junction.

- 3.1. You can continue directly from the **2.4** step with an ongoing project and skip to 3.3.
- 3.2. Alternatively, you can launch a new session of Free-D and **Open an existing MorphoLeaf project or create a new one** as described in section [2. Create a new MorphoLeaf project or load an existing MorphoLeaf project.](#)
- 3.3. Go to the **leaf contour extraction module** in the *MorphoLeaf* window, by clicking the **Extract Contour** icon on the left of the *MorphoLeaf* window.
- 3.4. Select the type of background of your picture in the **Background Selector**, and the channel which will be used to extract the leaf contour in the **Channel Selector**. Choose a **Fidelity** value, ranging from 10 to 100. A high fidelity value will make a contour that follows all the small details of the leaf margin and is recommended for very sharp pictures, while a low fidelity value will lead to a smoother contour and is more suitable for noisy pictures. It is recommended here to test different settings to identify those generating leaf contours that require the fewer editions.
- 3.5. Click **Process** to extract the leaf contours. This takes a few seconds to a few minutes depending on the number and size of the slices in the stack and will generate the leaf contour on each image that will appear as a thin blue line . You can switch the views between the

leaf, the leaf contour and both of them as described in [1.9](#). After contour extraction, a message appears reminding you to add two landmarks on each leaf corresponding to the blade-petiole junctions (see [3.7](#)).

3.6. You now have the opportunity to edit the leaf contour if necessary. You can check the quality of the extracted contours by navigating through the slices, zoom in and out of the picture (see [1.6. to 1.8.](#)). To edit the contours:

3.6.1. **Select the leaf contour by clicking on it:** all the points of the contour will become visible as small black squares. Alternatively, **right-click** anywhere around the leaf to open a contextual dialogue window and click **Select > Contours**

3.6.2. You may want to start by reducing the number of points of the contour to a manageable size, but be careful not to reduce it too much as it may lead to smoothing of the leaf contour. Typically 300-500 points is optimal, but this depends on leaf complexity and size. To reduce the number of points, **right-click** to open a contextual dialogue window, from which you can select the **Edit > Resample** to open the *Contour resampling* window. Alternatively, you can open the *Contour resampling* window by pressing **R** on your keyboard. In the *Contour resampling* window you can change the number of points (called Vertices) and validate by clicking **OK**.

3.6.3. You can manually edit any feature in a .sdf file. For this, choose the feature you want to edit, here the leaf contour, either by clicking it or **click right** to open a contextual dialogue window, from which you can select the **Edit > Select**. Next, **click right** to open again the contextual dialogue window and choose **Edit > Edit points** to open the *Vertex edition tools* window. Alternatively, you can open the *Vertex edition tools* window by pressing **E** on your keyboard. To move a point, select the **Move points** icon , click on a point and drag it with the mouse. To insert a new point, select the **Insert points** icon  and click on

the leaf contour where you want to insert the point. To remove a point, select the **Delete points** icon  and click a point to remove it (large numbers of points can also be deleted by dragging the mouse while keeping the mouse left button pressed-down). At the end of the edition process select **End edition** or press **Escape** on your keyboard. **Save** () the changes made in the .sdf file.

- 3.7. Once you have checked and/or edited the leaf contours you have to define the junction between the petiole and the blade by positioning two landmarks along the leaf contour. For this **right-click** to open the contextual dialogue window and select **Draw > Manual > Petiole**. Click successively on the **two positions** along the leaf contour that correspond to petiole/blade junction to draw the landmarks  and validate by pressing **Return** on your keyboard. These landmarks will be used to define the blade and the petiole parts on the leaf contour. If you need to edit these landmarks proceed as described in [3.6.3](#). Repeat steps 3.6 to 3.7 for every slice.

4. Extraction of the leaf tip and teeth sinuses

Here you will identify the sinuses on the leaf contours and have the opportunity to manually edit them if necessary. In the same step, the apex of the leaf will be automatically identified.

- 4.1. You can continue directly from step **3.7** with an ongoing project and skip to 4.3.
- 4.2. Alternatively, launch a new session of Free-D, open an existing .sdf file containing your pictures by clicking the  icon or by clicking the **File > Open stack**, selecting your file of interest in the *Open stack* window. **Open an existing MorphoLeaf project or create a new one** as described in section [2. Create a new MorphoLeaf project or load an existing MorphoLeaf project.](#) At this step, your .sdf file should contain validated leaf contours  and petiole/blade junction landmarks . If this is not the case proceed as described in section [3. Extraction of the leaf contour.](#)
- 4.3. Go to the **sinus extraction module** by clicking the **Extract Sinus** icon on the left of the *MorphoLeaf* window.
- 4.4. Sinuses are detected based on variations of the local curvature on the leaf contour. in a two-step procedure: first portions of the contour that contain a sinus are determined as continuous intervals with only local curvature values below a user-defined threshold, second within each of the obtained intervals, the point with the minimal curvature is selected as a sinus. You can adjust two parameters. **Half Neighborhood** corresponds to the number of pixels along the leaf contour that are used to calculate the local curvature. You may want lower this parameter to detect sinuses with a high frequency and increase it to detect sinuses with a low frequency. **Maximum Curvature Angle** is the limit value of the angle that determines the intervals containing a sinus. You may want to increase this parameter

to detect shallow sinuses and lower it to be more stringent and detect only sharper sinuses. Start sinus detection by clicking **Process**. You can then navigate through the stack to check the position of sinuses  and temporary sinus  (see below). You can test different values and parameters to find optimal detection conditions by clicking **Process** as many times as you like. Once optimal parameters have been identified you can edit the sinuses if necessary by proceeding as described in [3.6.3](#).

- 4.5. After sinus detection a warning window may pop-up indicating that a single sinus (that will appear as a temporary sinus ) has been detected on one or more slices of some leaves. Note: A tooth by definition must be surrounded by two sinuses therefore on each half leaf you must have two or more sinuses (or none if the contour is smooth). At this stage the pop-up window will prompt you to either keep or delete all the temporary sinuses. If you decide to keep them your next step is to go through the slices that have temporary sinuses and when required, manually add a second sinus by **right-clicking** and selecting **Draw > Manual > Primary sinus**. If such a correction is made, the temporary sinus will be automatically transformed into a sinus  in later steps. If you decide not to make such a correction the remaining single temporary sinuses will be automatically deleted in the step 5.4; there is no need to delete them manually.
- 4.6. The sinus extraction module also detects the apex of the leaf , as the point farther away from the petiole/blade junction. You should check that it is properly positioned in each slice and if necessary edit it by proceeding as described in [3.6.3](#).

5. Extraction of the teeth tips

Here you will identify the tips located between each pair of sinuses using one of two methods. The first method, more appropriate for sharp teeth, detects the point corresponding to the maximum of local curvature between two sinuses. The second method, that works better for tooth with rounder tips, is based on the maximization of a local symmetry criterion.

- 5.1 . You can continue directly from step **4.6.** with an ongoing project and skip to 5.3.
- 5.2. Alternatively, start Free-D, open an existing .sdf file containing your pictures by clicking the  icon or by clicking the **File** menu and **Open stack**, selecting your file of interest in the *Open stack* window. **Open an existing MorphoLeaf project or create a new one** as described in section [2. Create a new MorphoLeaf project or load an existing MorphoLeaf project.](#) At this step, your .sdf file should contain validated leaf contours  and petiole/blade junctions , sinuses  and leaf apex  landmarks. If this is not the case go back to previous steps.
- 5.3. Go to the **tip extraction module** by clicking the **Extract Tips** icon at the left of the *MorphoLeaf* window.
- 5.4. Two methods are available to detect the teeth tips. The **Local Curvature Maximum** method detects the point with the maximum local curvature between two sinuses. You may want to use this method for pointy tips. You have to choose the neighborhood on which the local curvature is calculated, expressed here as a percentage of the length of the tooth. The **Local Symmetry Maximum** method is based on the observation that towards their summit, teeth or lobes tend to be symmetrical and that the tip is located along the axis of symmetry. You may want to use this method for rather round structures. Start tip detection by clicking **Process.**

Once finished you can navigate through the stack to check the location of the teeth tips . You can test different values and parameters to find optimal detection conditions by clicking **Process** as many times as you like. Once optimal parameters have been identified you can edit the sinuses if necessary by proceeding as described in [3.6.3](#).

If single sinuses are still present on some half leaf contours, a warning window will pop-up. Before proceeding you should add sinuses or delete the temporary single sinuses using the procedure described in [4.5](#).

Note : If you choose palmate for the venation type ([2.4](#)) a tip will be positioned at the leaf tip while none will be positioned if you select pinnate.

6. Hierarchisation of the teeth

Here you will identify the hierarchical structure of teeth that is required to properly quantify parameters associated with the teeth. The aim is to identify and associate primary structures and the higher order structures they support. Two methods are available, the recursive method being more specifically dedicated to *Arabidopsis* leaves and the iterative method being more generic.

6.1 . You can continue directly from step **5.4.** with an ongoing project

6.2. Alternatively, start Free-D, open an existing .sdf file containing your pictures by clicking the  icon or by clicking the **File** menu and **Open stack**, selecting your file of interest in the *Open stack* window. **Open an existing MorphoLeaf project or create a new one** as described in section [2. Create a new MorphoLeaf project or load an existing MorphoLeaf project.](#) At this step, your .sdf file should contain validated leaf contours  and petiole/blade junctions , teeth sinuses  and tips , and leaf apex  landmarks. If this is not the case go back to previous steps.

6.3. Go to the **hierarchy module** by clicking the **Hierarchy** icon at the left of the *MorphoLeaf* window.

6.4. You can choose between two hierarchy detection methods. If you are unsure which method is most suitable for your samples we recommend you test the two methods and vary the parameters.

6.4.1. The **Hierarchy Recursive Method** is based on the idea that tooth inclination increases with rank making this method well adapted for leaves which have a rather regular, convex overall shape like those of *Arabidopsis thaliana*. The **Limit Angle** parameter allows you to set the stringency of the detection of the secondary structures. This parameter corresponds to the limit

of the angle at each sinus with the two neighboring sinuses under which a sinus is declared as a secondary sinus. You may want to lower the value of the limit angle to have a more stringent detection of secondary structures and increase it to allow the detection of less-pronounced secondary structures. In addition to the detection of secondary structures, this method also recognizes “distal structures” that are formed in some *Arabidopsis thaliana* leaves: whereas most of the teeth in this species are formed successively from the tip to the base of the leaf, some teeth may appear distally to the first-formed and largest tooth (see below). If you want to detect these distal structure activate the **Detect distal teeth** option. These distal structures are identified on each half blade as distal teeth to the largest tooth. Once you have defined detection parameters press **Process** to run the method. The outcome will be the change in the labeling of some landmarks with the identification of primary , secondary  and distal  structures. Note that some sinuses may have a double labeling being for instance those located between a primary tooth and a distal tooth. You can go back to the *MorphoLeaf* window and test different values for the Limit angle factor by clicking Process as many times as you like.

6.4.2. The **Hierarchy Iterative Method** is based on the idea that primary sinuses are more aligned with a smoothed leaf contour than secondary sinuses. The **Stringency** parameter allows you to set the stringency of the detection of the secondary structures. You may want to lower the value of the tolerance factor to be less stringent in the detection of secondary structures and increase it to be more stringent. Press **Process** to run the method. The outcome will be the change in the labeling of some landmarks with the determination of primary sinuses  and tips  and secondary sinuses  and tips . Note that here

each landmark will have a unique labeling. You can test different values for the tolerance factor.

6.4.3. If your samples have no hierarchical structure you can skip the detection of the hierarchy by clicking **No hierarchy** and **Process**. All the structures will be defined as primary .

7. Extraction of quantitative leaf and teeth parameters

Here you will extract quantitative parameters of the leaves.

- 7.1 . You can continue directly from step **6.4.** with an ongoing project and skip to 7.3.
- 7.2. Alternatively, start Free-D, open an existing .sdf file containing your pictures by clicking the  icon or by clicking the **File** menu and **Open stack**, selecting your file of interest in the *Open stack* window. **Open an existing MorphoLeaf project or create a new one** as described in section [2. Create a new MorphoLeaf project or load an existing MorphoLeaf project.](#) At this step, your .sdf file should contain validated leaf contours  and petiole/blade junctions , teeth sinuses  and tips , and leaf apex  landmarks, and if necessary the hierarchy should have been determined  and/or  . If this is not the case go back to previous steps.
- 7.3. Go to the **analysis module** by clicking on the **Analysis** icon at the left of the *MorphoLeaf* window. Start the quantification by clicking **Process**, which generates the two quantification files containing the quantifications of leaf and teeth parameters. If you wish, you can modify the **Prefix name** of the files and their location by changing the **Output path**. The section [10. List of measures](#) describes the measures performed.

8. Reconstruction of average shapes

Here you will reconstruct average leaf shapes using either of two methods. The first method computes average shapes of leaves grouped in successive bins based on their blade length (the bins can be either automatically calculated or defined by the user). The second method computes moving averages (the sampling can either be automatically calculated or the user can define the blade length of the average shapes). In this method, the user has to enter the neighbor rank, which defines the contribution of the different leaf samples to the construction of the average shapes. For each of these two methods you can decide which landmarks will be taken into account to compute the average shapes.

- 8.1 . You can continue directly from step **6.4.** with an ongoing project and skip to 8.3.
- 8.2. Alternatively, start again Free-D, open an existing .sdf file containing your pictures by clicking the  icon or by clicking the **File** menu and **Open stack**, selecting your file of interest in the *Open stack* window. **Open an existing MorphoLeaf project or create a new one** as described in section [2. Create a new MorphoLeaf project or load an existing MorphoLeaf project.](#) At this step, your .sdf file should contain validated leaf contours  and petiole/blade junctions , teeth sinuses  and tips , and leaf apex  landmarks, and if necessary the hierarchy should have been determined  and/or  . If this is not the case go back to previous steps.
- 8.3. Go to the **normalization module** by clicking the **Normalization** icon at the left of the *MorphoLeaf* window.
- 8.4. Start by defining the parameters of the **Resampling** by setting the landmarks that will be used to resample the contours before performing the normalization. In addition to the leaf apex and

petiole/blade junctions you can also include the primary sinuses and primary tips by clicking **Primary Sinuses** and **Primary Tips**, respectively.

8.5. Next choose the **Normalization Method** you want to use.

8.5.1 To construct average shapes of leaves grouped in successive bins based on their blade length, click on **Based on Bins**. The bins can be automatically calculated by clicking **Automatically-defined bins** and entering the **Number of bins**. Alternatively, the number and the limits of the bins can be defined by the user by clicking **User-defined bins** and **Uploading** a text file (.txt) containing the size limits of the bins. For instance in the file below, two bins are defined, the first including the leaves with a blade length from 200 to 500 (excluded) and the second from 500 to 1000 (excluded). Therefore two mean leaf shapes will be generated.

200
500
1000

8.5.2 To construct moving average shapes of leaves click on **Moving averaging**. You have to define the **Neighbor Rank**, which corresponds to the number of leaves contributing to the each average shape. A low Neighbor Rank will lead to a low number of leaves contributing to each average shape, which may appear noisy. Reciprocally, a high Neighbor Rank will lead to a high number of leaves contributing to each average shape and a smoother overall form. A text file named *bandwidths* is generated that containing for each average shape the bandwidth used to take into account the leaves contributing to it.

The sampling of the moving average can be automatically calculated by clicking **Automatically-defined sampling** and entering the **Number of averages (blades)**. Alternatively, the number and blade lengths of the average shapes to be

generated can be defined by the user by clicking **User-defined target lengths** and **Uploading** a text file (.txt) containing the blade lengths of the average shapes. For instance, if the following file is uploaded, 2 mean shapes with a blade length of 300 and 700 will be generated.

300
700

8.6. You can set the **Output** parameters by defining the **Output path** where the files will be saved. Click **Process** to generate the mean shapes, which depending on the method used may take a few minutes. After the process is finished the generated mean shapes will be grouped in a new .sdf file that can be viewed in Free-D, and in multiple .cv files that can be viewed with the Sviewer application (<http://free-d.versailles.inra.fr/html/freed.html> - download).

9. List of landmarks.



Contour



Petiole = blade/petiole junction



Apex = blade apex



Primary sinus



Temporary sinus



Primary tip



Secondary sinus



Secondary tip

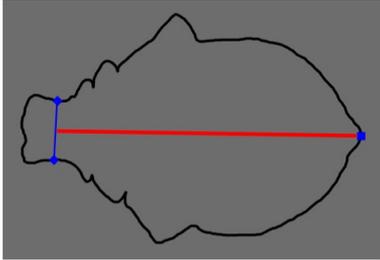


Primary distal sinus

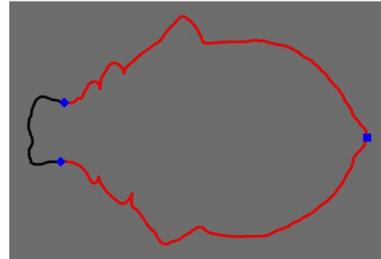


Primary distal tip

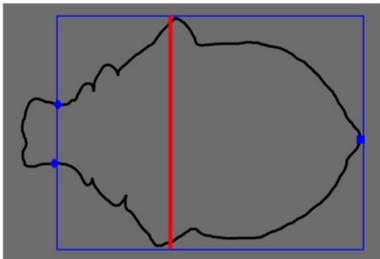
10. List of measures



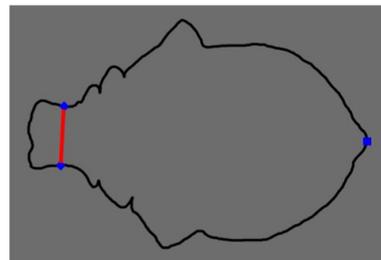
Blade_Length



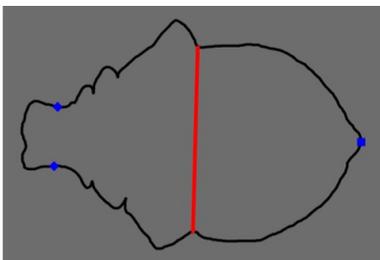
Blade_Perimeter



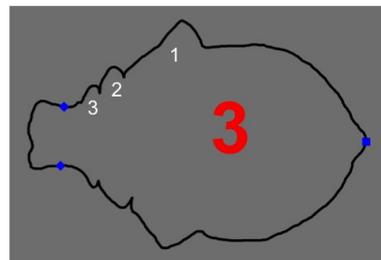
Blade_Width (BB)



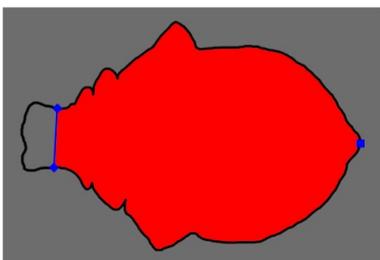
Petiole_Width



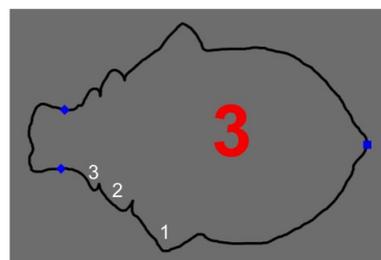
Blade_Width(IS)



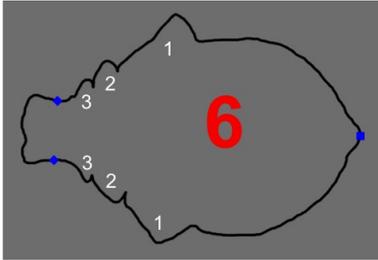
Upper_Teeth_Number



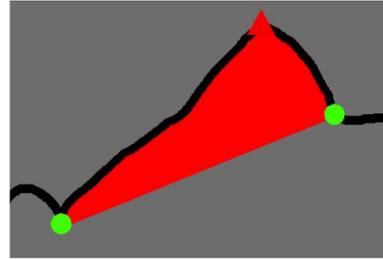
Blade_Area



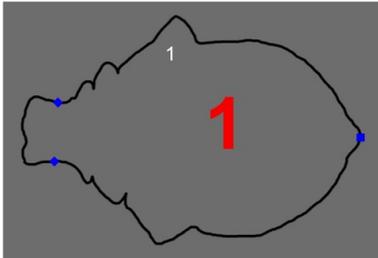
Lower_Teeth_Number



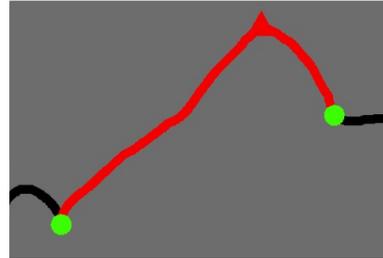
Total_Teeth_Number



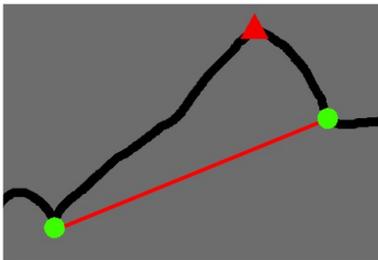
Tooth_Area



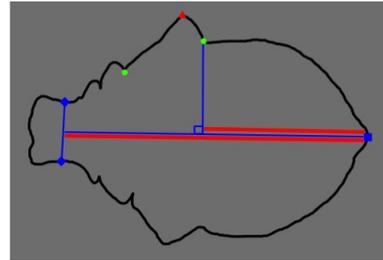
Tooth_Position



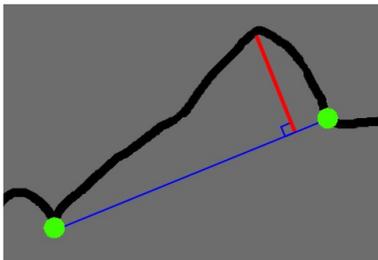
Tooth_Perimeter



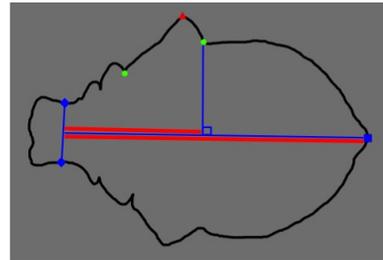
Tooth_Width



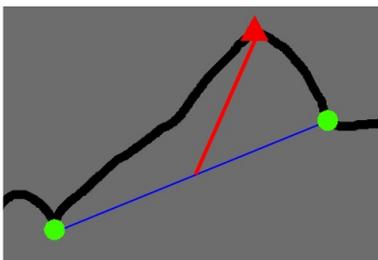
Tooth_Position_From_The_Tip



Tooth_Height (latitude)



Tooth_Position_From_The_Base



Tooth_Length (median)