A SHAPE ANALYSIS FRAMEWORK FOR SMALL ANIMAL PHENOTYPING WITH APPLICATION TO MICE WITH A TARGETED DISRUPTION OF *HOXD11*

Joshua Cates[*], *P Thomas Fletcher*[*], *Zachary Warnock*[**] *and Ross Whitaker*[*]

[*]Scientific Computing and Imaging Institute
University of Utah, Salt Lake City, UT, USA

[**]Howard Hughes Medical Institute
University of Utah, Salt Lake City, UT, USA

ABSTRACT

This paper proposes the use of high-dimensional, point-based shape models for the study of phenotype, which offer some important advantages over traditional morphometrics, including a more detailed representation of shape and a more systematic approach to shape statistics. Our approach allows for the analysis of shape at both global and local scales and can automatically extract lower-dimensional shape features useful for phenotypic characterization. We also demonstrate how covariates can be included in this framework. We apply the proposed modeling framework to a study of bone shape in mice with a targeted disruption of the *Hoxd11* gene.

***Index Terms*—** Image shape analysis, Genetics, Biomedical imaging

1. INTRODUCTION

Genetically modified mice have become commonplace models for the study of human development and disease. Modern gene targeting technology allows researchers to create specific alterations in a mouse genome that result in different patterns of anatomical growth and form—*phenotypes*. The phenotype of a mutant mouse population can be contrasted with that of a normal mouse population in order to gain insight into the functionality of the targeted genes. To measure differences in phenotype, researchers typically rely on light microscopy, histology, and traditional morphometric descriptions of anatomy, such as statistics of length and volume [1]. Increasingly, however, geneticists are exploring the use of 3D medical imaging technologies such as MRI and CT for phenotyping [2]. These imaging methods have the advantage that they are volumetric, non-invasive, and more easily adapted to high-throughput phenotypic screening. They also allow researchers to take advantage of a wide variety of image analysis algorithms, which has led to new morphometrics based on quantifying aspects of anatomical *shape*.

Anatomical shape from images can be represented and computed in many different ways. Typical representations of shape for phenotyping rely on explicitly chosen landmark positions to define relatively *low-dimensional* parameterized models. The variability in the shape of an insect wing, for example, might be measured as the variability in the positions where veins intersect, or the variability in animal skulls by the suture points between bones [3]. Researchers in the field of neuroimaging also conduct studies in comparative anatomy using shape, but have recently pioneered the use of *high-dimensional*, point-based models. Point-based models represent shape by automatically sampling each shape surface in a consistently ordered fashion so as to implicitly define surface point correspondences, which may be used to describe variability in a population of shapes [4, 5, 6]. Because point models may use arbitrarily many surface point samplings, they can provide a much more detailed description of anatomy than landmark-based models, and potentially offer more insight into the phenotypic expression of targeted genes. Furthermore, these methods are automatic, and thus are capable of processing larger datasets and producing results that are not influenced by the researcher's choice of landmark positions.

This paper proposes the use of high-dimensional, point-based shape modeling and analysis for small animal phenotyping. We describe a general shape analysis framework with relatively few free parameters that is based on recent shape analysis research and operates directly on volumetric images such as MRI and CT. We have applied this framework to the phenotypic study of several of the forelimb bones of mice with a targeted disruption of the *Hoxd11* gene. Phenotyping poses some new challenges for point-based shape modeling which we address in the context of the *Hoxd11* study. One major issue for phenotyping, for example, is how to detect subtle group differences in the presence of large anatomical variation, which is a common effect of targeted mutations. Another issue we discuss is how to control for covariates such as the differences in the developmental rate of mouse subjects. Our results from the *Hoxd11* study suggest that point-based shape modeling can be an effective tool for the study of mouse skeletal phenotype. We illustrate global and local group differences between normals and the *Hoxd11* mutants that are not observable with traditional metrics, and illustrate how we can use general linear models to control for covariates in global shape statistics. Finally, we show how an orthogonal decomposition of point-based shape models can inform the choice of lower-dimensional metrics of shape that may be more suitable for applications such as high-throughput phenotypic screening.

2. RELATED WORK

Recent research in image-based phenotyping using low-dimensional, landmark-based morphometrics is reviewed in [3], with a recent application to mouse skeletal phenotyping given by Dullin [7]. Several researchers have proposed higher-dimensional morphometric analysis of mouse brain phenotype using deformable registration between groups of mean MRI images [2], or deformable registration to a normal atlas [8]. These methods differ from the proposed point-based framework in that they represent shape only indirectly by the analysis of the deformation required to match an atlas, and they analyze regional patterns of variability, instead of the variability of specific anatomical structures. Frameworks that use point-based models for comparative studies of human brain anatomy have been proposed by Styner [4, 9] and Davies [5]. For this work, we use the particle-based modeling framework described by Cates, et al. [6], which has the advantage that it uses a nonparametric representation of shape and a parameter-free correspondence optimization algorithm.

3. METHODOLOGY

This section describes the proposed shape modeling and analysis pipeline and its application to the phenotype study of the *Hoxd11*-deficient mouse. The general overview of the pipeline is as follows. After breeding and imaging of the *Hoxd11* mutant and control specimens, segmentation of the CT volumes delineates the anatomy of interest. The segmentations are smoothed and aligned into a common coordinate frame using gross shape features. Point-based models are computed directly from the aligned volumes using a correspondence point optimization method, and statistical analysis is carried out on those models. The remainder of this section describes each of these stages in more detail. The results of the analysis are given in Section 4. The overall framework is general and modular, and thus some stages of this pipeline could be substituted with other methods. For instance, certain applications might warrant segmentation or correspondence methods that are different from those presented here.

3.1. *Hoxd11-/-* and Control Mice

The *Hox* complex of genes is known to play an important role in the proper development of the mouse, and a better understanding of the function of these genes may offer important insights into the cause of certain human birth defects. Through a series of gene targeting experiments, Boulet, Davis and Capecchi have previously shown that the *Hoxd11* gene in particular is especially important for the normal development and patterning of the appendicular skeleton [10, 11]. In [10], Davis prepares murine new born pup skeletons with alizarin red stain and measures the lengths of the autopod bones under digital light microscopy, a method of phenotype characterization that is the current standard for studies involving targeted disruption of gene sequences associated with skeletal development [1]. From these length measures, Davis, et al. hypothesize that mice deficient in the *Hoxd11* gene show significant differences in the average length of specific bones of the mouse forelimb.

For this study, 20 male mice homozygous for targeted disruption of the *Hoxd11* gene were bred from an existing colony of *Hoxd11-/-* female and *Hoxd11+/-* male mice. (Male *Hoxd11-/-* mice are infertile for unknown reasons). Details on creation of *Hoxd-11* mice by construction of a targeting vector and establishing a targeted ES cell line are described in [10]. *Hoxd-11-/-* was confirmed by PCR analysis and gel electrophoresis using tail DNA from each of the mice. *C57BL/6* (wild-type) male mice were used as the control group phenotype. Mice were sacrificed by carbon dioxide asphyxiation at 10 weeks and immobilized in a 50 mL centrifuge tube before scanning in order to minimize motion artifact and provide consistency in mouse position and placement for all scans. The eXplore Locus Small Animal MicroCT Scanner (GE Healthcare, London, Ontario), which utilizes a 3500 x 1750 CCD detector and Feldkamp cone-beam reconstruction, was used to perform high resolution 360 degree volumetric CT of each mouse.

3.2. Point-Based Shape Models

Following the breeding and imaging of the mice, three forelimb bones were segmented from the CT volumes by experts: the metacarpal (MC), the first phalange (P1), and the second phalange (P2) of digit 2 of the right forepaw of the mouse. These bones were chosen because the were identified in [10] as exhibiting significantly different average lengths in mutant and normal mice. The right humerus bone was also segmented as a variable to use in controlling for development rate, as indicated by [10]. For mouse bone segmentation, we use the Seg3D volume segmentation software (SCI Institute, University of Utah, www.seg3d.org) to compute a region-growing segmentation of the entire complex of forepaw bones, followed by a manual delineation of the boundaries between specific bones of interest.

The collection of shape segmentations must be aligned in a common coordinate frame for modeling and analysis. Where no a priori information is available to guide the proper alignment, we typically align segmentations with respect to their centers of mass and the orientation of their first principal eigenvectors. In the case of the mouse bones, we followed this automatic alignment step with a manual adjustment of the orientation around the principal axis, so that characteristic features of each each bone were in rough alignment. During the correspondence computation phase, this rough alignment is refined with respect to rotation and translation using a Procrustes algorithm applied at regular intervals in the optimization, which is an approach recommended in the shape analysis literature [12].

A binary segmentation contains an implicit shape surface at the interface of the labeled pixels and the background, but contains aliasing artifacts that must first be removed. We have found that the r-tightening algorithm given by Williams et al. [13] is effective in removing these artifacts without compromising the precision of the segmentation. Typically we follow the anti-aliasing step with a very slight Gaussian blurring to remove the high-frequency artifacts that can occur as a result of numerical approximations.

For correspondence point computation, we use the particle-based method described by Cates, et al. [6]. The general strategy of this method is to represent surfaces as discrete point sets that are distributed across an ensemble of similar shapes, via a gradient descent, so that their positions optimize the information content of the system. The optimization function is formulated so that it balances the entropy of individual surface samplings with the entropy of the shape model, maximizing the former for geometric accuracy (a good sampling) and minimizing the latter to produce a compact model. Point sets are modeled nonparametrically as dynamic particle systems, so that the method operates without any free parameters. For a full description of the correspondence point optimization method, the reader is referred to [6]. For the statistical analysis to follow, it is important that correspondences be computed without knowledge of the genetic classification of the shapes. We therefore compute shape models for a given anatomical object that include both the wild-type and mutant data, which we refer to as *combined* models.

For the *Hoxd11* study, we computed combined models for each of the three bones of interest, using 1024 correspondence points per shape and initializing with the splitting procedure described in [6]. We used the curvature-adaptive sampling strategy described in [6] to allow for oversampling in regions of higher curvature and thus a more detailed bone shape representation. Run times for each optimization using a *C++* implementation on standard 2GHz PC desktop hardware were between 2 and 3 hours.

3.3. Statistical Shape Analysis

Statistical analysis of shape for gene targeting is important for quantifying the abnormalities in growth and form of the targeted anatomy and identifying significant group differences between normal and mutant population (hypothesis testing). Using combined models, shape difference is quantified at both *global* and *local* feature scales by defining distance metrics in the shape space of the model that are used in traditional univariate and multivariate statistical analysis. The high dimensionality of the point-based model does not lend itself well to established statistical methods, and so shapes are typi-

cally first projected into a lower-dimensional, orthogonal decomposition of the shape space. A subset of the major modes of variation from a principal component analysis (PCA) may be used, for example. This type of analysis is intuitively appealing because a linear path through shape space along a major mode of variation describes a corresponding deformation of shape in the 3D image space. This deformation may characterize some interesting aspect of morphology, such as a stretching or bending.

For the global analysis of each of the three bones in the *Hoxd11* study, we perform a PCA of the shapes in the combined model. We consider the first principal component (PC1), i.e., the projection of each shape onto the major eigenvector of the covariance matrix, as a univariate measure of global shape. We then test the group differences in this measure between the mutant and and wild-type populations. It is important in this group analysis to take into account the variability in the rates of development of each mouse. We use the total humerus bone length, a bone not hypothesized to be affected by the *Hoxd11* gene, as a marker for overall developmental stage. Therefore, we test the group difference in the PC1 using an analysis of covariance (ANCOVA) model that includes the length of the humerus bone as a covariate. Finally, we wish to understand the relationship between the automatically chosen shape variable (PC1) and the morphometry-based shape variable (bone length) from the literature [10]. This is done by applying a linear regression to PC1 versus bone length and analyzing the correlation between the two measures. Bone length is measured automatically from the segmentations as the length of the projection of the segmentation onto its first principal component axis.

We define *local* shape features as regional patterns of variation on the shape surfaces, and analyze local group differences using the same combined models described above. The mean shape of each bone for the wild-type and mutant populations can be estimated as the Euclidean averages of their correspondence point positions. Point-wise vector-valued differences between the mean shapes are an indication of the directionality and magnitude of local shape differences. Styner et al. propose a point-wise hypothesis test for significance of the magnitude of local mean differences [9], with the null hypothesis that the distributions of correspondence point positions are the same regardless of group. The method is a nonparametric permutation test of the Hotelling T^2 metric with false-discovery-rate (FDR) correction to control for multiple-comparisons. For the mouse bone study, we applied an open-source implementation of the algorithm given in [9], using 20,000 permutations among groups and an FDR bound set to 5%.

For the *Hoxd11* data, we face a problem common to many phenotyping studies in that there is a dominant global shape effect induced by the gene targeting that obscures more subtle, local shape variations. The dominant effect is characterized by the first major mode of variation of the global shape analysis, which, for *Hoxd11* we show in Sect. 4 to be highly correlated with bone length. To analyze more subtle shape variation, it is helpful to remove the dominant effect in the local shape analysis. One approach is to simply normalize correspondences in the PC1 direction, but this approach does not remove the dominant effect on the correspondence optimization itself. For the mouse bone data, we instead perform a second correspondence point optimization, during which the correlated morphometric feature (bone length) is removed from the segmentations. This is accomplished by introducing a scaling transform for each shape that normalizes length along its principal eigenvector, and gives us a reasonable approximation to the point-based model with the dominant effect removed.

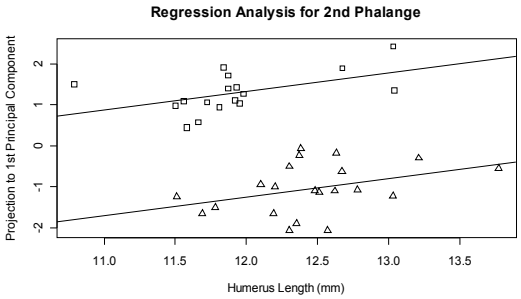

Fig. 1. ANCOVA for the PC1 measurements on the P2 bone. Wild-types are shown as squares and mutants as triangles.

4. RESULTS

As a part of performing the ANCOVA on the PC1 measurements, we first checked for interactions between group classification and the humerus length covariate. We found no significant interaction in all cases with p-values of 0.36, 0.68, and 0.99 for the MC, P1, and P2 bones, respectively. ANCOVA on the PC1 measurements for each bone shows significant differences in the group means of 8.54 mm for the MC, 4.02 mm for the P1, and 2.60 mm for the P2, all with p-values $\ll 0.01$. A graph of this analysis for the P2 bone is shown in Figure 1. (Graphs for MC and P1 are similar and are omitted here for brevity.) Correlation between bone length and the PC1 measures were high for the MC ($R^2 = 0.98$) and P1 ($R^2 = 0.95$), with less correlation seen in the P2 ($R^2 = 0.66$). All correlations were statistically significant at $p \ll 0.01$. These results support the hypothesis presented in [10] that reduced bone length is the dominant morphological effect of *Hoxd11*-deficiency, with the caveat that secondary effects may be influencing other bones such as the P2.

A local statistical shape analysis for the P1 bone is given in Figure 2, which shows the mean shapes of the mutant (a) and wild-type (b) populations. (Analysis of the MC and P2 bones are omitted for brevity.) The global length difference in the two means is easily observed from this comparison. Detailed views of the proximal and distal ends of the bone are shown to the right of the mean shapes. Arrow glyphs represent the orientation and relative magnitude of the mean shape differences ($a - b$), which, as a result of the dominant length effect, mostly point outwards along the long axis of the bone. Fig. 2c and d is a comparison of the mean shapes for mutant and wild-type populations when length is removed from the correspondence computation. The detailed views of the ends of these mean shapes now reveal more subtle shape differences that were not observed in a and b. At the distal end of the bone, for example, is a clearly visible indentation, which is indicated by the difference vectors ($c - d$) in that region. The proximal end shows a somewhat uniform difference in "thickness", with difference vectors generally pointing inwards towards the center axis of the bone. The p-values for the point-wise hypothesis tests indicate that group differences are significant ($p < 0.01$) everywhere for $a - b$. With the length effect removed, however, we see more interesting regional patterns of significant group differences ($c - d$), which are indicated by the more brightly-colored regions of Fig 2d. Note, for example, that the indentation at the distal end of the bone is now identified as a significant local shape difference between the two groups.

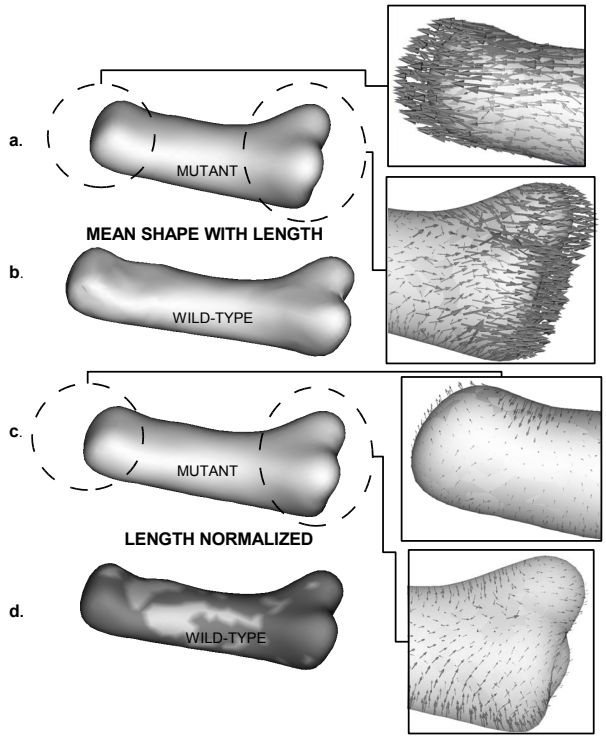

Fig. 2. Local statistical analysis of the P1 bone, showing the dominant effect of length and statistically significant local shape differences when length is removed.

5. DISCUSSION

Gene targeting is one of the most important tools for genetic study, and is widely used to examine the role that specific genes play in human development and disease. Because gene targeting studies often rely on metrics of shape to quantify phenotypic expression, more comprehensive and detailed representations of shape may allow for observations of genetic expression that have not been possible with current approaches. With traditional morphometrics, shape features are typically identified and parameterized in advance of statistical analysis. The analysis is therefore limited by the choice of features and by the feature scales. In this paper, we have proposed a high-dimensional, point-based modeling framework as an alternative tool for phenotypic analysis. This framework offers a more general and systematic approach to statistical shape modeling by requiring fewer assumptions about the model and representing shapes at much higher levels of detail. With point-based modeling, statistically interesting shape features are identified *automatically*, which is a key difference from traditional morphometrics and represents a major shift in the way shape is defined for gene targeting studies. Through our analysis of *Hoxd11*-deficient mice, we illustrate that point-based shape modeling is an effective approach to statistical analysis of group differences in both local and global shape features. Furthermore, global shape analysis can be used to identify effective lower-dimensional classifiers of genotype, which may be of interest in designing high-throughput screening protocols. Future work remains in order to explore the full potential of point-based shape modeling for phenotypic analysis. In particular, the study of methods for multivariate statistics, such as multivariate ANCOVA at the local shape scale and for multiple global features, is warranted.

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