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Variation in mouse pelvic morphology maps to locations enriched in Sox9 Class II and Pitx1 regulatory features

Variation in mouse pelvic morphology maps to locations enriched in Sox9 Class II and Pitx1 regulatory features

Running Title: Pelvic variation and gene regulation

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Abstract. Variation in pelvic morphology has a complex genetic basis and its patterning and specification is governed by conserved developmental pathways. Whether the mechanisms underlying the differentiation and specification of the pelvis also produce the morphological covariation on which natural selection may act is still an open question in evolutionary developmental biology. We use high-resolution Quantitative Trait Locus (QTL) mapping in the F_{34} generation of an advanced intercross experiment (LG,SM-G_{34}) to characterize the genetic architecture of the mouse pelvis. We test the prediction that genomic features linked to developmental patterning and differentiation of the hind limb and pelvis and the regulation of chondrogenesis are overrepresented in QTL. We find 31 single QTL-trait associations at the genome- or chromosome-wise significance level coalescing to 27 pleiotropic loci. We recover further QTL at a more relaxed significance threshold replicating locations found in a previous experiment in an earlier generation of the same population. QTL were more likely than chance to harbor Pitx1 and Sox9 Class II ChIP-seq features active during development of skeletal features. There was weak or no support for the enrichment of seven more categories of developmental features drawn from the literature. Our results suggest genotypic variation is channeled through a subset of developmental processes involved in the generation of phenotypic variation in the pelvis. This finding indicates the evolvability of complex traits may be subject to biases not evident from patterns of covariance among morphological features or developmental patterning when either is considered in isolation.

Keywords: Pelvis; *Sox9*; *Pitx1*; Evolutionary Genetics; Phenotypic Integration; Evolvability.

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Introduction

An outstanding question in evolutionary developmental biology and quantitative genetics is the extent to which development can produce covariation among traits in ways that potentially bias evolutionary trajectories over macro- and micro-evolutionary time scales (Cheverud, 1984; Hallgrímsson, et al. 2009; Rice, 1990; Wolf, 2002). An important step in realizing a unified account of genetics, development, and evolution is building developmentally explicit models of the ways in which developmental processes structure the transformation of genotypic and environmental influences into phenotypic variation (i.e. phenotypic integration) (Cheverud, 1984,1996; Hallgrímsson, et al. 2009, 2019; Pigliucci & Preston, 2004; Zeldich, 1988).

Comparative morphological and functional analyses of the pelvis have a deep history across multiple fields of study (Grabowski, 2013; Gregory, 1935; Gruss & Schmitt, 2015; Romer & Parsons, 1986). The pelvic girdle forms the anatomical interface between the hind limb and the axial skeleton and serves important roles in bearing the weight of and propelling an organism through its environment. In mammals, the pelvis can show strong sexual dimorphism including in aspects of the morphology of the birth canal, which plays important roles in parturition (Grunstra et al. 2019; McPherson & Chenoweth, 2012). Quantitative genetic investigations into pelvic morphology across several species (Carrier, Chase, & Lark, 2005; Chase et al., 2002, 2005; Kenney-Hunt et al., 2008; Kohn & Atchley, 1988) have provided insight into the genetic architecture of variation in this complex skeletal element. Like most aspects of the skeleton, morphology of the pelvis is moderately to highly heritable with robust genetic correlations among traits (Kohn & Atchley, 1988). Likewise, quantitative trait locus (QTL) and other gene mapping analyses show distributions of pleiotropic effects across pelvic traits typical of correlated metric morphological characteristics (Kenney-Hunt et al., 2008; Wagner et al., 2008). While these studies have served to enhance our understanding of the genetic basis of pelvic evolution, the QTL they identify are large and contain many genes, thus yielding limited insight into the location and identity of causative loci and their associated developmental mechanisms.

Most recently, these genetic approaches have been supplemented by studies in developmental biology with the aim of identifying the epigenetic processes involved in the specification, differentiation, and growth of the pelvic girdle (Capellini et al., 2011). Developmental investigations into pelvic form have given new insights into the mechanistic basis for the specification and differentiation of the hip bone (i.e. os coxa) and the adjacent,

articulated sacrum (Sears, Capellini, & Diogo, 2015; Young, Selleri, & Capellini, 2019). Together, they show three of the pelvis' constituent elements (less the sacrum), a cranially positioned ilium, a caudally/dorsally positioned ischium, and a ventrally located pubis, are specified early in embryonic development via the actions of key transcription factors, including *Pitx1* (Lanctot, Moreau, Chamberland, Tremblay, & Drouin, 1999; Marcil, Dumontier, Chamberlaind, Camper, & Drouin, 2003), *Pbx1-3* (Capellini et al., 2006, 2011; Selleri et al., 2001), and *Islet1*(Itou et al., 2012), which partition the cells of the early somatopleuric field into distinct cranial and caudal domains. After cell-fate specification, the action of other transcription factors, such as *Sox9* (Bi et al., 2001) and *Emx2* (Malashichev, Borkhvardt, Christ, & Scaal, 2005; Malashichev, Christ, & Prols 2008; Pellegrini et al., 2001), in cartilaginous anlagen then lead to cellular differentiation and the onset of endochondral ossification. Numerous signaling molecules interacting between the somatopleure, mesenchymal condensations, and surrounding tissues aid in the development and maturation of the ilium, ischium, and pubis (Young et al., 2019). While these studies provide vitally important insights into the genes and/or regulatory sequences involved in pelvic development, they do not provide an account of how development structures heritable phenotypic variation in pelvic form.

Here, we leverage the combined power of high-resolution QTL mapping and functional genomics to investigate the genetic architecture and developmental basis of heritable variation in the mouse pelvis. We integrate quantitative genetic and functional genomic approaches to test hypotheses about the mechanistic basis of the generation of genetic variation on which evolutionary processes might act to effect evolutionary change. Using genotyped and pedigreed individuals from the F_{34} generation of an advanced intercross design, we first establish the genetic basis of covariation in, and the effects of sex and diet on, eight linear morphological traits reflecting different aspects of ilium, ischium, and pubis morphology (Figure 1). We then identify QTL contributing to individual differences in morphology. Together, these provide a statistical first impression of the genotype-phenotype map for specific parts of the pelvis. Using bioinformatics on developmental genetic and functional genomics features, we next test hypotheses about the relative enrichment of QTL for genes with known roles in the development of the pelvis or bony tissue in general and several classes of regulatory features known to be active in the development of the pelvis and/or hind limb. These tests allow us to generate a refined picture of the phenotypic integration of the pelvis by identifying candidate mechanisms

for the conversion of genomic variation into phenotypic variation in pelvic morphology.

Materials and methods

Animal subjects and care. All experiments using mice were approved by and conducted in accordance with the standards of the Institutional Animal Care and Use Committee (IACUC) of Washington University School of Medicine, St. Louis. Mice used in this study were acquired from the F_{34} generation of an advanced intercross (AI) experiment (LG,SM-G34) descended from an initial cross of LG/J females and SM/J males obtained from The Jackson Laboratory. F_1 hybrids from this cross were then intercrossed to produce an F_2 generation. From the F_2 generation onwards, the animals were mated at random except to avoid brother sister pairs and minimize variation in the contribution of full sibships to the next generation. Half of the individuals in each sex in each litter were fed a high fat (#TD88137, Harlan Teklad) or low fat diet (#D12284, Research Diets) with similar caloric content starting at weaning (at 3 weeks of age). Detailed explanations of the breeding and handling of the mice can be found in Norgard et al. (2011). The F_{34} generation includes ≈ 990 skeletonized individuals from 137 full sibships depending on the measured characteristic (see Table 1 for sample size and summary statistic information). Carcasses were skinned and skeletonized using dermestid beetles.

Genotyping. Each individual mouse was genotyped for 2,842 single nucleotide polymorphisms distributed across the autosomal genome. These SNPS were drawn from the Oxford/CTC SNP set (http://www.well.ox.ac.uk/mouse/INBREDS/) and are all polymorphic between LG/J and SM/J inbred mouse strains. The allelic states of SNPs were assessed using the Illumina GoldenGate Bead Array (Illumina, San Diego, CA) at the Center for Inherited Disease Research. The SNPs were spaced at intervals averaging ≈ 0.5 centiMorgans (cM) scaled to the F_2 map and ≈ 8.5cM scaled to the F_{34} map.

Phenotyping. Individual hip bones (ossa coxae) were embedded in florist foam forms and subjected to micro-CT scanning at 34.5 µ resolution using a Skyscan 1172 (Bruker) µCT. The images were reconstructed in NRecon (Bruker) and then processed, visualized, and scored for three-dimensional coordinate data at landmarks using AMIRA (ThermoScientific). A set of eight linear distances among landmarks was used in this analysis. These were chosen to cover

dimensions of the pelvic bone to reflect overall form and function of the pelvis, to capture dimensions related to the structure of each pelvic sub-element (i.e., ilium, ischium, and pubis), and to capture traits figuring prominently in arguments about the evolution of the pelvis in vertebrates, especially humans and other mammals (Figure 1). All measurements on all individuals were taken by a single individual. In the case of several traits, multiple inter-landmark distances were averaged to minimize the effect of intra-observer measurement error. These include the lengths of the ischium, ischium to caudal iliac blade, pubis length, ischium to pubis length, and pubis to cranial ilium (see Figure 1 for details). A subset (n = 72) of the bones were scanned, reconstructed, and measured twice to assess the repeatability of the results. Repeatabilities in the form of within individual cross-replicate variance were estimated for each trait using ANOVA (Sokal & Roff, 1995). Individual traits were highly repeatable, nearly always exceeding 95%, and the averaged traits showed even higher repeatabilities.

Quantitative genetic analysis. Additive genetic variance-covariance matrices were estimated using a mixed effects model fit with the MCMCglmm package (Hadfield, 2010) in the R statistical computing environment (R Core Team, 2018). An additive genetic relationship matrix among individuals obtained using a pedigree was used to model the random effect of relatedness. Covariates consisted of terms for diet (either high- or low-fat), sex (male or female), whether an individual came from a large or small litter (a number of pups equal to or fewer than vs. greater than the number of nipples on a dam), and age at sacrifice (in days). We used a weakly informative prior with a degree of belief parameter equal to the number of traits assuming a heritability of 0.5 for all traits and no covariance among them.

Posterior distributions for the model parameters were obtained in each case by sampling over 1,000,000 iterations using a thinning interval of 500 after a burn-in time of 500,000 iterations. We used the posterior distributions to calculate estimates of narrow sense heritability (h^2), evolvability (*e*, Hansen & Houle, 2008), and the additive genetic (**G**) and environmental (**E**) covariance. Convergence was assessed by inspecting the plots of the traces of the Markov chain and ensuring auto-correlation across samples was acceptably low for all terms ($r \approx 0.1$).

QTL mapping. We estimated the locations of QTL using a mixed model extension of the Haley-Knott (Haley & Knott 1992) method, tailored for use in advanced intercross experiments using

the QTLRel package (Cheng, 2011). Probabilities of genotypic scores were interpolated between scored markers at 1 cM (on the F_{34} scale. ≈ 0.06 cM on the F_2 scale) intervals between loci containing scored SNPs. We fit models at each marker location and each imputed intervening cM location. The covariates age, litter composition, sex, and diet were included in the model. Additive (*a*) and dominance (*d*) genetic effects and their standard errors were estimated using a generalized linear model controlling for covariates and relatedness among individuals. Unlike in the F_2 generation of an intercross experiment, later generations have family structure. As such, we include the combined polygenic effect of all genes on individual differences in the model. We did so by using additive and dominance relationship matrices drawn from the same pedigree used to estimate the genetic covariance among the traits.

We fit genetic models including additive and dominance effects on each trait at each genotyped locus and imputed intermediate position. We compared the fit of the basic model for each trait at each imputed location in the genome to the fit of a null model including no genetic terms using a likelihood ratio test and expressed the differences in fit as a LOD score.

Thresholds for acceptance of a region as a QTL were estimated by adjusting the minimum acceptable LOD value using a Bonferroni correction based on the effective number of loci on each chromosome (Li & Ji 2005). The seventeen-fold increase in the length of the genetic map of the F_{34} generation over the map of the F_2 generation of the same population means some individual chromosomes in the F_{34} are about as long as the entire F_2 genome. As such, we followed the suggestion of Chen and Storey (2006) and chose the chromosome-wise threshold to accept QTL. The chromosome-wise thresholds ranged from 3.66 on chromosome 1 to 2.92 on chromosome 19 and the genome-wise threshold was 4.72 (Table 2). We used a relaxed threshold to evaluate the LOD scores within regions identified in a study of pelvic traits in the F_2 generation of the same population (Kenney-Hunt et al., 2008). Given the subtle differences in the traits and the ways in which they were measured, we set the relaxed threshold to LOD = 2.8, reflecting eight chances from eight traits to find an effect within the bounds of a single QTL discovered in the F_2. This allows evaluation of the replication of results across generations and provides an expanded set of regions to test for enrichment of genomic properties (see below). We used a 1.5 LOD drop-off criterion to identify the confidence regions for QTL (Manichaikul et al. 2006).

The additive and dominance effects of QTL on each trait and their standard errors were

estimated using a generalized linear model. In cases where there were pleiotropic effects of a QTL across multiple traits, each with a slightly different peak location within the QTL, the effects on the respective traits were estimated using the location corresponding to their individual highest LOD score. The genomic location of each QTL interval was expressed in terms of mouse genome version mm9.

Functional genomics analysis. Each QTL was queried against the MGI Gene Expression Data resource (Smith et al., 2014) to identify those genes: a) Expressed in the developing pelvic girdle; b) Expressed in skeletal tissues in general; and c) In the MGI Mutant Allele Function resource (Eppig et al. 2015) of mutants affecting the pelvis or the growth of bone. We also drew on published gene enhancer locations (see below) known to be associated with limb formation and another set of the same verified in VISTA (Infante, 2015). We recorded which elements lay within the bounds of each of the QTL by category to achieve a qualitative sense of the overlap between genes suspected to play central roles in pelvic/bone development and those regions of the genome we identify as contributing to individual differences in pelvic morphology.

In a second analysis, we tested to see if the QTL identified were enriched for these features relative to random locations in the genome. We examined potential Pbx1 and Emx2 dimerization motifs (Capellini, et al. 2011), ChIP-seq identified Pitx1 binding peaks (Infante, Park, Mihala, Kingsley, & Menke, 2013), ChIP-seq identified Sox9 binding peaks (Generic and Class I and II. Ohba, He, Hojo, & McMahon, 2015; Liu et al., 2015), Sox9 SuperEnhancers (Liu & Lefebvre, 2015; Ohba et al., 2015), H3K27ac marked regulatory elements (of the flank and hind limb expressed at age E11.5. Infante et al., 2013), and DNase I hypersensitivity data from the ENCODE database generated on hind limb and flank tissues (ascertained at age E11.5. ENCODE Consortium et al., 2007).

These different sets of genomic features were systematically assembled and examined for intersections with QTL using the UCSC Table Browser Intersection Tool (Karolchick, 2004), as opposed to being collected from the literature and is thus not subject to biases inherent to happenstance collection. We used a χ^2 test to see if each class of elements was represented in the QTL more often than we would expect if drawn from the entire genome by chance. We conducted separate tests on the results derived from the F_{34}-only results and the results including replicated results across the F_2 and the F_{34}. We performed a total of 13 tests for each set of QTL,

one for each of the class of features suspected to be involved in the development of the pelvic girdle, bone development, and/or hind limb and flank development, and one for the occurrence of known or suspected genes based on functional effect or expression. We judge significance using a Bonferonni-adjusted significance threshold for 13 tests ($\alpha \approx 0.0038$).

Data availability statement The phenotypes, covariates, pedigree, and genotypes used in this study will be made available on the Dryad repository in the event that the paper is accepted. These data may also be obtained on request to the corresponding author.

Results

As is the case with most morphological features, we find there is ample genetic variation in pelvic traits underlain by many loci, each with a small effect. The QTL identified in the F_{34} strongly replicate those found in an earlier generation (F_2) of the same experimental population. We find few genes with known or suspected roles in development of pelvis/hind limb in the QTL. The QTL are, however, enriched in some classes of regulatory features known to be active in pelvis and hind limb development.

Ample genetic variation in the pelvis. Summary statistics for the linear pelvic traits in the population are presented in Table 1. All traits were moderately to highly heritable and had evolvabilities well within the range of values observed for morphological traits (Table 1), especially those reflecting skeletal form (Cheverud, 1988; Hansen, Pélabon, & Houle, 2011). The evolvability estimate for Ilium Width stood out as being considerably larger than those calculated for other traits. Otherwise, the relative values of the estimates of the magnitude of additive genetic variation were not particularly different among pelvic traits. The genetic and phenotypic correlations showed strong similarities in their distribution among traits (Matrix correlation of R = 0.92 with a posterior credible interval of 0.86 to 0.96) as is typical for morphological traits when sample sizes are large (Cheverud, 1988).

Multiple loci of small effect contribute to genetic variation in the pelvis. For the analysis using only results from the F_{34} generation, we detected 31 single trait QTL significant at the genome- or chromosome-wise significance threshold across 12 of the 19 autosomes, which coalesce into 27 pleiotropic QTL (see Table 3 for a summary of their locations and their effects). Six QTL

reached genome-wise significance (LOD⩾ 4.7). The QTL spanned regions ranging between 1.1 and 10.0 Mb. Most of the QTL affected only single traits at this level with two having significant effects on two traits (QTL *Pelvis* 4.4 affecting Ischium to Caudal Iliac Blade and Pubis to Ilium Length; *Pelvis* 12.2 affecting Ilium Length and Ischium Length) and one affecting three traits (QTL *Pelvis* 13.2 affecting the traits Ischium to Caudal Iliac Blade, Ischium Length, and Ischium to Pubis Length). Most loci, however, had significant effects on other traits at a pointwise ($p = 0.05$) level in detected QTL, indicating some degree of pleiotropy. These results are similar to those obtained by previous studies of skeletal traits, which resulted in a small number of highly pleiotropic QTL (Wagner et al., 2008).

In general, QTL had statistically significant additive effects with the Lg/J derived allele tending to increase the value of the trait. Of the 31 single trait QTL in the F_{34} generation, 27 had statistically significant additive effects, 21 of which indicated the allele derived from the Lg/J strain increased the value of the trait relative to the Sm/J derived allele. The magnitude of the additive effects averaged 1.1% of the mean of their respective traits, ranging from 0.2% to 3.9%. Dominance effects were also evident, with 12 of 31 single trait QTL in the F_{34} exhibiting statistically significant dominance effects and three displaying signs of under- or over-dominance. Dominance effects averaged 0.75% of the mean and ranged from 0% to 3.5%. On average, each single QTL accounted for 3.1% of the phenotypic variance of its respective trait, with a minimum of 1.9% and maximum of 12.3%.

Strong replication of results across generations. Comparing the results of the present study to a similar study conducted on the F_2 generation of the same population (Kenney-Hunt et al., 2008) yielded substantial overlap between the two studies. Of 27 QTL (discounted for pleiotropy) identified in this study, 18 of them replicated results from the F_2 generation of the same population (Table 3). Moving from the F_2 generation to the F_{34} generation, 42 of 58 of the Kenney-Hunt (Kenney-Hunt et al., 2008) QTL replicated for at least one pelvic measurement in the F_{34} with a LOD ≥ 2.3 (Supplementary Table 1). However, a conspicuous mismatch between the two studies is apparent in the results on chromosome 12, where it appears that several QTL with opposite additive effects not apparent in the F_2 now emerge in the F_{34} generation. Recombination in the intervening generations might have led to them segregating with sufficient independence to become distinguishable in the F_{34} generation.

Few genes with known roles in bone development reside in QTL. In the QTL identified in the F_{34} analysis and the QTL from the combined F_2/F_{34} analysis, we found there was a modest representation of genes with known roles in pelvic or bone development, as assessed by intersections with databases on gene expression and function (Table 4 and Supplementary Table 2). Interestingly, many of the genes implicated as having core roles in the early patterning of the pelvic girdle, which include genes known to affect the patterning of the pelvis (Young et al., 2019) including the ilium (*Tbx4*, *Emx2*, *Fgf10*, and *Pbx1/2*), the pubis (*Alx1/4*, *Prrx1*, and *Twist1*), and possibly the ischium (*Pax1*) were absent from QTL. However, residing within these intervals are two factors playing notable roles throughout pelvic development particularly at mesenchymal condensation and chondrogenesis stages. The first is *Sox9*, present on chromosome 11 in a QTL influencing Ischium Length (QTL *Pelvis 11.3*), which reaches chromosome-wise significance (LOD = 3.56). The second is *Pitx1*, present on chromosome 13 at 335 cM, well within the bounds of the QTL influencing Ilium Width detected using the combined F_2/F_{34} analysis (*Pelvis F2/F34 13.01*, Supplementary Table 2).

QTL are enriched with regulatory features involved in aspects of pelvic development. We tested whether our identified QTLs are enriched for genomic features known to be involved in the development of pelvic, limb, and bone development. To carry out these analyses we first mined several published transcription factor and histone marker ChIP-seq, DNase I HS, and *in silico* transcription factor prediction datasets on developmental regulation of limb development (see methods) and matched their locations with QTL intervals and the genome-wise distribution of elements. Judged against a Bonferroni-corrected significance level ($p = 0.0038$), five of twelve classes of features known or predicted to play a role in the regulation of pelvic girdle, skeletal, and limb development were more frequent in the QTL identified in the F_{34} alone than was expected by chance (Table 5). In addition, six of twelve classes were overrepresented in QTL from the combined F_2/F_{34} analysis (Table 5). Specifically, we found Pitx1 ChIP-seq signals, reflective of the locations in the genome where Pitx1 physically binds during hind limb development, were particularly strongly enriched within the QTL. Likewise, Sox9 Class II ChIP-seq peaks, indicative of Sox9 binding in chondrocytes, were also highly enriched relative to chance in both tests. In the results from the F_{34} alone, generic Sox9 ChIP-seq peaks, as identified

by Liu et al. (2015), were also enriched at the Bonferroni-corrected level but only at the single test ($p = 0.05$) level in the combined F_2/F_{34} QTL. Conversely the Sox9 Class I ChIP-seq peaks were not overrepresented in QTL from the F_{34} only analysis but were in the combined F_2/F_{34} QTL. With respect to histone ChIP-seq assays on the E11.5 limb and flank using H3K27ac, typically considered a marker of active enhancers, only those called hind limb peaks were enriched and only in the combined F_2/F_{34} QTL analysis. Despite their hierarchical roles in pelvic patterning, *in silico* predicted Pbx/Emx2 binding sites were not enriched in either the F_{34} QTL alone or in the combined F_2/F_{34} results when multiple tests were taken into consideration. Finally, known genes were overrepresented in QTL in both cases.

Discussion

The principle innovation of this study lies in the connection between variation (QTL) and developmental genetic (i.e., regulatory mechanisms) accounts of pelvic morphology. Our first goal was to characterize the genetic basis of variation in the mouse pelvis using statistical quantitative genetic and QTL mapping techniques. A second goal was to assess the extent to which a QTL-based quantitative genetic account of the generation of individual differences showed signs of being structured by different classes of developmental processes. This was accomplished by way of testing to see if the QTL identified here contained genes from a suite known to be involved in pelvic or limb development. Likewise, we tested to see if the QTL identified here were enriched for a series of features that were drawn from the developmental literature and suspected to be involved in pelvic development.

We found variation in the mouse pelvis is a complex interplay of many genetic and environmental influences acting through the life course, as has been the case for other morphological characteristics (Cheverud, 1988; Kruuk, Slate, & Wilson, 2008). Moreover, not all developmental pathways involved in the development of the pelvic girdle appear to channel the genetic influence responsible for generating individual differences in this population. This conclusion, however, is tempered by our relative dearth of knowledge on the developmental genetic mechanisms governing the pelvis, as compared to the limb for example (Sears et al., 2015; Young et al., 2019). Thus, knock-out and other experiments using pelvic gene mutations of large effect may not afford clear insight into the mechanisms that can vary in natural populations.

It is important to emphasize that our results depend on the particular QTL we identify. If

360 animals are drawn from different original stocks, they will likely display different patterns of
361 genetic variation. As such, other experiments might identify different regions of the genome as
362 being important for pelvic variation and these regions might contain different genomic elements
363 than those identified here. Whether these alternate suites of QTL might display rather different
364 patterns of association with genomic elements is an empirical issue resolvable by replicating this
365 study in another experimental population.
366
367 *Variation in pelvic morphology is caused by many loci of small effect.* With respect to the
368 additive genetic covariation, the morphology of the pelvis is highly to moderately evolvable and
369 heritable and shows substantial genetic correlations among traits. As is typical for metric
370 morphological traits, the QTL tend to be detected for a single trait with chromosome- or genome-
371 wise significant effects (Wagner et al., 2008) with few highly pleiotropic loci. Each QTL also
372 accounted for a small proportion of the total genetic variance in each trait with no one trait's
373 variance being fully accounted for by the effects of QTL.
374 The additive effects of QTL show the substitution of an allele derived from the large
375 founder strain for one from the small strain tended to increase the value of the trait, which is
376 consistent with the evolved differences between the founding strains. There are, however, a fair
377 number of exceptions in which the SM/J derived allele imparts an increase on the trait value. The
378 change in genomic background and developmental context brought on by the crossing of the two
379 strains might have led to novel epistatic interactions and changes in the effects of the alleles.
380 Alternatively, alleles with effects contrary to the direction of selection in either founder strain
381 may have been fixed by random genetic drift or drafted along with linked alleles of stronger
382 effect during the selection used to produce the strains.
383
384 *Skeletal growth rather than patterning may generate evolvability of pelvic morphology*. To date,
385 most of what is known about the developmental genetics of the pelvis relates to the roles of early
386 transcription factors and signaling molecules during the bone's patterning stage. Indeed, this
387 stage has been most intensively studied because of the finding that the pelvic field is closely
388 affiliated with the early limb field (Capellini et al., 2011; Sears et al., 2015). Thus, it is not
389 surprising that our understanding of the patterning mechanisms of pelvic development has been
390 influenced by targeted studies in limb development, which have characterized factors necessary

for the development of both structures.

Conspicuously absent from our pelvic QTL are many genes with known involvement in the basic patterning of the pelvic girdle even though there are several transcription factors with specific roles in the development of the individual pelvic elements. This may arise from a partial separation between the action of gene regulatory networks and other developmental pathways responsible for laying out the basic patterning of a developing structure and those influencing variation through growth among non-pathological adults. Of a set of genes identified as playing a crucial role in the patterning of the mouse pelvic girdle by Young et al., (2019), Sears et al. (2015), and others (Capellini et al., 2006, 2011; Itou et al., 2012; Lanctot et al., 1999; Marcil et al., 2003; Selleri et al., 2001), including *Emx2*, *Fgf10*, *Pbx1/2*, *Pitx1*, *Sox9*, and *Tbx4* in ilium patterning, *Alx1/3/4*, *Islet1*, *Prrx1*, and *Twist1* in pubis patterning, *Pax1* and *Islet1* in ischium patterning, only *Sox9* was included in a QTL identified in the F_{34}-only analysis while *Pitx1* was found using additional results from the F_2. These results are contingent, in part, on the particulars of the population in which we mapped the QTL. Different experiments on crosses of other mouse strains might well lead to different QTL being discovered and different sets of genes might be represented in those QTL.

The lack of known genes with central roles in patterning marking the early development of the pelvis may indicate the patterning stages are not periods of development during which proper function of the organism can tolerate variation and thus constitute constraints on evolution via internal stabilizing selection (Cheverud, 1984). This difference in variability (i.e. the propensity for a system to generate variation) between early processes of patterning and later processes including endochondral ossification is supported at the genomic level in part by a lack of enrichment in many genomic elements related to the patterning stage of limb and pelvis development. We interpret this result as indicating that variation in the skeletal morphology of the mouse ilium, ischium, and pubis may not be generated during early stages of pelvic patterning but rather at later stages involved in endochondral bone growth and ossification (See also Sanger et al., 2011). Future work targeting both developmental windows for the locations of important transcriptomic and epigenomic signatures along with functional and biomechanical analysis will address this issue more concretely.

Comparing variational and functional genomic results. Little is known about the developmental

processes that permit the generation of variation in the pelvis. The results of our investigation into the developmental correlates of variation demonstrate that not all classes of genomic features known to play some role in the development of the pelvis and/or limb appear to be enriched in the QTL we identify as contributing to variation in the pelvis in this population. As elaborated on above, we emphasize "in this population" as the particular associations between genes and other genomic features and regions of the genome identified in QTL analyses may differ across experiments.

In the case of Pbx/Emx2 sites, where we do not find significant enrichment of the QTL after considering multiple tests, Capellini et al. (2011) reported that mutations to the different *Pbx* genes often resulted in complex pelvic phenotypes (i.e., those influencing both cranial and caudal pelvic structures, and more often than not resulting in complete loss of pelvis rudiments during development), not simply by additively regulating variation in pelvic girdle morphology. In combination with our results, this would suggest that while Pbx family members hierarchically regulate various downstream factors, including *Emx2*, and factors responsible for ilium and pubis patterning (Capellini et al., 2011), phenotypic variation arises through other inputs into these systems. In this scenario, genetic influences resulting in variation in adult phenotypes may not be introduced into the population through this set of mechanisms, perhaps because of this (i.e., Pbx/Emx2) network's overarching effects on both cranial and caudal pelvic structures. On the other hand, influences acting through *Sox9* and potentially its target genes during chondrogenesis appear to be important for the generation of genetic variation in this population. We also found that E11.5 HL/Flank H3K27ac signals, which serve to mark active enhancers, were also not overrepresented in our QTL. This may indicate that, while this kind of epigenetic modification may be important for the specification of the hind limb and pelvic girdle, it may not serve as a mechanism by which genetic variation in pelvic form is generated.

While Sox9 SuperEnhancers do not appear to be overrepresented in our QTL and we get mixed results for generic Sox9 ChIP-seq results (Liu et al., 2015), we have a clear signal for the enrichment of Sox9 Class II ChIP-seq peaks (Ohba et al., 2015) in our QTL in both the F_{34} only analysis and in the combined F_2/F_{34} analysis. The Sox9 Class II features are known to be highly tissue specific and involved in regulating chondrocytes through the direct binding of Sox9 complexes to the DNA itself (Pellegrini et al., 2001), thus making the Sox9 Class II regulatory mode a prime candidate mechanism for understanding how individual differences in pelvic form

are generated. In this scenario, allelic variation that modifies Sox9 binding events may be governing pelvic variation. As Sox9 is a key regulator of chondrogenesis, these results also support the hypothesis that endochondral ossification and the growth it promotes may more likely be targeted by evolutionary processes than early patterning.

The *Pitx1* gene has an important role in specifying the hind limb or pelvic fin structures across vertebrates and has been implicated as a primary contributor to variation in the pelvic apparatus in populations of stickleback fish enabling them to adapt quickly to different environments (Chan et al. 2005; Thompson et al., 2018). Moreover, mutations to *Pitx1* in mice result in ilium dysmorphologies among other more subtle pelvic and hind limb alterations (Marcil et al. 2003). The weight of evidence from developmental investigations in mammals suggest *Pitx1* plays multiple roles in regulating the endochondral bone growth in the hind limb through both the proliferation of chondrocytes and their terminal differentiation thus laying down the conditions for ossification. This makes it a prime candidate for a process important for driving the generation of phenotypic variation (Infante et al. 2013; Marcil et al. 2003).

Our results support this contention as all classes of Pitx1 ChIP-seq features were overrepresented in the QTL identified here indicating regulation of growth through this set of mechanisms may contribute to individual differences in this population of mammals. In the case of *Pitx1* and the evolution of the pelvic apparatus in stickleback fish, the *Pitx1* gene itself shows signs of being highly conserved, with adaptive differences among groups being driven by natural selection acting on variation in its associated regulatory features (Chan et al., 2005; Thompson et al., 2018). Our results support the position that *Pitx1 cis*- and *trans*-regulation is an important regulator of phenotypic integration in the pelvic girdle and may be important for rendering the pelvic girdle evolvable across many species of vertebrates. This latter point is supported by our finding of a QTL affecting Ilium Width in the F_2/F_{34} replicated set containing the *Pitx1* gene (QTL *Pelvis F_2/F_{34} 13.01*). Ilium Width is the most evolvable structure in our dataset and the only trait standing out from the rest in terms of its variational properties.

Conclusion

Mechanisms underlying the basic patterning of morphology may not always be involved in the generation of covariation among traits available for natural selection and random genetic drift to

cause evolutionary change. Here, we demonstrated Pitx1 and Sox9 Class II regulation are important mechanisms for the phenotypic integration of the mouse pelvis. That Pitx1 regulation may be an important developmental mechanism for the generation of covariation in this mouse population is particularly exciting given its role in structuring variation allowing adaptation of the pelvic apparatus of populations of stickleback fish to new environments (Chan et al., 2005; Thompson et al., 2018).

While the genetic basis of covariation in the mouse pelvis is complex and the product of many overlapping influences acting through development, developmental mechanism-specific channeling of genetic influences on morphology may lead to strong biases in the ways in which covariation can occur in populations given different distributions of segregating alleles and interactions with the environment (Cheverud, 1984). These biases in the generation of covariation through development might have both constrained and enabled the evolutionary trajectories leading to the diversity of pelvic girdle morphology we see today.

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