

Table S1. Genomic loci reported to be associated with split-hand/foot malformation.

Gene symbol	Gene name	Entrez Gene number	Chromosome locus	Mendelian Inheritance in Man phenotype number	Evidence for involvement in limb development in animal models
<i>BHLHA9</i>	Basic helix-loop-helix family member a9	727857	17p13.3	612576	<i>Bhlha9</i> knockdown leads to truncated pectoral fins in zebrafish (Klopocki et al., 2012)
<i>CDH3</i>	Cadherin 3	1001	16q22.1	225280	<i>Cdh3</i> is expressed in the apical ectodermal ridge of mice (Kjaer et al., 2005)
<i>DLX5</i>	Distal-less homeobox 5	1749	7q21.3	183600; 220600	Double knockout of <i>Dlx5</i> and <i>Dlx6</i> in mice phenocopies split-hand/foot malformation (Merlo et al., 2002; Robledo et al., 2002)
<i>DLX6</i>	Distal-less homeobox 6	1750	7q21.3	183600	Double knockout of <i>Dlx5</i> and <i>Dlx6</i> in mice phenocopies split-hand/foot malformation (Merlo et al., 2002; Robledo et al., 2002)
<i>EPS15L1</i>	Epidermal growth factor receptor pathway substrate 15 like 1	58513	19p13.11	-	Double knockout of <i>Eps15l1</i> and <i>Eps15</i> in mice leads to absent limbs (Milesi et al., 2019)
<i>FGFR1</i>	Fibroblast growth factor receptor 1	2260	8p11.23-p11.22	615465	<i>Fgfr1</i> -deficient mice have truncated limb buds (Xu et al., 1999)
<i>FGFR2</i>	Fibroblast growth factor receptor 2	2263	10q26.13	-	<i>Fgfr2</i> deficiency in mice leads to absent limb buds (Xu et al., 1998)
<i>HOXD13</i>	Homeobox D13	3239	2q31.1	606708	Deletion of both <i>Hoxd13</i> and <i>Hoxa13</i> in mouse embryonic limbs leads to truncated limbs (Kmita et al., 2005)
<i>IGF2</i>	Insulin like growth factor 2	3481	11p15.5	-	<i>Igf2</i> is expressed in the apical ectodermal ridge of mice (Cao et al., 2019)

<i>MAP3K20</i>	Mitogen-activated protein kinase kinase 20	51776	2q31.1	616890	<i>Map3k20</i> inactivation in mice leads to duplicated hindlimbs (Spielmann et al., 2016)
<i>TP63</i>	Tumor protein p63	8626	3q28	605289	<i>P63</i> -deficient mice have truncated forelimbs and absent hindlimbs (Mills et al., 1999; Yang et al., 1999)
<i>PRDM1</i>	PR/SET domain 1	639	6q21	-	<i>Prdm1</i> is expressed in the apical ectodermal ridge of chick limb bud (Ha & Riddle, 2003), and <i>Prdm1</i> loss impairs fin outgrowth and patterning in zebrafish (Lee & Roy, 2006)
<i>UBA2</i>	Ubiquitin like modifier activating enzyme 2	10054	19q13.11	619959	<i>Uba2</i> knockout leads to truncated pectoral fins in zebrafish (Schnur et al., 2021)
<i>WNT10B</i>	Wnt family member 10B	7480	12q13.12	225300	<i>Wnt10b</i> is expressed in the apical ectodermal ridge of mice (Witte et al., 2009)
Unknown	Unknown	-	10q24	246560	Retrotransposon insertion in an orthologous genomic region in mice phenocopies split-hand/foot malformation (Chai, 1981; Kano et al., 2007)
Unknown	Unknown	-	Xq26	313350	Some genes in this region are expressed in the embryonic limb: <i>Fgf13</i> is expressed in chick limb bud mesenchyme (Munoz-Sanjuan et al., 1999); <i>Fhl1</i> is expressed in the interdigital regions of mouse and rat limb buds (Wang et al., 2008; Williams et al., 2005); <i>Zic3</i> is expressed in mouse limb bud mesenchyme (Quinn et al., 2012)

Table S2. TaqMan probes used in droplet digital PCR assays, National Birth Defects Prevention Study, 1997-2011.

Genomic location targeted by probe [†]	Description of location	Probe ID
chr10:103285832	Exon 6 of <i>BTRC</i> gene	Hs02207908_cn
chr19:16568651	Intron 1 of <i>EPS15L1</i> gene	Hs07148439_cn

[†] Location in GRCh37/hg19 coordinates.

Table S3. Droplet digital PCR assay development results for control DNA samples at various concentrations, National Birth Defects Prevention Study, 1997-2011.

Assay	Concentration of DNA sample in assay (ng/μL)	Copies/μL of target gene / RNaseP gene [†]	% Above quality threshold [‡]	Target:RNaseP ratio [§]	Number of droplets with data [¶]
Assays to evaluate droplet digital PCR performance with control DNA samples at 5 ng/μL					
QuantStudio 3D Reagent Control Kit	Control reagent	854.88 / 869.98	90.30	0.98	16,407
Hs02207908_cn	30	655.72 / 676.84	89.00	0.97	15,743
	20	487.65 / 498.52	91.90	0.98	16,868
	10	249.34 / 236.25	87.30	1.06	15,890
	5	120.95 / 120.95	85.00	1.00	15,174
QuantStudio 3D Reagent Control Kit	Control reagent	949.51 / 967.74	92.72	0.98	16,546
Hs07148439_cn	30	753.79 / 810.97	88.40	0.93	15,858
	20	424.45 / 469.11	88.72	0.90	16,639
	10	187.98 / 231.62	84.91	0.81	15,874
	5	104.99 / 115.72	89.30	0.91	16,651
Assays to evaluate droplet digital PCR performance with control DNA samples at 1 ng/μL					
Hs02207908_cn	5	124.45 / 123.55	52.59	1.00	9,796
	5	114.56 / 124.46	85.30	1.00	16,727
	5	126.51 / 124.59	89.89	0.99	16,177
	1	123.59 / 138.14	93.55	0.89	17,708
	1	124.68 / 123.20	91.68	1.01	18,041
	1	120.73 / 129.86	93.37	0.93	17,801
Hs07148439_cn	5 ^{††}	25.37 / 23.31	88.94	1.09	16,071
	5 ^{††}	22.20 / 21.02	88.96	1.06	16,008
	1	28.98 / 27.89	87.52	1.04	16,125
	1	24.56 / 26.38	95.80	0.93	16,369
	1	24.34 / 22.86	87.99	1.06	18,567

[†] The target gene was *GLA* for the control reagent, *BTRC* for the Hs02207908_cn probe, and *EPS15L1* for the Hs07148439_cn probe.

[‡] “% Above quality threshold” should be $> 50\%$.

[§] For the control reagent, the GLA:RNaseP ratio was expected to be $1 \pm 10\%$.

[¶] Values for “Number of droplets with data” were expected to be $\geq 10,000$.

^{††} One replicate assay for the control DNA sample at a concentration of 5 ng/μL failed; therefore, the results of only two assays were reported.

Abbreviation: PCR, polymerase chain reaction.

Table S4. Primers used for targeted sequencing of single nucleotide variants and small deletions, National Birth Defects Prevention Study, 1997-2011.

Gene	Target genomic location[†]	Forward primer	Reverse primer
<i>PHIP</i>	chr6:79711789	ATTTTAACAGTTTCGGTGGGAAG	CACGTGATTTCATTATGCATT
<i>HOXB8</i>	chr17:46690675-46690678	AGGGGAGGGGCATGGGGG	CTCTGTCTCGCCCTTTCCC
		AGACAATTGCACAAGGAG	ACCTACAGCCGCTACCAG
<i>SPOP</i>	chr17:47677801	CGCCTAAACTGAATCCCCAC	GCCTGGGGAACATTCTGCTT
<i>UBA2</i>	chr19:34921514-34921518	TCAGTTAGTGTTTACCTCGACA	AAGAGGCCATGAAAGTTACACC
<i>RBM10</i>	chrX:420709	ACCTACCTCTCCCCACCCACT	TCCCGCCCATACCCACCAC
		CCCCAAAAGGACCCCATGGAGA	TTCTCCCGCCCATACCCACCAC
<i>FGF13</i>	chrX:137939821-137939822	TATAACACTTTCACCAGCCAG	GGATATTAGCCAGTTTAACCT
<i>ILF3</i>	chr19:10782133	CGAGCAGGCAGAGTCCGATA	AGACGTGAAGTTTATGCCCAA
<i>ILF3</i>	chr19:10794396	TTTTCCTGACACCCCTCTCG	CCCCTTCTACAGCCTCCTC

[†] Location in GRCh37/hg19 coordinates.

Table S5. Types of birth defects present in the children of 1,191 parents included as controls in case-control analyses, National Birth Defects Prevention Study, 1997-2011.

Birth defect	Number (%) of control parents[†]
Anophthalmia/microphthalmia	132 (11.1)
Anterior chamber defect with or without glaucoma	48 (4.0)
Biliary atresia	108 (9.1)
Bladder exstrophy	52 (4.4)
Cloacal exstrophy	42 (3.5)
Colonic atresia	18 (1.5)
Ebstein anomaly	95 (8.0)
Glaucoma without anterior chamber defect	76 (6.4)
Heterotaxy	186 (15.6)
Hypoplastic left heart syndrome	275 (23.1)
Multiple intestinal atresia	8 (0.7)
Sacral agenesis	64 (5.4)
Tricuspid atresia	87 (7.3)

[†] Percentages may not sum to 100% because of rounding.

Table S6. Case-control analysis results for common variants located in the exons of previously reported candidate genes for split-hand/foot malformation, the National Birth Defects Prevention Study, 1997-2011.

dbSNP ID	Variant location [†]	Gene	Variant	Variant type	Alternate allele frequency [‡]	Unadjusted <i>p</i> -value [§]	Adjusted <i>p</i> -value [§]
rs8049247	chr16: 68712730	<i>CDH3</i>	ENST00000264012.4:c.612C>A (p.Ile204)	Synonymous	0.1387	0.73	0.99
rs34494880	chr16: 68719113	<i>CDH3</i>	ENST00000264012.4:c.1430G>A (p.Arg477His)	Missense	0.0681	0.13	0.98
rs33914440	chr19: 16547778	<i>EPS15L1</i>	ENST00000248070.6:c.342G>T (p.Pro114)	Synonymous	0.0643	0.59	0.98
rs2304000	chr8: 38286811	<i>FGFR1</i>	ENST00000484370.1:c.*3G>C	3-Prime untranslated region	0.2023	0.71	0.99
rs811925	chr6: 106547372	<i>PRDM1</i>	ENST00000369089.3:c.207C>G (p.Asp69Glu)	Missense	0.1678	0.96	1.00
rs1010273	chr6: 106555025	<i>PRDM1</i>	ENST00000369089.3:c.1740G>A (p.Pro580)	Synonymous	0.0965	0.44	0.98

[†] Location in GRCh37/hg19 coordinates.

[‡] Alternate allele frequency from gnomAD v4.1.0 database.

[§] *p*-values from logistic regression analysis comparing alternate allele frequencies between case children and controls. Adjusted *p*-value obtained using the Benjamini-Hochberg method to control the false discovery rate at 0.05.

Abbreviation: dbSNP, National Center for Biotechnology Information database of Single Nucleotide Polymorphisms; *CDH3*, cadherin 3; *EPS15L1*, epidermal growth factor receptor pathway substrate 15 like 1; *FGFR1*, fibroblast growth factor receptor 1; *PRDM1*, PR/SET domain 1.

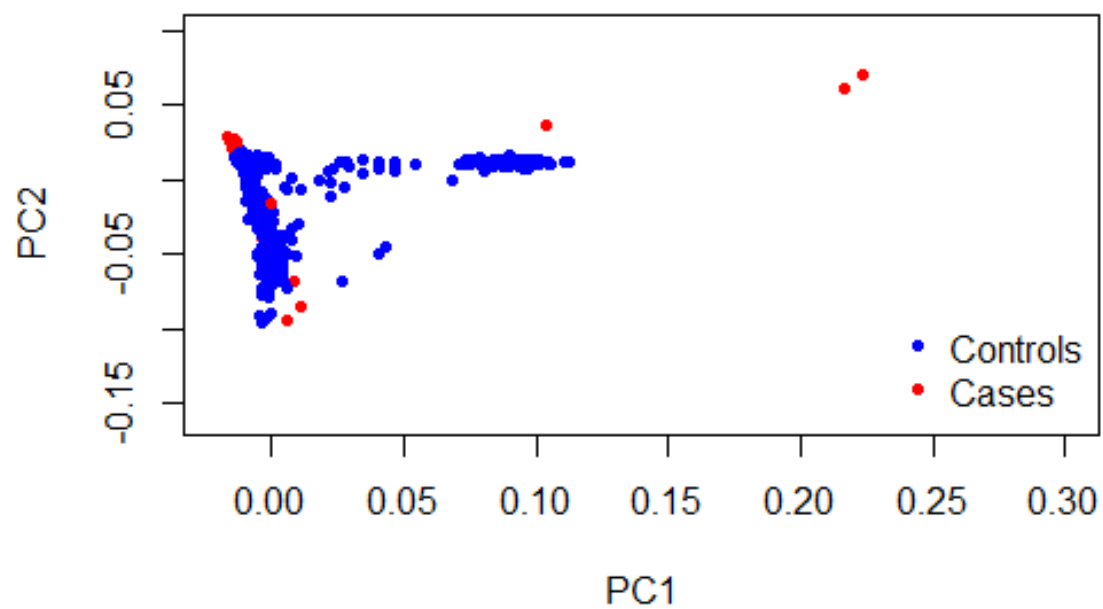


Figure S1. Plot of the first two principal components between 26 cases with split-hand/foot malformation and 1,040 controls in the National Birth Defects Prevention Study, 1997-2011. The controls were matched to cases on ancestry using the PCAmatchR program.

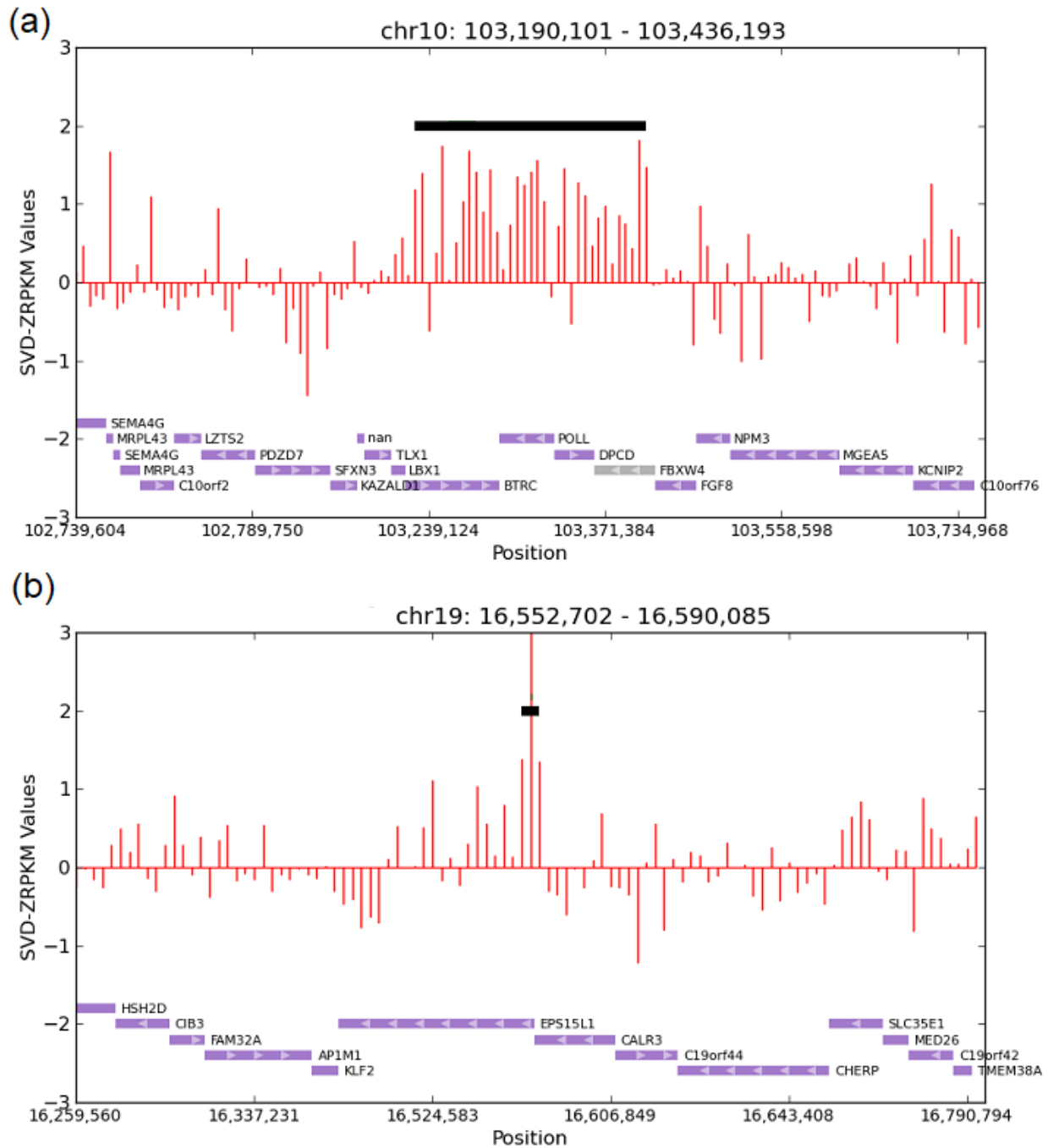


Figure S2. Copy-number variants in cases with split-hand/foot malformation, National Birth Defects Prevention Study, 1997-2011. Plots show (a) a copy-number gain at chromosome 10q24 in case 8 and (b) a copy-number gain at chromosome 19p13.11 in case 9 predicted from exome sequence data using Conifer. The x-axis shows the genomic location in GRCh37/hg19 coordinates. The y-axis shows normalized read depth, which is an indicator of the signal strength of the copy-number variant call. The horizontal red line across each plot is a reference line

indicating a signal strength of zero. The height of the vertical red bars represents the signal strength at each position in the region, with bars above the reference horizontal line indicating duplications and bars below the reference horizontal line indicating deletions. The black bar demarcates the copy-number variant call in each plot. Genes in the region are shown as purple bars in the bottom half of each plot.

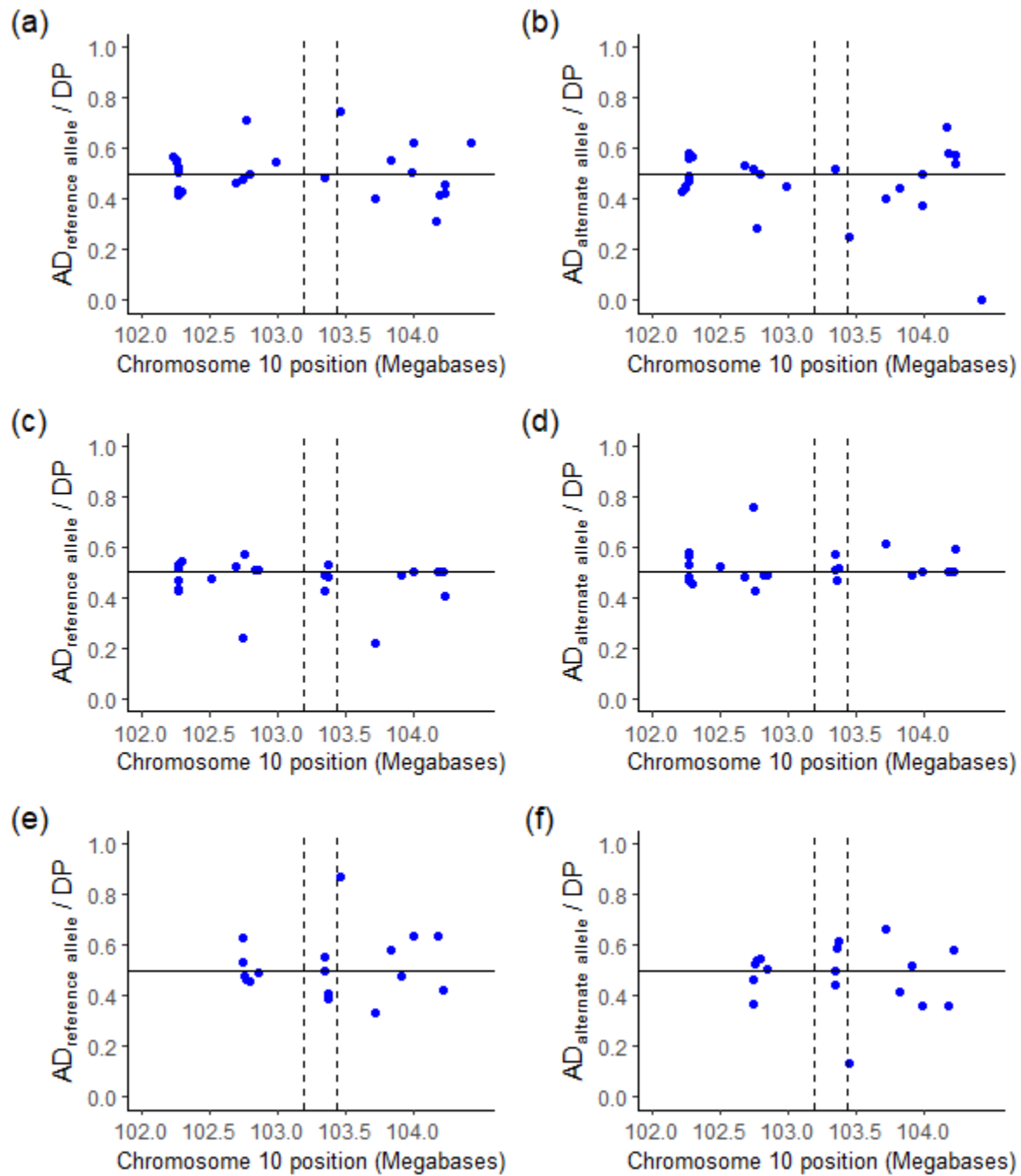


Figure S3. Allele balance in heterozygous variants in the chromosome 10q24 region in split-hand/foot malformation family trio 8, National Birth Defects Prevention Study, 1997-2011. The genomic region shown in each plot is chr10:102190101-104436193, which encompasses the predicted 10q24 copy-number gain in case 8 and one megabase upstream and downstream of the gain. The two vertical dashed lines in each plot demarcate the boundaries of the copy-number gain in the child, based on exome sequencing data. The x-axis shows the genomic location in

GRCh37/hg19 coordinates. The y-axis shows allele balance of heterozygous variants within the genomic region. The allele balance was calculated from exome sequence data as allele depth (the number of reads supporting an allele) divided by read depth (the total number of filtered reads supporting the variant). On the y-axis, allele depth is represented by the abbreviation, AD, and read depth by the abbreviation, DP. Plots of allele balance were generated separately for the reference and alternate alleles of heterozygous variants in the mother (a) and (b), the father (c) and (d), and the child (e) and (f). The number of heterozygous variants in each plot was 25 for the mother, 22 for the father, and 17 for the child. In each plot, the solid horizontal line at an allele balance of 0.5 is a reference line that represents the expected allele balance of heterozygous variants in diploid species.

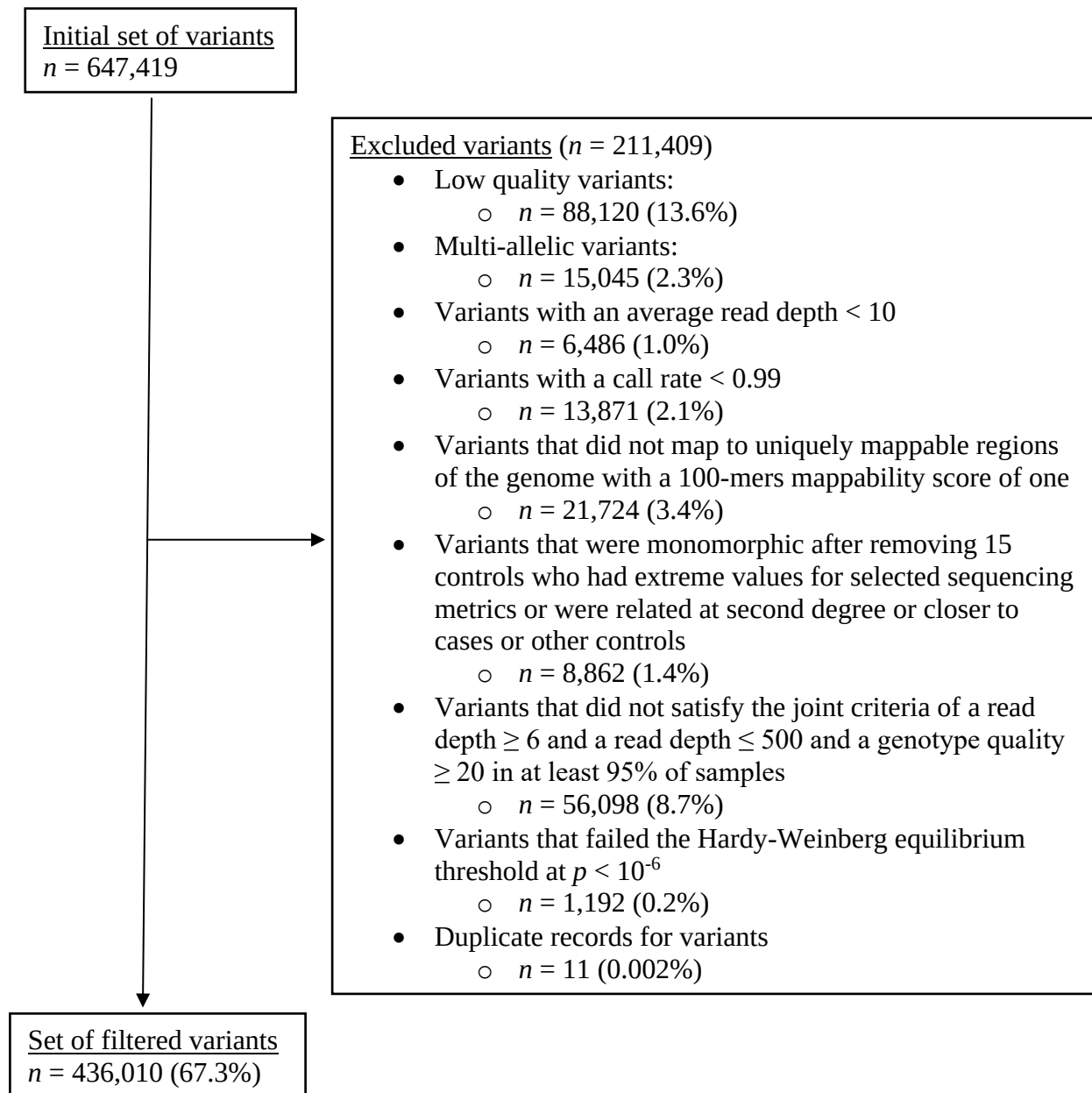


Figure S4. Filtration of variants in exome sequence data for case children with split-hand foot malformation and controls (parents of children with non-limb birth defects), National Birth Defects Prevention Study, 1997-2011.

Supplementary References

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