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**Title**

New disease gene location and high genetic heterogeneity in idiopathic scoliosis

**Running title**

Gene location in idiopathic scoliosis

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1 **Abstract**

2 Idiopathic scoliosis is a spine disorder of unknown origin with 1.5-3% prevalence in the  
3 general population. Besides the large multifactorial form sample of idiopathic scoliosis, there  
4 is a good evidence for the existence of a monogenic subgroup in which the disease is inherited  
5 in a dominant manner. However, results from literature suggest a strong heterogeneity in the  
6 locations of the mutated genes. Using a high resolution genome-wide scan, we performed  
7 linkage analyses in three large multigenerational idiopathic scoliosis families compatible with  
8 dominant inheritance including 9 to 12 affected members or obligate carriers. In two of these  
9 families, our results suggested intra-familial genetic heterogeneity whereas, in the other, we  
10 observed a perfect marker disease co-segregation in two regions at 3q12.1 and 5q13.3. We  
11 can state that one of these two locations is a novel idiopathic scoliosis disease gene locus,  
12 since the probability of having this perfect co-segregation twice by chance in the genome is  
13 very low ( $p=0.001$ ). Lastly, in all three families studied, linkage to the previously mapped  
14 dominant idiopathic scoliosis loci on chromosomes 19p13.3, 17p11.2, 9q34, 17q25 and 18q is  
15 unlikely, confirming that there is a high genetic heterogeneity within the subgroup of  
16 dominant forms of idiopathic scoliosis.

17

## 18 **Introduction**

19 Idiopathic scoliosis (IS) is a spine deformity of unknown cause commonly defined by a  
20 rotation of vertebral bodies leading to a structurally fixed lateral curvature of the spine with a  
21 Cobb's angle of at least 10°. Idiopathic scoliosis affects up to 3% of the population <sup>1</sup>, girls  
22 being more commonly affected than boys <sup>2 3</sup>. Adolescent idiopathic scoliosis (AIS, MIM  
23 181800) occurs in otherwise healthy adolescents and accounts for approximately 85% of IS  
24 cases, other cases being infantile or juvenile (15%). About 3 per 1,000 children present with  
25 severe AIS forms over 30° which may require braces or surgical treatment and may have  
26 severe functional and aesthetic consequences.

27 Aside from idiopathic forms, scoliosis may also occur secondary to other hereditary disorders  
28 including defects in extracellular matrix components (e.g. Marfan syndrome, Ehlers-Danlos  
29 syndromes), neuromuscular disorders (e.g. Friedreich ataxia, muscular dystrophies),  
30 osteochondrodystrophies (e.g. osteogenesis imperfecta, neurofibromatosis), vertebral  
31 segmentation defects or other malformation syndromes including CHARGE syndrome and  
32 Smith-Magenis (del17p11.2) syndrome.

33 Several population studies established that up to 40% of IS are familial <sup>4</sup> and that there is a  
34 high concordance rate of this disease in monozygotic twins <sup>5</sup>. Many studies concluded that the  
35 most likely mode of inheritance was multifactorial, postulating that predisposing alleles were  
36 required together with environmental factors for phenotypic expression <sup>6</sup>. Although the exact  
37 mode of inheritance remains unknown in many IS cases, there is now evidence for a  
38 monogenic subgroup. A segregation analysis using a model with age and gender effects  
39 established a significant contribution of a major causal gene transmitted according to an  
40 autosomal dominant inheritance with sex-dependent incomplete penetrances <sup>7</sup>. A small  
41 number of model-dependent linkage analyses using an autosomal dominant model in large  
42 multigenerational IS families have been performed successfully to date. Indeed such large

43 multiplex IS families compatible with dominant inheritance are very rare. A genome-wide  
44 scan of seven unrelated multiplex families of Chinese descent with AIS concluded that there  
45 was a linkage to chromosome 19p13.3 (IS1, MIM 181800)<sup>8</sup>. This finding was replicated in  
46 several IS multiplex families in Alden et al's study<sup>9</sup>. A similar study in an Italian family  
47 mapped a second locus to 17p11.2 (IS2, MIM 607354)<sup>10</sup>. Another study reported on the  
48 mapping of two loci for IS on chromosome 9q31.2-q34.2 (IS4, MIM 612238) and 17q25.3-  
49 qtel (IS5, MIM 612 239), the latter by combining information obtained from two unrelated IS  
50 families<sup>11</sup>. Recently, a locus for IS and pectus excavatum was found in a single large family  
51 on chromosome 18q<sup>12</sup>.

52 In the present study, we performed model-dependent linkage analyses in three large IS  
53 families compatible with autosomal dominant inheritance of the disease trait with a high  
54 number of informative meioses. We compared our results to published linkage data.

55

## 56 **Families and Methods**

### 57 *Patient and Family Inclusion*

58 We selected large multiplex IS families and we genotyped the largest families, i.e. those  
59 including the highest number of affected individuals or obligate carriers. A positive diagnosis  
60 for IS was reached by combining clinical examination of the spine, including the forward  
61 bending test (Adams test), and the Cobb's angle measurement on radiographic evaluation.  
62 Both a Cobb's angle above 15° (in order to minimize the risk of phenocopies) and vertebral  
63 rotation were required for the positive diagnosis of IS. Patients harbouring a spine curvature  
64 but no rotational component were assigned an "unknown status", as were those with any other  
65 associated anomaly of the spine or, more generally, with atypical IS. Patients with normal  
66 clinical examination and strictly normal spine on radiograph were considered "unaffected".  
67 All individuals underwent clinical examination by at least one clinical geneticist (PE) and one

68 orthopaedist (BB and/or JCB). Each radiograph was carefully and often repeatedly checked,  
69 by the clinical geneticist and one or both orthopaedists.

70 Pedigrees of the three selected IS families are shown in the figure. All families are three-  
71 generation pedigrees suggestive of the transmission of an autosomal dominant mutation with  
72 incomplete penetrance. Clinical data on patients belonging to the families studied are  
73 provided in table 1.

74

#### 75 *DNA sample collection*

76 We selected for genotyping 57 individuals including 27 affected patients or obligate carriers  
77 belonging to the three families presented here. Blood samples were collected from each  
78 participant (IS patients and their first-degree affected or unaffected relatives) after providing  
79 them with an information sheet describing the research study and obtaining their informed  
80 written consent. This research study was approved by the local Ethical Committee and was  
81 promoted by the Hospices Civils de Lyon, France. Genomic DNA was extracted from blood  
82 samples according to standard procedures. EBV-transformation of lymphocytes was  
83 performed in at least one affected individual per family. After the cell-lines were established,  
84 transformed lymphocytes were stored frozen in liquid nitrogen. Normal standard  
85 chromosomal studies on blood lymphocytes were obtained from at least one affected  
86 individual in each family ruling out gross chromosomal abnormalities.

87

#### 88 *Genotyping*

89 Considering the large number of possible IS candidate genes with regard to their function and  
90 the wide genetic heterogeneity of IS, we decided to favour genome-wide approaches rather  
91 than candidate loci mapping strategies. We genotyped the three large multiplex IS families  
92 using a high density 1,000 microsatellite DNA marker set, with an average spacing of 3.7 cM  
93 between each marker, where genetic locations are based on the deCODE map<sup>13</sup>.

94

95 *Statistical analyses*

96 The classical method used for mapping a disease gene under monogenic transmission is the  
97 Morton Lod Score analysis (1955). A lod score value of 3.00 (for autosomal loci) means that  
98 the hypothesis of linkage between the disease trait locus and the marker locus is 1,000 times  
99 more likely than the hypothesis of no linkage. This method is very powerful when the disease  
100 is due to a mutation of the same gene in all families with full penetrance for mutation carrier.  
101 In the present situation, it is realistic to assume that the disease is due to segregation of a  
102 dominant mutation in the families selected for this study. However, the results from literature  
103 suggest a strong heterogeneity in the mutated genes. In addition, penetrance values may differ  
104 according to the genes and mutations involved. Clerget-Darpoux et al <sup>14</sup> showed that a  
105 misspecification of the penetrance values results in a lod score decrease and hence in a loss of  
106 the power to detect linkage.

107 To overcome the interfamilial genetic heterogeneity, we performed a lod score analysis  
108 separately in each family. Analyses were performed assuming the segregation of a dominant  
109 mutation, with and without the possibility of phenocopies. In linkage analysis, marker  
110 segregation was considered only in affected individuals. The status of other family members,  
111 including unaffected individuals, was assumed to be unknown. In such an analysis, the results  
112 do not depend on the absolute penetrance values, but only on the relative ones (probability to  
113 be affected for a non mutation carrier versus this probability for a mutation carrier).

114 In a first step, we measured in each family the lod score value that could be reached at the  
115 disease gene locus in the situation of no phenocopy and full informativity. This theoretical  
116 maximum lod score value  $Z_{i\max}$  can be simply inferred from the number  $T_i$  of disease allele  
117 transmissions to affected individuals in each family. If we denote  $M_i$  the number of meioses

118 between a common ancestor and all affecteds,  $n_i$  the number of common ancestors and  $T_i =$   
119  $M_i - n_i$ , in each family  $F_i$  :

120  $Z_{i\max} = \log_{10} [ L(\theta=0)/L(\theta=0.5) ]^{T_i} = - T_i * \log_{10} (1/2)$  where  $L(\theta=0)$  is the likelihood of H1  
121 (hypothesis of linkage at  $\theta=0$ , i.e. no recombination event between the marker and the disease  
122 locus) and  $L(\theta=0.5)$  is the likelihood of H0 (hypothesis of no linkage).

123 In a second step, we estimated the probabilities under H0 to reach the maximum lod score on  
124 each arm of autosomes 0, 1 or more than once. This maximum occurs according to a Poisson  
125 distribution with a parameter  $\lambda$  equal to the probability of reaching  $Z_{i\max}$  if hypothesis H0 is  
126 true =  $(0.5)^{T_i}$ . On the whole genome (44 chromosome arms), this parameter  $\lambda$  is equal to  
127  $44 * (0.5)^{T_i}$ .

128 In a third step, we computed maximum Morton lod scores using Merlin software <sup>15</sup> separately  
129 in each of the selected families.

130 In a final step, we tested in each of the three families studied the compatibility with linkage to  
131 the five loci previously considered to be IS loci (19p13.3, 17p11.2, 9q34, 17q25, 18q), using  
132 both the genetic model without phenocopy described here and a more flexible model allowing  
133 for various rates of phenocopies (0.01, 0.03, 0.10 and 0.020).

134

## 135 **Results**

136 Table 2 gives the values  $M_i$ ,  $n_i$ ,  $T_i$  and the expected maximum lod score  $Z_{i\max}$  in each family  
137  $F_i$ . It also gives the probabilities to reach it 0, 1 or more than once under the H0 hypothesis in  
138 whole genome analysis. Expected maximum lod scores are 1.51, 3.01 and 2.71 in families F1,  
139 F2 and F3 respectively. The probability of never reaching  $Z_{i\max}$  under H0, and thus only once  
140 (under H1), in whole genome analysis, is 25% for F1, 96% for F2, and 92% for F3.

141 Table 3 gives, for each family F1, F2 and F3, the maximum lod score (max Z) and marker  
142 informativity at this location. In families F1 and F3, the maximum lod scores obtained after



143 whole genome scan were significantly lower than the expected  $Z_{max}$  previously estimated.  
144 Affected individuals never shared a same transmitted allele in any chromosome arm.  
145 Consequently, the hypothesis that the disease is due to the transmission of a dominant  
146 mutation has to be reconsidered in these families. The variable severity in these two families  
147 suggests a possible intra familial genetic heterogeneity. In family F1, the severity of IS in  
148 affected individuals is highly variable since, in several patients of this family, Cobb's angles  
149 are between  $16^\circ$  and  $24^\circ$  and over  $50^\circ$  in four patients. Similarly, patient III1 of family F3 is  
150 affected with a severe form of IS (Cobb's angle of  $65^\circ$ ), whereas angles for the five other  
151 patients are between  $15^\circ$  and  $24^\circ$ . When patient III1 of family F3 is removed from the linkage  
152 study, the lod score reaches the theoretical maximum value of 2.11 at the D15S979 locus.  
153 In family F2, the lod score  $Z_{max}$  of 3.01 is reached both on chromosome 3 (3q12.1) and on  
154 chromosome 5 (5q13.3). As noted in table 2, the probability of having twice obtained this  
155 value randomly (under  $H_0$ ) is very small ( $p=0.001$ ). It means that there is strong evidence that  
156 a disease gene lies in one of these two regions. The lengths of chromosome intervals shared  
157 by all patients at the two possible disease gene locations are 7cM and 12cM respectively  
158 (table 3). The boundaries of these intervals, ]D3S3690-D3S3045[ and ]D5S2851-D5S1397[  
159 respectively, were deduced from recombination events in affected individuals as observed on  
160 haplotypes. To test whether the study of unaffected members of family F2 may help in the  
161 discrimination between these two locations, we performed a mod score analysis<sup>16</sup> with all  
162 genotyped family members (affected and non affected) using the software Genehunter-  
163 Modscore 2.0.1<sup>17</sup>. The results from four unaffected individuals in this family, II7, II8, III6,  
164 III7 (see the figure) proved to be informative. The mod scores obtained at chromosome 3 and  
165 5 loci respectively were identical and did not allow us to discriminate between the two  
166 possible locations.

167 Lastly, we considered lod scores obtained in families F1, F2 and F3 on the 5 regions claimed  
168 to be idiopathic scoliosis loci. All lod scores were negative when assuming no phenocopy and  
169 a maximum value of 0.00 was reached when the model allowed for phenocopies.

170

## 171 **Discussion**

172 Using a set of 1000 polymorphic markers (decode set), we performed a genome-wide linkage  
173 analysis under a dominant mode of transmission in three large multiplex IS families. Several  
174 pitfalls such as inter-familial genetic heterogeneity, possible phenocopies or unknown  
175 penetrance for mutation carrier were taken into account. First, lod score analyses were  
176 computed for each family independently. Second, in order to decrease the risk of  
177 phenocopies, individuals with a Cobb's angle of at least 15°, instead of the widely accepted  
178 threshold of 10°, were considered as affected. Indeed, mild scoliosis or even mild curvature  
179 and/or rotation of the spine are likely to be caused by a combination of environmental and  
180 minor gene effects rather than by a major gene effect <sup>7</sup>. In addition, previous reports and our  
181 own observations showed that there are important variations in Cobb's angle measurements of  
182 up to 5° among different physicians <sup>18,19</sup>.

183 This study shows the mapping of a new locus for dominant IS in a large family (F2) either on  
184 5q13-q14 or on 3q11-q13. Indeed, in this family, the observed Zmax reached the expected  
185 Zmax value of 3.01, on both genomic locations. Since the probability of obtaining a Zmax  
186 value in the genome twice by chance is very low (p=0.001), we can conclude that there is  
187 strong evidence that a novel IS disease gene lies in one of these two locations. This IS gene  
188 still remains to be identified. Among the genes in the two chromosomal regions, many are  
189 involved in cellular signalling, some are coding for hypothetical/uncharacterized proteins yet  
190 to be discovered, and others were cloned but their function is not yet fully documented.  
191 Mutation screening using either exome sequencing or targeted high throughput sequencing,

192 gene expression studies, and *in vivo* functional analysis of candidate genes within these loci  
193 need to be performed.

194 In this study, we also provide further evidence for a high heterogeneity among monogenic  
195 forms of IS. In each of the three families studied, the lod scores are either negative or  
196 inconclusive, depending on the phenocopy rate assumption, at the five previously suggested  
197 locations for IS genes, namely 19p13.3, 17p11.2, 9q34, 17q25 and 18q. Such high genetic  
198 heterogeneity favours the view that computing IS families independently, when performing  
199 linkage analyses based on a monogenic model hypothesis, is critical to obtaining accurate  
200 results.

201 We also show that the selection of multiplex IS families diagnosed with strict clinical criteria  
202 does not warranty that the apparently dominant inheritance is simply explained by the  
203 segregation of a unique gene mutation. We have to consider the difficulty of selecting  
204 families with a high number of informative meioses without introducing intra-familial  
205 heterogeneity or phenocopies, to be able to detect linkage at a family level. It seems that the  
206 criteria of a minimum angle of  $15^\circ$  is not sufficient to ensure intra-familial genetic  
207 homogeneity, and we hypothesize that clinical variability between family members also has to  
208 be minimized. Indeed, in both families F1 and F3, the  $Z_{max}$  values obtained were far beyond  
209 the values expected under the assumption of a single causative mutation segregating in all  
210 affected members. In family F1, IS patients have a wide range of Cobb's angle values  
211 extending from  $16^\circ$  to  $130^\circ$ , and in family F3, one patient is affected with a severe form of IS  
212 (Cobb's angle of  $65^\circ$ ), whereas angles for the five other patients are between  $15^\circ$  and  $24^\circ$ .  
213 When the patient with the Cobb's angle of  $65^\circ$  from family F3 is removed from the analysis,  
214 we observe a perfect co-segregation of the disease and the D15S979 marker. Therefore, in  
215 such studies, we suggest paying careful attention to the clinical aspects of scoliosis, possibly  
216 including Cobb's angle values, even within single families. Such stratification by the severity

217 of the phenotype or by other discrete clinical aspects of scoliosis may minimize the risk of  
218 intra-familial heterogeneity. The results provided in our study, supporting a high degree of  
219 inter- and intra-familial genetic heterogeneity in IS, raise questions regarding several  
220 previously reported mapping studies based on linkage data obtained by pooling together  
221 independent IS families. It should be noted that *a posteriori* selection of families compatible  
222 with linkage to a specific locus may lead to false primary linkage and/or fine mapping. Thus,  
223 in genetically heterogeneous disorders in which a different mutated gene in each family may  
224 be dealt with, pooling linkage data obtained from unrelated families should be considered  
225 with caution. Accurate linkage mapping studies are required to allow the subsequent  
226 identification of dominant IS genes. To date, no dominant IS gene has been identified, and we  
227 hope that the present study will prove to be an important step toward this goal. Also, although  
228 dominant IS genes, yet to be identified, may account for a restricted number of IS cases, they  
229 may provide insights into the genetic bases of multifactorial IS, which remain largely  
230 unknown<sup>20</sup>. Future genetic and functional analyses of candidate genes located within these  
231 defined regions will hopefully reveal gene defects accounting for IS, which will allow, in  
232 turn, the identification of secondary molecular factors that may point towards yet  
233 unrecognized IS pathophysiological pathways.

234

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241

242 **Competing interests:** none

243 **Web resources**

244 Online Mendelian (OMIM), <http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>

245 National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>

246 deCODE Genetics, <http://www.decode.com/>

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## **Figure Titles and Legends**

Title to Figure. Simplified pedigrees for families 1-3.

Figure Legend: Simplified pedigrees are drawn for families 1-3. All pedigrees are compatible with autosomal dominant inheritance of the disease trait. Cobb's angles are indicated. Antero-posterior spine radiographs are shown for affected individuals. Question marks indicate uncertain status. ND: status not determined. Several unaffected individuals, who have been genotyped, are not included since they were not useful in linkage analysis. In family 2, four unaffected individuals are included since they contributed to the mod score analysis.

## **Titles to tables**

Table 1. Clinical data for patients included in this study

Table 2. Informativity studies performed in each family. Only affected individuals have been taken into account.

Table 3. Maximum lod score values obtained in each family using only affected individuals.



**Table 1**

Family	Patient	Age at diagnosis (years old)	Cobb's angle on radiograph (age: years old)	Cobb's angle	Apical vertebrae	Therapy
<b>F1</b>	IV-2	13	76° (65)	right thoracic	D9	physiotherapy
	IV-3	adolescence	55° <sup>a</sup> (61)	right thoraco-lumbar	D12	spinal surgery
	IV-4	fortuitous	18° (50)	right lumbar	L2	none
	IV-6	12	35°/28°(64)	right thoracic/left lumbar <sup>b</sup>	D8-D9/L2-L3	bracing
	IV-7	6	90°/93° (20)	right thoracic/left lumbar <sup>b</sup>	D7/L2	unknown
	IV-8	9	21° <sup>a</sup> (26)	right thoracic/left lumbar <sup>b</sup>	D8/L3	spinal surgery
	V-1	15	16° (15)	left lumbar	L3	none
<b>F2</b>	II-1	fortuitous	27° (73)	right thoraco-lumbar	D12-L1	none
	II-3	fortuitous	19° (74)	right lumbar	L1	none
	II-4	fortuitous	23° (71)	right lumbar	L2	none
	II-6	adolescence	41° (67)	right thoraco-lumbar	L1	none
	III-3	adolescence	15° (49)	right thoraco-lumbar	L1	bracing
	III-4	fortuitous	29° (46)	right thoraco-lumbar	D12	none
	III-8	fortuitous	15° (42)	right thoraco-lumbar	L1	none
	IV-1	adolescence	29°/29° (20)	right thoracic/left lumbar <sup>b</sup>	D8/L2	physiotherapy
	IV-2	fortuitous	15° (16)	right thoracic	D7	none
	IV-3	fortuitous	14°/16° (19)	right thoracic/left lumbar <sup>b</sup>	D8/L1	none
	IV-4	12	18° (15)	left thoraco-lumbar	L1	physiotherapy
	<b>F3</b>	II-3	fortuitous	14°/16°(58)	right thoracic/left lumbar <sup>b</sup>	D8/L1
II-8		fortuitous	24°(66)	right thoracic	D8	none
III-1		adolescence	65°(18)	right thoracic-left lumbar	D8	spinal surgery
III-2		fortuitous	15°(32)	right lumbar	L2	none
IV-1		9	22°(15)	right thoracic	D8	physiotherapy
IV-2		10	20° (15)	right thoraco-lumbar	L1	bracing

a Cobb's angle after arthrodesis

b Double major curve

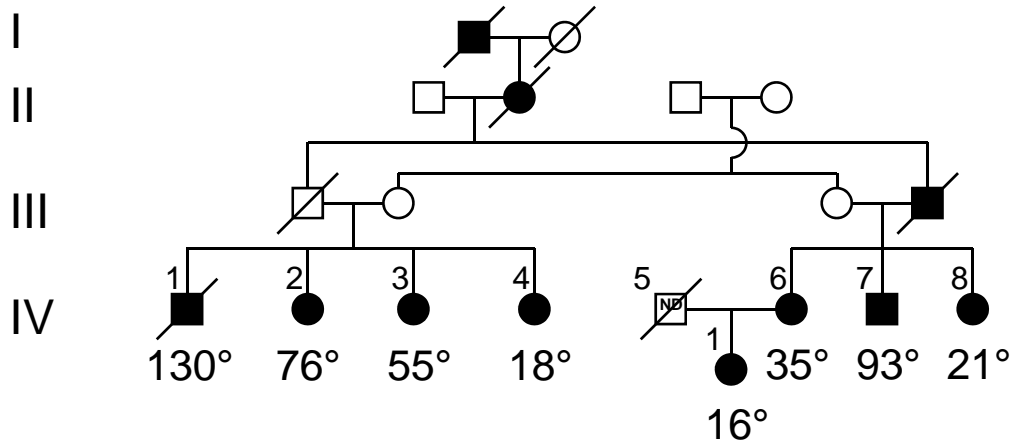
**Table 2**

Family	Number of meioses between a common ancestor and all affected individuals	Number of common ancestors	Number of disease allele transmissions to affected individuals	Expected Maximum lod score	Probabilities to reach $Z_{i_{max}}$ under $H_0$ , 0, 1 or more than once in whole genome analysis		
$F_i$	$M_i$	$n_i$	$T_i = M_i - n_i$	$Z_{i_{max}} = -T_i * \log_{10}(0.5)$	0	1	>1
F1	9	4	5	1.51	0.253	0.346	0.401
F2	12	2	10	3.01	0.958	0.041	0.001
F3	10	1	9	2.71	0.918	0.079	0.003

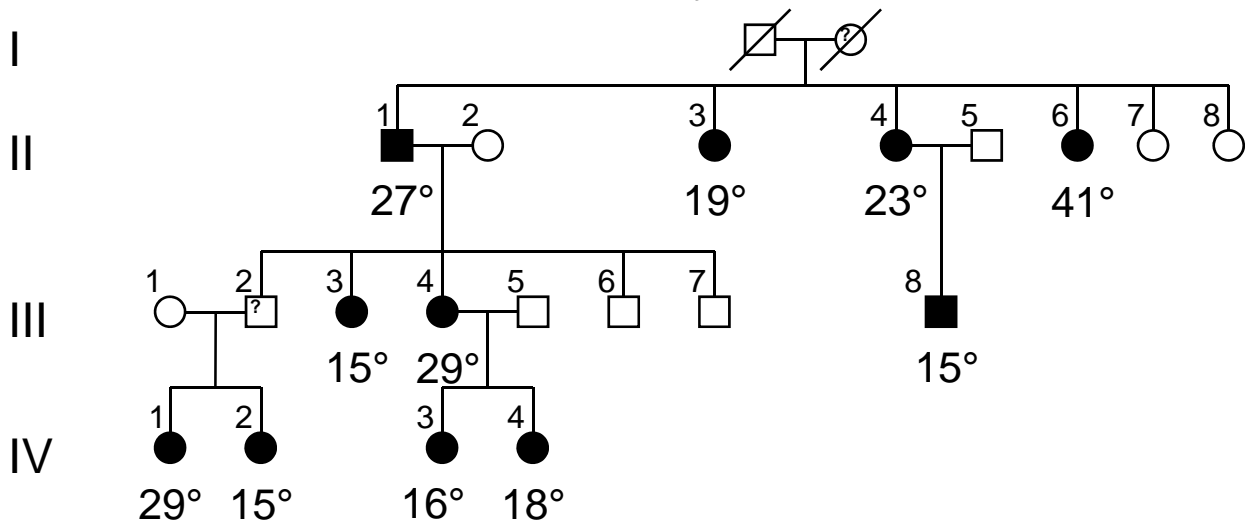
**Table 3**

family	Expected Z <sub>max</sub>	Observed Z <sub>max</sub>	informativity	Localisation (interval length)	Chromosomal location
F1	1.51	0.85	0.79	D14S274	14q23.1
F2	3.01	3.00	0.99	D5S2003 (D5S2851-D5S1397, 12 cM)	5q13.3
	3.01	3.01	0.99	D3S2462 (D3S3690-D3S3045, 7 cM)	3q12.1
F3	2.71	1.23	0.87	D15S979	15q25.1

### Family 1



### Family 2



### Family 3

