REPORT

Linkage Analysis Identifies a Novel Locus for Restless Legs Syndrome on Chromosome 2q in a South Tyrolean Population Isolate

Irene Pichler, Fabio Marroni, Claudia Beu Volpato, James F. Gusella, Christine Klein, Giorgio Casari, Alessandro De Grandi, and Peter P. Pramstaller

Restless legs syndrome (RLS) is a common neurological condition with three loci (12q, 14q, and 9p) described so far, although none of these genes has yet been identified. We report a genomewide linkage scan of patients with RLS (n = 37) assessed in a population isolate (n = 530) of South Tyrol (Italy). Using both nonparametric and parametric analyses, we initially obtained suggestive evidence of a novel locus on chromosome 2q, with nominal evidence of linkage on chromosomes 5p and 17p. Follow-up genotyping yielded significant evidence of linkage (nonparametric LOD score 5.5, $P \leq .0000033$; heterogeneity LOD score 5.1; $\alpha = 1.0$) on chromosome 2q. Three families (S01, S05, and S016) were shown to descend from a common founder couple. A disease haplotype shared between family S01 and family S05 defines a candidate region of 8.2 cM; in addition, the single affected individual in family S016 shares three linked alleles at neighboring markers, which suggests a reduced candidate interval of only 1.6 cM. Two-point linkage analysis in this 10-generation pedigree provided significant evidence of a novel RLS locus in this region (LOD score 4.1). These findings reemphasize the genetic heterogeneity of the disorder and strongly support the identification of a novel locus for RLS on chromosome 2q.

Restless legs syndrome (RLS [MIM 102300]) is a common and frequently underdiagnosed neurological disorder. Estimated prevalence rates vary widely, from 2% to 15% of the general population.[1,2] RLS can be diagnosed by the clinical presence of four essential criteria developed by the International Restless Legs Syndrome Study Group[3] and revised in a National Institutes of Health consensus statement[4]: (1) an urge to move the legs, usually accompanied by uncomfortable sensations (paresthesias, dysesthesias), (2) occurrence of symptoms primarily while at rest, (3) partial or total relief with movement, and (4) worsening of symptoms in the evening or night. RLS is generally classified into idiopathic and symptomatic forms. In idiopathic RLS, which includes both sporadic and familial cases, results of physical and neurological examinations are normal. Symptomatic (secondary) forms can be caused by many conditions, such as polyneuropathy, iron deficiency, anemia, kidney disease, or thyroid dysfunction.[5]

A substantial genetic contribution to idiopathic RLS has been consistently recognized from population and family studies. A family history of RLS was described in 40%–90% of patients,[6] and the high concordance rate of 83.3% between identical twins was reported.[7] In a set of multiplex families from North America, the heritability of RLS was estimated to be 0.60.[8] Most reports of familial cases have suggested autosomal dominant transmission,[9] although broad intrafamilial phenotypic expressivity, with a variable age at onset and disease course, has been reported.[10] To date, linkage studies have identified three loci on chromosomes—12q,[11] 14q,[12] and 9p[8]—in RLS-affected families from different populations. However, no disease-causing gene has yet been identified. These molecular findings, together with the reported variable expressivity of the phenotype, suggest substantial genetic and clinical heterogeneity of RLS.

One way to reduce the genetic complexity, and potentially also the environmental heterogeneity, is the use of isolated populations. Here, we report a genomewide linkage analysis of patients with RLS collected from one small village that has conserved a high degree of isolation in the western Alps of South Tyrol (Italy). It reveals increased background linkage disequilibrium (LD) due to the peculiar demographic history that has led to genetic drift and founder effects.[13] The village originates from an old settlement, has few founders, and experienced slow expansion over time. Apart from these demographic characteristics relating to the population history and therefore associated with the genetic background, historical documents allowed the reconstruction of extended pedigrees as far back as the 17th century.[14] The study is part of a larger ongoing genomic research program (GenNova) investigating the South Tyrolean population.

The RLS phenotype was clinically assessed in two steps. First, a detailed questionnaire-based interview and a med-

From the Institute of Genetic Medicine, European Academy (I.P.; F.M.; C.B.V.; A.D.G.; P.P.P.), and Department of Neurology, General Regional Hospital (P.P.P.), Bolzano, Italy; Molecular Neurogenetics Unit, Center for Human Genetic Research, Massachusetts General Hospital, Boston (J.F.G.); Departments of Neurology (C.K.; P.P.P.) and Human Genetics (C.K.), University of Lübeck, Lübeck, Germany; and Vita-Salute University and San Raffaele Scientific Institute, Milan (G.C.)

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Address for correspondence and reprints: Dr. Peter P. Pramstaller, Institute of Genetic Medicine, European Academy, Viale Druso 1, 39100 Bolzano, Italy. E-mail: peter.pramstaller@eurac.edu

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Table 1. Clinical Features of RLS in the 16 Patients of Families S01, S05, and S016

Family and Patient	Sex	Age (years)		Severity (IRLS/JHRLSS)[a]	Awakenings	Involvement of Upper Limbs	Lateralization of Symptoms	Course	Haplotype Status (c2q/c5p)[b]
		At Study	At Onset						
S01:									
VIII-1	F	70	12	32/2	−	−	Left	Progressive	+/+
VIII-2	F	76	12	31/3	+	+	−	Progressive	+/+
VIII-5	M	79	60	NA	−	−	Left	Stable	+/+
VIII-7	M	68	30	10/1	−	−	−	Remission	+/−
VIII-11	M	63	50	11/2	−	−	Right	Stable	+/−
IX-14	M	43	20	18/2	+	−	−	Progressive	+/+
IX-16	M	39	15	15/2	+	+	Right	Remission	+/+
IX-17	F	51	41	20/2	+	+	−	Stable	+/+
IX-19	F	34	29	10/1	−	−	Left	Progressive	+/+
IX-22	M	36	31	7/2	−	−	−	Stable	+/−
IX-24	M	27	25	7/2	+	+	−	Stable	+/+
X-27	F	14	13	6/1	−	−	−	Stable	+/+
S05:									
VIII-1	M	64	49	13/1	+	−	−	Remission	+/+
VIII-2	F	59	52	12/2	−	−	−	Stable	+/+
VIII-3	M	63	58	NA	+	−	−	Stable	+/+
S016:									
IX-3	M	39	35	8/1	−	−	−	Stable	+/+

[a] NA = not assessed.

[b] Individuals sharing the haplotypes on chromosomes 2q and 5p are identified with +/+; those sharing the haplotype only on chromosome 2q are identified with +/−.

ical screening exam of 530 voluntary study participants were performed. The questionnaire included the four minimal criteria for RLS.[3] A blood sample was taken for DNA extraction and routine clinical chemistry analysis. In the second step, subjects presumed to have RLS were reexamined by a movement-disorder specialist. A standardized neurological examination was performed, and a detailed interview covered age at onset of symptoms, family history, localization of symptoms, lateralization, and sleep problems. The severity of symptoms was rated using both the International Restless Legs Syndrome Rating Scale (IRLS)[15] and the Johns Hopkins Restless Legs Severity Scale (JHRLSS).[16] The final diagnosis of RLS was made according to currently accepted diagnostic criteria.[4] Secondary causes of RLS were determined by electrophysiology and biochemical tests, including laboratory parameters for iron metabolism; renal, hepatic and endocrine function; and diabetes. None of the subjects reported the use of medication known to affect sleep or sensory or motor functions. Thirty-seven patients with idiopathic RLS whose ancestry was from this village were identified; three patients (members of families S01, S02, and S03) were designated as having uncertain status. Nine individuals with symptoms associated with secondary RLS were excluded from the present study. Twelve affected individuals clustered in one large and extended family (S01), and 17 other families consisted of 1–5 affected members each. Six of the families (S01, S02, S03, S05, S06, and S08) were informative for linkage; the remaining 12 were subsequently included for haplotype analysis. Of the 18 families, 61 clinically unaffected members were available for the study. Disease status of 31 unaffected individuals was set as "unknown," because they participated only in the first step of the phenotype assessment. Of 14 fully assessed healthy members of S01, one was set as "unknown," because his age was lower than the mean age at onset plus 1 SD, as obtained from the sample of affected individuals in this family. Study participants gave written informed consent. The study was approved by the local ethics committee.

We performed a 4-cM genomewide linkage scan on DNA from all 530 study participants, using 1,000 microsatellite markers (i.e., STRs) genotyped at deCODE Genetics. The data were analyzed by multipoint model-free and parametric linkage analyses with use of the software GENEHUNTER 2.1.[17] For the model-free analysis, the NPL score was calculated using the S_{all} statistic to estimate the allele sharing among all affected individuals in the pedigrees. Because of program memory constraints, 3 affected and 13 unaffected individuals of the largest family (S01) were dropped from the analysis. The parametric analysis was performed under the assumption of an autosomal dominant model, which allowed for locus heterogeneity. The disease-allele frequency was set at 0.001, and penetrances were set at 0.7, 0.7, and 0.01 for homozygotes, heterozygotes, and noncarriers, respectively. Marker-allele frequencies were estimated on the basis of the 530 study participants, and recombination rates for males and females were assumed to be equal.

The results of the genomewide multipoint nonparametric and parametric analyses are presented in figure 1. The highest signal among the 22 scanned autosomes was observed for marker *D2S117*, with an NPL score of 3.79

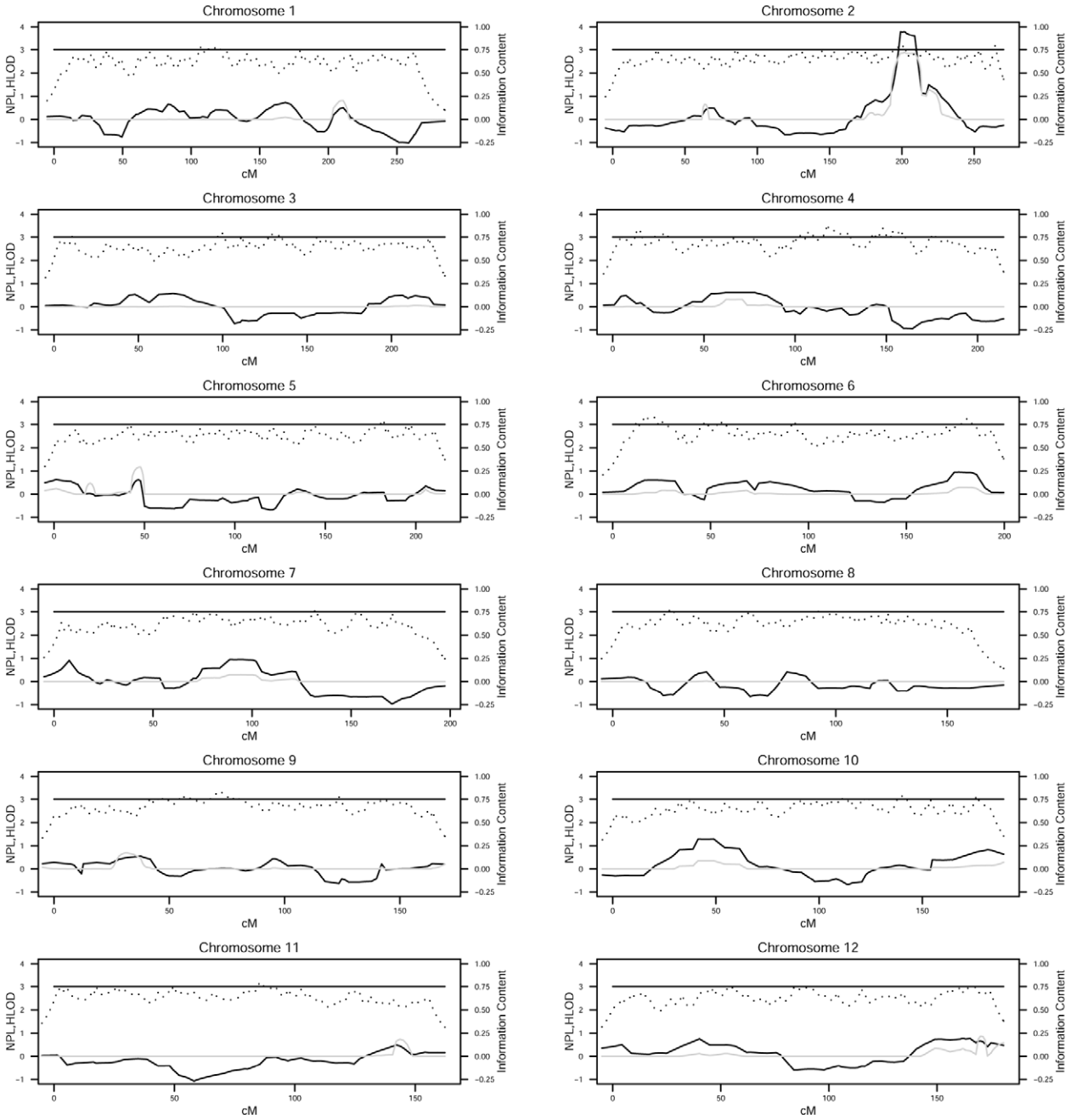
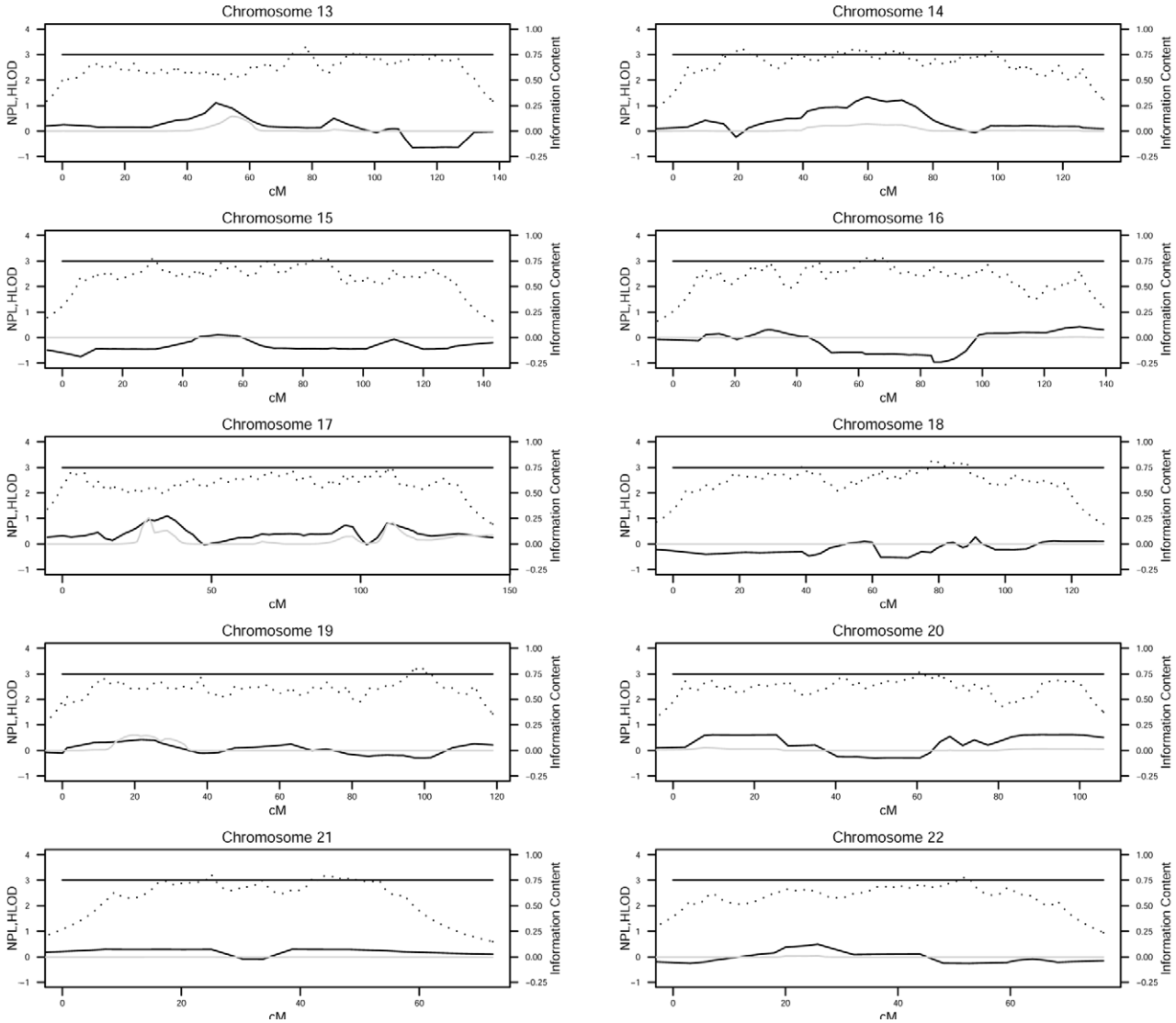


Figure 1. Results of the genomewide scan for each chromosome. The black curve represents NPL scores, the gray curve represents HLOD scores, and the dotted curve represents STR marker informativity. Genetic distances are based on the deCODE Genetic map.

($P = .0042$) and a LOD−1 interval of 18.47 cM. The highest heterogeneity LOD (HLOD) score (2.88) was detected between markers *D2S311* and *D2S116* ($\alpha = 1.0$). Despite the fact that linkage analysis in the presence of heterogeneity is robust and powerful for complex diseases, the estimate of the proportion of linked families, α, is not accurate in this circumstance.[18–20] The largest multigenerational family (S01) contributed the bulk of the score for the linkage peak on chromosome 2q (LOD score of 2.34 at marker *D2S117* and corresponding NPL score of 12.5 [$P = .0019$]). In addition, two other regions of the genome showed nominal evidence of linkage to RLS in the parametric multipoint analysis: *D5S2848* (HLOD 1.2; $\alpha = 0.43$) and *D17S804* (HLOD 1.0; $\alpha = 1.0$).

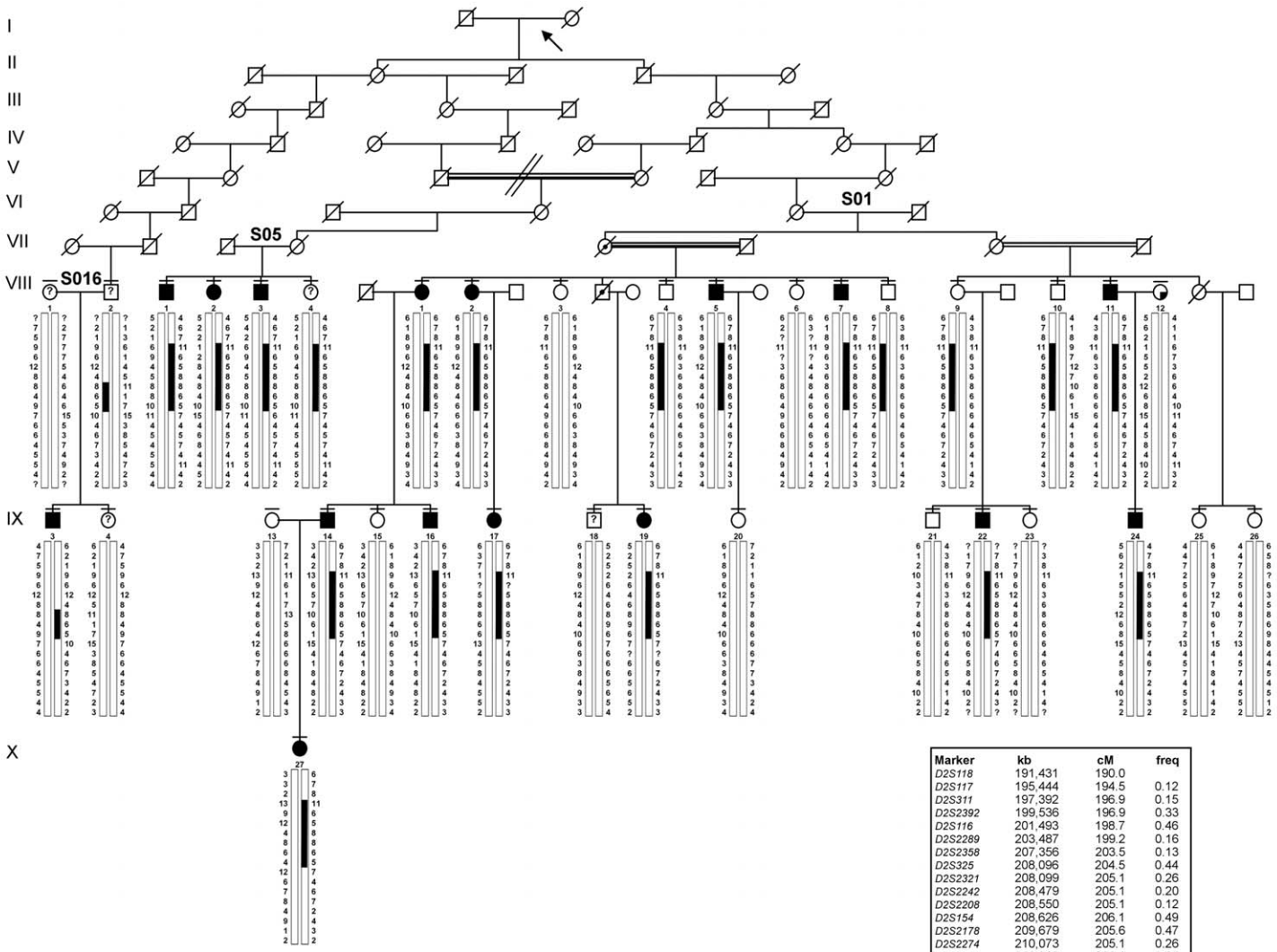
To fine map the chromosome 2 candidate region, we chose a set of 11 additional microsatellite markers (*D2S2392*, *D2S2289*, *D2S325*, *D2S2242*, *D2S2208*, *D2S154*, *D2S2178*, *D2S2274*, *D2S1369*, *D2S157*, and *D2S371*) from



the University of California–Santa Cruz (UCSC) Genome Browser 2004 human genome assembly (UCSC Genome Bioinformatics). Marker order was based on the physical map, and genetic distances were based on the sex-averaged Marshfield genetic map (Center for Medical Genetics Web site) (fig. 2). We estimated allele frequencies for these markers from 88 unrelated (during at least the last 3 generations) individuals of the same village. We genotyped these markers in 101 individuals from the 18 RLS-affected families, using a 3100 Sequencer (Applied Biosystems), and we assigned genotypes using Genemapper version 3.7 software.

For the detailed mapping of the chromosome 2 candidate region, we calculated multipoint model-free and parametric LOD scores using SIMWALK2,[21,22] which allowed the inclusion of all individuals of S01. A two-point analysis was performed using the MLINK program (LINKAGE software package).[23] The same parameters as in the genomewide multipoint analysis were used. The SIMWALK2 analysis (fig. 3) provided strong evidence of a candidate region between markers *D2S311* and *D2S317* (11.7 cM), with a peak NPL score of 5.5 ($P \leqslant 3.33 \times 10^{-6}$) and a peak HLOD score of 5.1 at markers *D2S325, D2S1369,* and *D2S157*. These data, which exceed the genomewide significance level defined by Lander and Kruglyak (LOD 3.6; $P = .000022$),[24] indicate the presence of an RLS locus in this region of chromosome 2q.

We next used genotype data for 20 markers on chromosome 2q to examine the pattern of haplotype transmission for all individuals in the 18 families, in an attempt to identify haplotype sharing among the 37 idiopathic RLS cases. We found that a common 7-marker haplotype (11-6-5-8-8-6-5) in the chromosome 2q33 region is shared identical by descent by all 15 affected members of fami-



Marker	kb	cM	freq
D2S118	191,431	190.0	
D2S117	195,444	194.5	0.12
D2S311	197,392	196.9	0.15
D2S2392	199,536	196.9	0.33
D2S116	201,493	198.7	0.46
D2S2289	203,487	199.2	0.16
D2S2358	207,356	203.5	0.13
D2S325	208,096	204.5	0.44
D2S2321	208,099	205.1	0.26
D2S2242	208,479	205.1	0.20
D2S2208	208,550	205.1	0.12
D2S154	208,626	206.1	0.49
D2S2178	209,679	205.6	0.47
D2S2274	210,073	205.1	0.26
D2S1369	210,369	206.1	0.08
D2S157	211,031	206.1	0.10
D2S371	212,138	206.7	0.14
D2S317	213,327	208.6	

lies S01 and S05 (fig. 2). Obligate recombination events defined a candidate region of 8.2 cM between markers *D2S311* and *D2S2208*. Notably, the single affected individual of family S016 shares the alleles of the disease-chromosome haplotype for only the three distal markers (8-6-5) (fig. 2). From genealogical studies, we found that families S01, S05, and S016 descended from a common founder couple 10 generations ago (fig. 2). Two-point linkage analysis of this large genealogy resulted in a maximum LOD score of 4.1 at $\theta = 0.0$ at marker *D2S2242*. The remaining 15 families do not descend from a common ancestor; in those families, no common haplotype was observed, and the maximum LOD score we obtained was 0.4 in S06.

The three families S01, S05, and S016 also share a common haplotype for the chromosome 5p region, with the exception of three affected members of S01 (VIII-7, VIII-11, and IX-22), but not for the chromosome 17p region. Proximal and distal recombinants allowed us to define the common genomic region as spanning 11.8 cM (between markers *D5S1954* and *D5S2848*), although the potential significance of this region is unclear.

Clinical data from all 16 affected members of families S01, S05, and S016, together with the haplotype status on chromosome 2q33 and chromosome 5p15-p14, are reported in table 1.

We performed our model-based analyses with the assumption of autosomal dominant inheritance with a reduced penetrance of 70%. Formal segregation analysis in a large set of families has argued for a single autosomal dominant major gene with a multifactorial component in families with early disease onset (i.e., onset age of <30 years).[25] The reported penetrance value varies from full penetrance[26] to 70% for individuals showing only periodic leg movements during sleep but who are otherwise without RLS symptoms.[27] Additionally, in children and mildly affected individuals with intermittent symptoms, it can be difficult to recognize the RLS phenotype. To overcome this possible drawback, we opted for a reduced penetrance in our linkage analysis. However, reanalysis of our data with different model parameters, such as increasing the estimates of penetrance, did not substantially change the results (e.g., two-point LOD score of 3.3 with 0.9 penetrance and 0.01 phenocopies). Similarly, an affected-only analysis yielded a two-point LOD score of 3.4.

Three other loci have been reported elsewhere for RLS.[8,11,12] Linkage to the chromosome 12q and chromosome 14q loci was recently replicated.[28–30] On the other hand, linkage to chromosome 12q could be replicated neither in two South Tyrolean families[31] nor in two northern Italian families.[32] Furthermore, when the same village investigated in the present report was studied, no evidence of linkage of RLS was found[14] to the three RLS loci described elsewhere.[8,11,12] These data, together with the substantial variability of symptoms, suggest that RLS may have a polygenic basis, with contributions of major genes, modifier genes, and complex interactions of genes and environment. In geographically or culturally isolated populations that descended from a small number of founders, a more restricted number of susceptibility loci and disease alleles at each locus can be reasonably expected. In fact, the RLS locus on 12q was identified in the French Canadian population, for which a founder effect has been described.[11] Recently, it was shown that 31.6% of the total number of families identified in the French Canadian population were linked to the chromosome 12q locus.[28]

LD and historical data from the South Tyrol region and the small isolated village suggest that individuals may share a small number of disease alleles.[13] The shared disease haplotype in related families S01 and S05 effectively narrows the candidate region to 8.2 cM. Using the program PHASE,[33] which has been shown to provide accurate estimates of haplotype frequencies from population data,[34] we estimated haplotype frequencies from 88 unrelated individuals in the village. The extended shared haplotype from families S01 and S05 was present in only 0.93% of the control individuals, which strongly indicates that its shared presence in the affected individuals points to the presence of an RLS disease allele. By contrast, the population frequency of the 3-marker haplotype identified in the distantly related S016 was 7%, which makes reliance on this single individual for definition of a reduced candidate region of 1.6 cM more risky. In the 1.6-cM region, there are currently 11 annotated genes, of which 3 are predicted genes with unknown function. Among some plausible candidate genes, we recognized (1) *CPO* with carboxypeptidase activity, (2) *CREB1,* which encodes a cAMP-responsive element-binding protein, and (3) *KLF7,* Kruppel-like factor 7, with transcription factor activity. The larger 8.2-cM interval contains >100 annotated genes. The hap-

Figure 2. Ten-generation genealogy of the 16 affected individuals of families S01, S05, and S016 and 2q locus haplotypes. Patients with idiopathic RLS are indicated by blackened symbols; one individual of family S01 with uncertain disease status is indicated by a symbol with the bottom right corner blackened; RLS suspected by family history is indicated by a dot; examined individuals are denoted by a bar. Disease status of individuals indicated with a question mark was set as "unknown" for one healthy member of family S01 because his age was lower than the mean age at onset plus 1 SD in this family and was set as "unknown" for four unaffected members of families S05 and S016 because they participated only in the first phase of the phenotype assessment. For computational reasons, one consanguinity loop had to be broken in the linkage analysis. The arrow depicts the common founder couple. The disease haplotype is denoted by the blackened vertical bar; the normal haplotype is marked by an unblackened vertical bar. The inset shows marker map positions and allele frequencies (freq).

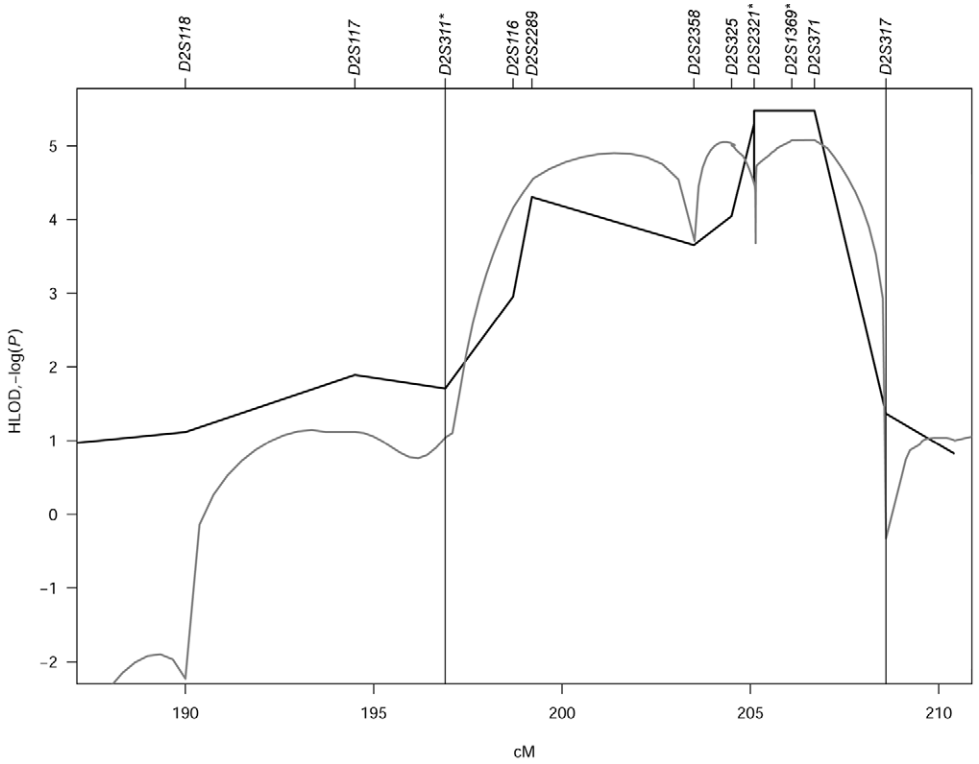


Figure 3. HLOD scores and −log(*P* value) of NPL scores for markers in the 2q region after fine mapping. The black line indicates the −log(*P* value) of NPL scores, and the gray line indicates the HLOD score. Marker *D2S311** is at the same position as marker *D2S2392*, marker *D2S2321** shares the same position as markers *D2S2242* and *D2S2208*, and marker *D2S1369** is close to markers *D2S154*, *D2S2178*, *D2S2274*, and *D2S157*; thus, these additional markers are not represented on the graph.

lotype frequency of the chromosome 5 region was estimated to be 23%, suggesting that this interval does not play a major role in the etiology of RLS in our population.

In summary, our linkage data encompassing 37 idiopathic RLS cases from this isolated village and the haplotype-sharing data of patients of three families provide strong evidence for the identification of a new major susceptibility locus for RLS at 2q33 in a South Tyrolean population isolate. The novel locus provides further evidence that RLS is a genetically highly heterogeneous disorder. In addition, it can be expected that the identification of the underlying gene defect at 2q33 will contribute to our current understanding of the underlying biological mechanisms of RLS.

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Web Resources

The URLs for data presented herein are as follows:

Center for Medical Genetics, http://research.marshfieldclinic.org/genetics/ (for Marshfield genetic map)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for RLS)
UCSC Genome Bioinformatics, http://genome.ucsc.edu/

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