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Family-based association mapping provides evidence for a gene for reading disability on chromosome 15q

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Family-based association mapping was used to follow up reports of linkage between reading disability (RD) and a genomic region on chromosome 15q. Using a two-stage approach, we ascertained 101 (stage 1) and 77 (stage 2) parent–proband trios, in which RD was characterized rigorously. In stage 1, a set of eight microsatellite markers spanning the region of putative linkage was used and a highly significant association was detected between RD and a three-marker haplotype (*D15S994*/*D15S214*/*D15S146*: *P* and empirical $P < 0.001$). A significant association with the same three-marker haplotype was also observed in the second-stage sample ($P = 0.009$, empirical $P = 0.006$). Our data therefore provide strong evidence for one or more genes contributing to RD being located in the vicinity of the region including *D15S146* and *D15S994*. In addition, our results provide support for association analysis being a useful method to map susceptibility loci for complex disorders.

INTRODUCTION

Reading disability (RD), or dyslexia, is a common condition that affects between 5 and 10% of children of school age (1) and produces effects that continue into adult life (2). There is no doubt that RD can have a serious social impact (2,3). Although a century of research has failed to uncover its pathophysiology (4), there is now overwhelming evidence that genes play a significant role in the aetiology of RD (5,6). The familial nature of RD was recognized when the condition was first identified (7) and has since become well established (5,8,9). Twin studies have shown that the tendency for familial clustering is primarily due to genes, rather than shared environmental factors, with heritability estimates ranging up to 0.71 (5,9). It is likely that multiple genes of small to moderate effect are involved, with some contributing to general and others to specific deficits (10), a view that is also supported by segregation analysis (11).

Reading is a complex task and disability could arise from deficiencies in one or more associated cognitive processes. A number of global information processing routes essential for reading have been identified and these are best characterized as either phonological or non-phonological. Processing in the first category is based on the relationship between letters or letter groups and sounds. An example of the processing involved in the latter category occurs through an orthographic route which exploits whole-word information. Although the majority of twin studies have revealed a significant genetic contribution to both phonological and non-phonological processing deficits, they have also provided evidence that the phonological route is influenced more strongly by genes (12,13), a finding also supported by twin studies of specific language impairment (14).

Linkage studies have previously identified regions likely to contain genes contributing to RD, in particular the long arm of chromosome 15 (15–17), the short arm of chromosome 6 near the human leukocyte antigen region (15,18–21), and regions on chromosomes 1 and 2 (22,23). Smith *et al.* (16) first implicated chromosome 15q reporting linkage to RD in ~20% of families tested. Subsequent studies, some of which included a proportion of the original pedigrees, failed to replicate this finding (24,25), a result not unexpected for complex disorders even if the first result was a true positive. However, a lod score >3 was recently observed for the original region of linkage in six independent families by Grigorenko *et al.* (15). They also observed that linkage was strongest among poor single-word readers. In addition, Schulte-Korne *et al.* (17) reported supportive evidence for linkage between a spelling component of RD and markers in the same region of chromosome 15 (maximum lod score = 1.78).

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Several basic strategies are available for positional cloning in complex traits such as RD but, in essence, all of them involve first assigning an approximate map position by linkage. The hope is then to determine more precise map positions via association analyses, exploiting potential linkage disequilibrium (LD) in relatively dense marker maps, followed by mutation analyses of genes that map to the identified regions (26,27). Recent reports have cast doubt on the utility of association mapping for detecting genes contributing to complex disorders (28,29). Kruglyak (29) has suggested that LD might only be detectable over 3 kb using single nucleotide polymorphisms (SNPs), although others have shown LD to exist over distances of up to 1 cM in restricted populations (30) and up to 400 kb in outbred populations (31–34), using microsatellites or SNPs.

Although some evidence for the successful application of association mapping has come from studies of insulin dependent diabetes mellitus (30,34–36) and psoriasis vulgaris (33), data for other complex disorders are sparse. We used the association approach to follow up the putative linkage between RD and 15q in an outbred population, adopting a family-based design so as to mitigate against the possible confounding effects of population stratification (37). The study was carried out in two stages involving samples of 101 (stage 1) and 77 (stage 2) parent–proband trios in which RD was defined conservatively. Since LD can be increased further by use of haplotypes of several markers, as highlighted by Kruyglyak (29), we tested for association with multi-marker as well as single-marker haplotypes.

RESULTS

Stage 1

Six markers from the 8 cM region of putative linkage on chromosome 15q were used for initial association analysis (Fig. 1). Comparing the allele distributions on transmitted and non-transmitted parental chromosomes in trios from stage 1, one marker (*D15S994*) showed a significant difference (P = 0.007, empirical P = 0.006; Table 1; P = 0.04 with Bonferoni correction for six tests). Although haplotype relative risk (HRR) (38) analysis of trios is robust against population stratification, it may be confounded by non-random mating and therefore an extended transmission disequilibrium test (eTDT) was also used to test the observed association with RD for statistical significance (39). As anticipated, the two methods gave similar results and both detected a significant association for *D15S994* only (eTDT, P = 0.004; Tables 1 and 2). Since evidence for an association was obtained at one end of the analysed region, two additional markers proximal to *D15S994* were typed (*D15S146* and *D15S1044*). No evidence for an association with RD was obtained for either of these additional markers (Table 1).

To increase informativity, we next performed pairwise HRR analysis of RD using the HAPMAX computer program (Table 3). A significant association with RD was observed for all pairwise combinations of markers that included *D15S994*, with the exception of haplotypes involving the most distal markers *D15S1028* and *D15S121*. The most significant finding (P = 0.001, empirical P < 0.001, P = 0.03 with conservative Bonferoni correction for 28 tests) was obtained for marker

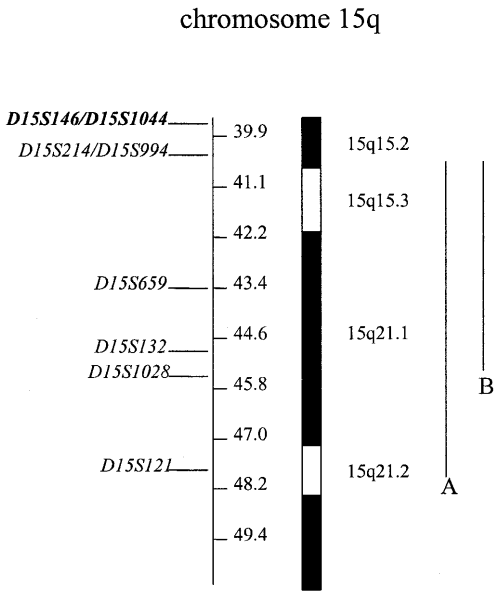

Figure 1. Schematic presentation of chromosome 15 including (from left to right) marker names, marker positions in cM (taken from Marshfield Sex-Averaged Linkage Map) and cytogenetic map. All data were extracted from the Genome Database (http://www.gdb.org/gdb/). Line A indicates the region of suggested linkage by Grigorenko (15). B is the region suggested by Schulte-Korne (17). Six markers were chosen from the 8 cM region of putative linkage on chromosome 15q and used for initial association analysis. These markers are indicated in plain typeface. Markers in bold type were not part of the initial screening set.

Table 1. Association between RD and single markers on chromosome 15 (stage 1)

Marker	HRR χ^2 (df)	P value	Empirical P value	TDT P value
D15S146	10.88 (6)	0.09	0.09	0.16
D15S1044	2.66 (6)	0.85	0.87	0.95
D15S214	7.88 (7)	0.34	0.39	0.50
D15S994	20.97 (8)	**0.007**	**0.006**	**0.004**
D15S659	10.85 (8)	0.21	0.24	0.10
D15S132	7.92 (7)	0.34	0.34	0.50
D15S1028	11.22 (9)	0.26	0.29	0.30
D15S121	1.24 (3)	0.74	0.75	0.70

HRR, haplotype relative risk; TDT, transmission disequilibrium test. Significant associations are shown in bold.

combination *D15S994/D15S214*. Marker *D15S214* was also the only one to significantly increase the observed single-marker association when combined with *D15S994* (χ^2 = 40.58, df = 25, P = 0.013). We also observed significant evidence for an association between RD and haplotypes of the centromeric marker combinations *D15S146/D15S214* (P and empirical P = 0.002) and *D15S146/D15S1044* (P = 0.044, empirical P = 0.048). In addition, all combinations of three markers (the maximum number that could be dealt with by HAPMAX due to the large number of potential microsatellite haplotypes) from the region between *D15S146* and *D15S994* revealed significant evidence for association. The strongest evidence was obtained for *D15S146/D15S214/D15S994* (χ^2 = 133.06,

Table 2. Transmission status of parental *D15S994* alleles in RD trios

Allele	1	2	3	4	5	6	7	8	9
Transmitted	1	7	15	95	12	18	26	1	1
	(1)	(7)	(13)	(46)	(10)	(18)	(23)	(1)	(1)
Non-Transmitted	0	8	29	79	17	7	28	8	0
	(0)	(8)	(27)	(30)	(15)	(7)	(25)	(8)	(0)

Numbers in parentheses refer to heterozygous matings used for extended transmission disequilibrium test analysis.

81 df, P and empirical $P < 0.001$, $P = 0.03$ with conservative Bonferoni correction for 29 tests) which was significantly stronger than the single-marker association of *D15S994* alone ($\chi^2 = 133.06 - 61.55 = 71.51$, 81 – 31 = 50 df, $P = 0.025$). We were unable to identify any specific haplotypes preferentially transmitted or not transmitted to RD patients, respectively.

Stage 2

We attempted to replicate the most significant single-marker (*D15S994*), two-marker (*D15S994/D15S214*) and three-marker (*D15S146/D15S214/D15S994*) haplotype associations from stage 1. However, although the three-marker result could indeed be confirmed ($P = 0.0091$, empirical $P = 0.0059$), we failed to replicate the most significant single- and two-marker relationships (Table 4). We were also unable to identify any specifically associated haplotypes.

A different pattern of three-marker haplotype distribution emerged for the second compared with the first sample when non-transmitted haplotypes from the two stages were compared ($P < 0.001$). A weaker, albeit significant, haplotype frequency difference was also observed between the transmitted chromosomes of stages 1 and 2 ($P = 0.010$). On further *post hoc* analysis of the second sample, we observed a suggestive association between RD and *D15S146* ($P = 0.039$, empirical $P = 0.055$) which did not remain significant after multiple testing had been taken into account (Bonferoni correction for three tests). We also noted a significant association of *D15S146/D15S994* with RD ($P = 0.013$, empirical $P = 0.017$) which was also significant in stage 1 ($P = 0.016$, empirical $P = 0.019$) but was only of borderline significance in stage 2 after correction for multiple testing (Bonferoni correction for three tests).

DISCUSSION

Linkage studies have identified regions likely to contain genes contributing to RD but, as is generally expected for complex phenotypes, these areas are still large. We therefore sought supporting evidence for an RD susceptibility locus mapping to 15q (15–17) and have attempted to refine its map position using a two-stage, family-based association mapping approach.

A significant association was observed between RD and one of six markers (*D15S994*) spanning the region of interest. The evidence in favour of an association of this region to RD was further supported by pairwise haplotype analysis. The most significant pairwise association was observed with markers *D15S994/D15S214*. Furthermore, all marker pairs centomeric to *D15S994* that did not include *D15S994* were significantly associated with RD whereas telomeric marker haplotypes were not. Combinations of three markers from the region between *D15S146* and *D15S994* revealed significant evidence for association, with the strongest evidence obtained for *D15S146/D15S214/D15S994*. This three-marker association was significantly stronger than the association observed with *D15S994* alone.

We sought to replicate the most significant single-, two- and three-marker haplotype associations from stage 1 in an independent sample of trios. Again, we observed a significant three-marker haplotype association with RD in the second sample, but failed to replicate the single- (*D15S994*) or two-marker haplotype associations (*D15S994/D15S214*). On subsequent analysis, we observed haplotype frequency differences between the two samples. This difference was most pronounced for the non-transmitted haplotypes of samples 1 and 2, but less pronounced between the chromosomes inherited by probands of the two samples. It is noteworthy that the two populations were generally from different parts of the UK and we could speculate that the moderately high mutation rates of microsatellites may have given rise to different allele distributions in the two population samples, but that sufficient levels of potential LD were independently maintained with

Table 3. Pairwise haplotype association with RD on chromosome 15 in stage 1 sample

	χ^2 (df) (lower half)				*P* value (upper half)			
MKs	146	1044	214	994	659	132	1028	121
146		**0.044**[a]	**0.002**[b]	**0.016**[c]	0.091	0.027	0.183	0.687
1044	45.59 (31)		0.643	**0.004**[d]	0.117	0.461	0.425	0.550
214	55.12 (29)	21.88 (25)		**0.001**[e]	0.320	0.138	0.411	0.488
994	57.63 (37)	58.83 (33)	61.55 (31)		**0.025**[f]	**0.006**[g]	**0.041**[h]	0.062
659	59.23 (46)	49.70 (39)	41.50 (38)	75.06 (53)		0.143	0.123	0.100
132	46.56 (30)	30.10 (30)	38.48 (30)	63.46 (38)	56.28 (46)		0.205	0.506
1028	59.95 (31)	43.08 (42)	36.30 (39)	78.00 (58)	82.81 (69)	54.70 (47)		0.152
121	17.40 (21)	18.57 (20)	19.53 (20)	36.65 (25)	40.27 (30)	22.24 (23)	41.32 (33)	

MKs, chromosome 15 markers.
Significant associations are shown in bold.
Empirical *P* values: [a]0.048; [b]0.002; [c]0.019; [d]0.005; [e]<0.001; [f]0.046; [g]0.006; [h]0.064.

Table 4. Association (HRR) between RD and marker combinations on chromosome 15 (stage 2)

Marker(s)	χ^2 (df)	*P* value	Empirical *P* value
994	7.83 (8)	0.45	0.48
214/994	36.94 (31)	0.21	0.29
146/214/994	114.10 (81)	**0.009**	**0.006**

Significant associations are shown in bold.

susceptibility loci within each population sample. It is also possible that we were observing the effects of more than one susceptibility mutation and/or multiple founders. Even so, both our samples produced evidence implicating the same region of chromosome 15. Our data are thus consistent with the hypothesis that a susceptibility gene for RD maps to chromosome 15q15–q21. Furthermore, since LD is not usually expected to extend over large regions of the genome in outbred populations (29), our data suggest that the most likely location of such a gene is within 1 cM of the region between *D15S994* and *D15S146*.

Regions of linkage in complex disorders are typically large and therefore contain many potential susceptibility genes for further analysis. For example, the 8 cM region of putative linkage we have screened here can be expected to contain 250 genes on average (under the simplistic assumption of ~100 000 genes equally distributed across the genome). Association mapping across regions of linkage using moderately dense microsatellite maps in outbred populations is one of the main hopes for isolating susceptibility genes underlying complex phenotypes. Alternative strategies such as genome-wide association studies (27) or high-density SNP mapping (29) are as yet not technically feasible and, initially, are likely to be extremely expensive. However, although LD has been demonstrated between microsatellites over distances up to 1 cM in relatively isolated (30) and outbred populations (31,32), association mapping has only been applied successfully to two complex phenotypes in outbred populations, namely insulin dependent diabetes mellitus (30,34–36) and psoriasis vulgaris (33). The approach thus remains the subject of controversy (28,29). Our findings provide important and timely support for association mapping as a method of gene localization although a definitive proof of utility will only come from the identification of a susceptibility gene *per se*.

MATERIALS AND METHODS

Two samples comprising 101 and 77 parent–proband trios, were ascertained through contacts with Local Education Authorities across South Wales (stage 1) and English schools specialized in the education of children with reading difficulties (stage 2). We used the stage 2 sample as a replication sample to test the most significant single-, two- and three-marker haplotype associations with RD, detected in stage 1.

Sample 1 was ascertained through initial contact with 742 families. Replies were received from 252, of which 223 children were tested and 146 met our IQ and RD criteria (65% of those tested). DNA was collected from 101 families who formed complete trios. The proband ages ranged from 8 years 6 months to 16 years 11 months [mean age: 13 years 3 months, standard deviation (SD) = 2.20)] and comprised 86 males and 15 females, with IQs ranging from 85 to 134 (mean = 101, SD = 10.82). All proband parents were Caucasians originating from the UK.

The second sample consisted of 77 RD parent–proband trios. Sixty-four of these were ascertained through contact with English schools, all but one of which were registered with Crested (the Council for the Registration of Schools Teaching Dyslexic Pupils). The remaining 13 families were ascertained in the same way as the first sample, that is, through Local Education Authorities from South Wales. The total target sample comprised 584 families and replies were received from 185. Of the 167 children tested, 108 met our IQ and RD criteria (65% of those tested). DNA was collected from 77 families (67 male probands, 10 female) who formed complete trios. The proband ages ranged from 9 years 9 months to 17 years 1 month (mean age = 13 years 10 months, SD = 1.83). The proband IQs ranged between 85 and 131 (mean = 103, SD = 11.43). All proband parents were Caucasians originating from the UK.

We defined the general RD phenotype in relation to reading ability and estimated IQ. The inclusion criteria for the current study required that probands had an IQ >85 and were at least 2.5 years behind their chronological age in reading. This is a severe degree of reading impairment and is likely to represent the lower 5th percentile of children. We used four subtests from WISC III UK (40) to provide a pro-rated full-scale IQ score: Vocabulary, Similarities, Block Design and Picture Completion. We used the accuracy score from the Neale Analysis of Reading Ability (41) to determine reading age. The difference between proband's chronological age and accuracy score ranged from 2 years 6 months to 9 years 11 months (mean difference = 5 years, SD = 1.66) in the stage 1 sample, and 2 years 6 months to 9 years 8 months (mean difference = 5 years 5 months, SD = 1.75) in the second sample. The RD definition used in this study is based on prose reading but is directly comparable to the single-word recognition measure used by Grigorenko *et al.* (15) and others (20,21). Similarly, the Gray Oral Reading Test (GORT) used by Smith *et al.* (16) is also based on prose reading and is also directly comparable to ours. However, Schulte-Korne *et al.* (17) defined affection status as spelling performance at least 1 SD below that predicted from IQ score, which is likely to produce a higher prevelance rate than our measure but to be correlated (5).

After informed consent, venous blood was taken from parents and DNA extracted by standard procedures. DNA was extracted from 25 ml saline mouthwash samples from children by centrifugation at 2000 *g* for 10 min, followed by incubation with proteinase K, SET buffer and SDS at 50°C for 12 h. The DNA was then isolated by standard phenol–chloroform extraction. The chromosomal interval used for association mapping was selected on the basis of the two recent studies supporting linkage to 15q (15–17), yielding an area of ~8 cM (Fig. 1). We aimed to select an initial marker set spaced roughly at 1 cM intervals (Fig. 1, plain typeface). Due to the scarcity of markers this was not possible in the region between *D15S994* and *D15S659*. We also included two additional markers adjacent to *D15S994*, following the detection of an association in this region. Primer pairs were obtained from MWG Biotech (Ebersberg, Germany) and Genset (Paris,

France) with the forward primer of each pair labelled with FAM, TET or HEX fluorescent dyes. Polymerase chain reactions (PCRs) were performed in 12 µl reaction volumes containing 48 ng of genomic DNA, 5 pmol of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 200 µM dNTPs and 0.3 U of *Taq* polymerase (Qiagen, Hilden, Germany). All PCRs were performed on Hybaid (Teddington, UK) PCR Express thermocyclers or MJ Research (Braintree, UK) PTC-100 thermocyclers with an initial denaturation stage at 94°C for 5 min followed by 30 cycles (30 s at 94°C, 30 s at 50–65°C, 30 s at 72°C) and a final extension step at 72°C for 10 min. Reactions for each marker were performed separately, with products being multiplexed into size-specific sets prior to gel electrophoresis. TAMRA-labelled molecular weight markers were run in each lane. Markers were typed on ABI373 sequencers using the Genescan and Genotyper software (Applied Biosystems, Warrington, UK).

We tested for allelic association between individual markers and RD using an HRR method and the eTDT method with Monte Carlo simulation implemented to obtain empirical significance levels (39). Multimarker as well as single-marker HRR analyses were undertaken using the computer program HAPMAX (http://www.uwcm.ac.uk/uwcm/mg/download). This program was first used to estimate marker haplotype frequencies on transmitted and non-transmitted chromosomes in cystic fibrosis families (38), employing an expectation maximization algorithm to allow for phase unknowns. The resulting maximum likelihood estimates of haplotype frequencies were tested for significant differences making use of the fact that twice the log-likelihood difference between the models involved approximates to a χ^2 distribution. Since the number of degrees of freedom became large for haplotypes including more than one marker, however, empirical *P* values were also determined by simulation. To this end, maximum likelihood estimates of haplotype frequencies obtained under the assumption of no difference pertaining to transmitted and non-transmitted chromosomes were used each time to simulate 10 000 trio sets of the original size and genotyping efficiency. For these sets, χ^2 values were calculated using HAPMAX and the number of times the simulated values exceeded the original χ^2 was recorded. This figure, divided by the number of simulations (i.e. 10 000), represents an empirical *P* value for the original χ^2. In addition we have taken a very conservative approach to taking account of multiple testing by adjusting for six tests for single-marker associations, 28 tests for two-marker and 29 tests for multi-marker haplotype associations (stage 1), although we recognize that the latter tests are not independent of each other and would thus underestimate the significance of the findings. As stage 2 analyses were mainly replication tests, Bonferoni corrections were only applied to unplanned tests of association.

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